RNA transmission and expression from inert HIV candidate vaccine virus-like-particles

Ziyaad Valley-Omar

Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Molecular and Cell Biology

UNIVERSITY OF CAPE TOWN

August 2008

Declaration

The work described in the thesis was performed both in the Department Molecular and Cellular Biology and the Institute of infectious diseases and molecular medicine of the University of Cape Town, under the general supervision of Professor Edward Rybicki, Dr Ann Meyers and Professor Enid Shephard. This work is my own and where the use of other is included, their contributions have been acknowledged.

……

Ziyaad Valley-Omar

August 2008

Acknowledgements

I wish to express my thanks to the following:

My parents **Abeeda** and **Mohamed** for their constant support, m&m's and always encouraging me to let my studies and research take precedence.

My sweety, **Nadia Isaacs** for her unwavering support, understanding and particularly for demystifying the intricate and more complex workings of Microsoft Word formatting.

My Supervisors, **Professor Edward Rybicki** and **Dr Ann Meyers** for their advice, guidance and extreme patience exercised throughout my research in the virology laboratory, which has instilled in me a greater sense of confidence during research and importantly a greater understanding and appreciation of the ethos of science.

Professor Enid Shephard for her invaluable advice and guidance during my immunology and cell uptake studies.

All members of the Molecular and Cellular Biology department, virology division past and present for creating a pleasant and enjoyable working environment.

Mr Mohamed Jaffer for his patience and guidance provided during electron microscopy work.

Mr Zaahier Isaacs (Z1), **Ms Desiree Bowers**, **Ms Shireen Galant**, **Ms Anke Binde** and **Mr Rodney Lucas** for making the "dull-natured" UCT medical school more bearable and for their valuable assistance during my mammalian cell culture and mouse immunology studies

The **South African AIDS Vaccine Initiative** and the **National Research Foundation** for their generous financial support.

Table of Contents

Abbreviation list

AcMNPV	*Autographa Californica* multiple nucleopolyhedroviruses
AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral
BEVS	Baculovirus Expression Vector System
BHK-21	Baby Hamster Kidney Cell Line
BMH	Branched multiple hairpin
BV	Budded virus
CA/P24	Capsid protein
CAT	Chloramphenicol acetyltransferase
CMV	Cytomegalovirus
CpG-ODN	CpG-Oligonucleotides
CTL	Cytotoxin T-lymphocyte
DC	Dendritic cells
DIS	Dimerization initiation site
ELISA	Enzyme Linked Immunosorbent
ELISPOT	Enzyme Linked Immunosorbent Spot
EM	Electron microscopy
Env	Envelope glycoprotein
FCS	Foetal calf serum
FIV	Feline Immunodeficiency Virus
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
Gp41/120	HIV envelope glycoprotein
HEK 293	Human Embryonic Kidney Cell Line
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
IFN-	Interferon-
Ig	Immunoglobulin
IL-	Interleukin-
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
LDI	Long-distance Interaction
LTNPs	Long-term-non-progressors
LTR	Long terminal repeat
MA/P17	Matrix protein
MDDCs	Monocyte-derived dendritic cells

MHC-	Major histocompatibility complex
MHR	Major homology region
MLV	Murine leukemia virus
NC/P7	Nucleocapsid protein
NPV	Nucleopolyhedrovirus
OV	Occluded virus
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PH	Polyhedrin
PRRs	Pattern recognition receptors
RAW 264.7	Mouse macrophage cell line
RT-PCR	Reverse transcriptase polymerase chain reaction
SF21	*Spodoptera frugiperda 21*
SFU	Spot forming units
SFV	Semliki forest virus
SL	Stem-loop
SN	Supernatant
TEM	Transmission electron microscopy
Th-	T helper-
TLR-	Toll-like-receptor
US FDA	United States food and drug administration
UV	Ultra violet
VLP	Virus-like-particle
VSV	Vesicular stomatitis virus
WT	Wild type
WSSV	White spot syndrome virus

Abstract

HIV-1 Gag virus-like-particles (VLPs) produced in various expression systems are potent stimulators of both cellular and humoral immune responses in animal models. The encapsidation of large concentrations of random cellular RNA species is known to accompany the assembly of HIV virus particles. This RNA plays a crucial role by serving as a molecular scaffold for the assembly of Gag structural proteins into particles.

Non-pseudotyped VLPs that do not present any HIV envelope glycoproteins are regarded as inert particles as they contain no replicative nucleic acid and are presumed to be unable to deliver encapsidated RNA for expression in inoculated individuals. Live virus cellular entry studies have shown that non-pseudotyped Gag particles are destined for degradation in acidified vesicles subsequent to receptor independent cellular entry. In addition to host cell RNA incorporation, Gag VLPs produced in insect cell-based, baculovirus expression systems have been observed to incorporate the baculovirus-derived Gp64 envelope glycoprotein. Gp64 has been shown to be efficient at enabling the delivery and expression of genes from recombinant baculoviruses and other Gp64 pseudotyped live viruses in mammalian cell lines both *in vivo* and *in vitro*. This study, therefore, set out to establish for the first time whether inert, baculovirus-derived (Gp64 pseudotyped) Gag VLPs could mediate delivery and expression of randomly encapsidated RNAs in mammalian cell lines.

Gp64 quantitation analyses on VLP extracts showed the incorporation of constant, high concentrations of Gp64, ~1650 Gp64 molecules/VLP. This suggested that Gp64 could mediate VLP cellular entry and possibly the expression of encapsidated RNA. I co-expressed a CAT reporter RNA species in the VLP expression system for VLP encapsidation to determine if randomly encapsidated RNAs could be expressed in mammalian cell lines. Real-time RT-PCR results demonstrated that the levels of encapsidated CAT RNA were highly variable between VLP extractions (0.1-11 copies/VLP) and were thus representative of randomly encapsidated cellular RNA.

VLP cell-uptake assays were conducted using several mammalian cell lines (BHK-21, HeLa, HEK 293 and RAW 264.7), to detect the expression of randomly

encapsidated CAT RNAs. The use of a CAT ELISA post VLP uptake demonstrated for the first time that inert VLPs were capable of entering mammalian cell lines via a non-degradative pathway and CAT RNAs were expressed at comparable levels between the cell lines.

Established virus inactivation techniques were investigated to prevent the undesired transmission and expression of VLP-encapsidated RNAs in mammalian cells. Virus inactivation by heat treatment demonstrated the greatest success in neutralizing CAT RNA expression, possibly functioning through the disruption of Gp64 envelope glycoproteins. The heat treatment of VLPs blocked all CAT expression in some cell lines, while other cell lines appeared unaffected by the VLP heat treatment. Heat treatment thus did not prevent RNA expression in all cell lines but rather appeared to limit the range of cells that the VLPs can enter and express their RNAs.

Cellular entry mediated by functional envelope glycoproteins enables the stimulation of cellular immune responses, effective at controlling HIV infection. Heat treatment and hence Gp64 disruption was therefore expected to disrupt the cellular immune response. This study assessed whether there were any changes to VLP-stimulated immune responses subsequent to heat treatment. Mice were inoculated with VLP-only (heated or non-heated) or DNA vaccine prime/VLP boost (heated or non-heated) regimens. Mice inoculated with heated VLP only, displayed a significant reduction in cellular immune responses compared to unheated VLPs (60%-80% reduction). On the contrary, DNA prime/heated VLP boost inoculation regimens stimulated substantially enhanced cellular immune responses. Additionally, cellular immune responses stimulated by slightly higher concentrations of heat-treated VLP boosts, were very similar to responses stimulated by the non-heated VLP boost.

These novel findings suggest that VLPs can be heat-treated to reduce transduction and expression of encapsidated RNAs in mammalian cells. To retain VLP immunogenicity, the only condition would be the use of slightly higher inoculation doses of the heated VLPs in a prime/boost inoculation regimen.

Chapter 1

Introduction and Literature review

1.1 Current HIV infection statistics

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

This year (2008) marks 25 years since the reported isolation of *Human immunodeficiency virus* (HIV) as the causative agent of acquired immunodeficiency syndrome (AIDS). In spite of the continued efforts and investment in the battle against HIV, infection remains rife and the epidemic continues to spread. Current statistics of HIV infection highlight the problem the medical world is faced with. As of the end of 2007 the estimated number of individuals living with HIV was approximately 33.2 million worldwide (Figure 1.1 (A, C)). More than two thirds of these people (~22.5 million) are located in sub-Saharan Africa, where approximately 1.7 million new infections occurred in 2007 (Figure 1.1 (B, D, E)). On a global scale an estimated 2.5 million new HIV infections occurred during 2007. This translates to 6850 infections per day, almost 70% of which occurred in developing countries (UNAIDS website, 2008). Life expectancy in Southern Africa has thus fallen from 61 to 49 years over the last 20 years (Fauci 2007). Despite these alarming statistics however, the high infection rate does appear to have levelled off in the last 7-8 years (Figure 1.1 (A, B)).

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

response pathways are interlinked, the host's immune response is effectively crippled by the depletion of these cells. Most fatalities that arise from AIDS are therefore a consequence of opportunistic infections, which are sufficient to kill the host due to the weakened immune system.

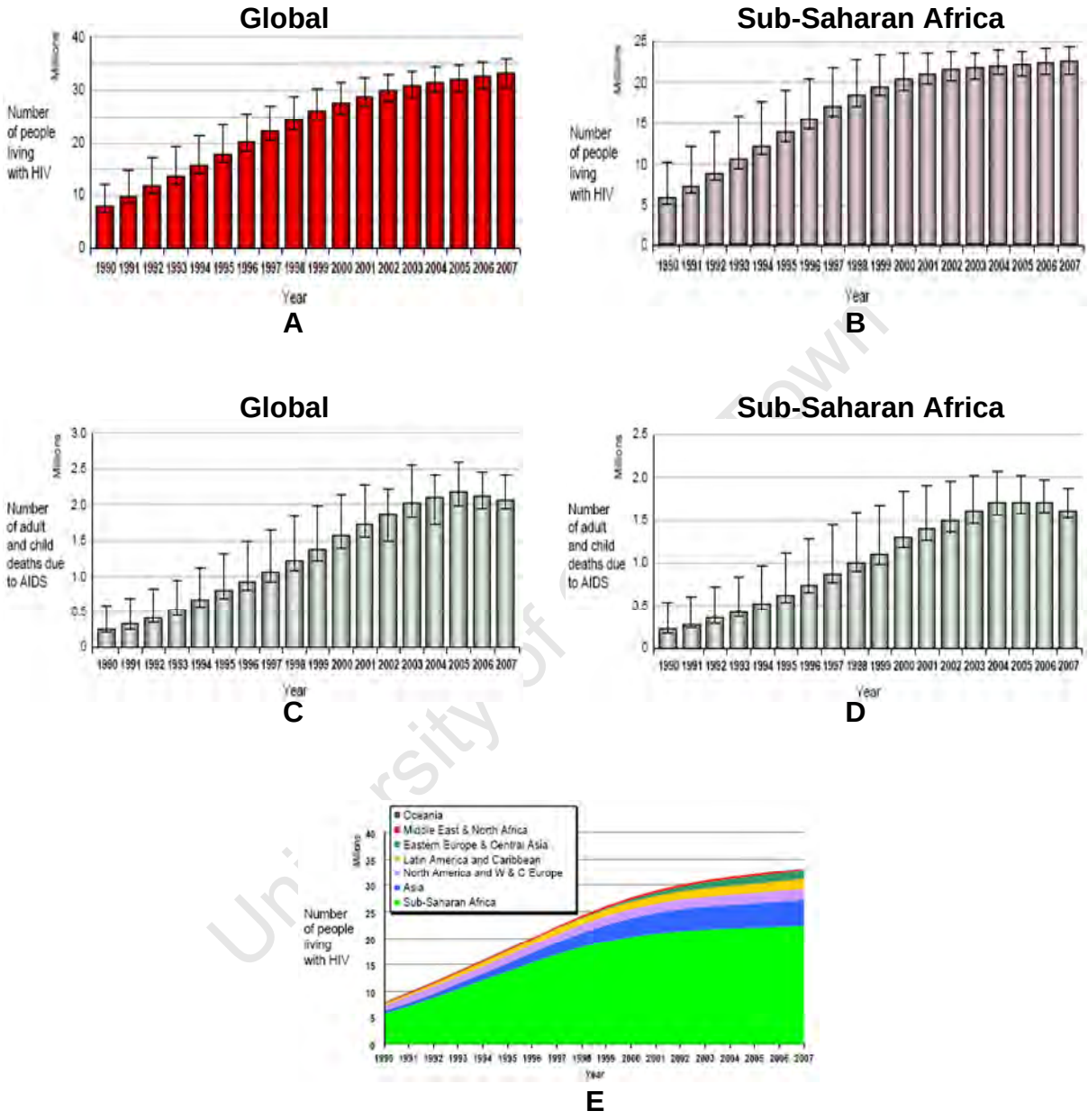


Figure 1.1 Graphs illustrating the number of people living with HIV and deaths due to AIDS between 1990 and 2007. (A) People living with HIV Globally and (B) in Sub-Saharan Africa. (C) Adult and child deaths due to AIDS globally and (D) in Sub-Saharan Africa. E: Estimated number of adults and children living with HIV by region (UNAIDS website, 2008)

An immune system which has been primed with an HIV vaccine however, would hopefully allow early recognition of the infecting virus and subsequent

destruction of virus particles before they have an opportunity to multiply in the host (Mascola *et al.*, 2000; Baba *et al.,* 2000; Letvin, 2002).

1.2 Drug therapy

Drug therapy for the treatment of HIV-infected people is the alternative to vaccination (Figure 1.2). Currently more than 25 highly effective antiretroviral drugs (ARVs) exist, which include protease inhibitors (ritonavir, saquinavir), nucleoside-analogue reverse transcriptase inhibitors (AZT, lamivudine) and non-nucleoside reverse transcriptase inhibitors (nevirapine). These have contributed significantly to reducing AIDS-related morbidity and mortality (AEGIS website, 2008). The primary drawbacks of this form of therapy are that it is generally very expensive, often has unpleasant side effects and its use is generally limited only to those who can afford it. The effect of ARVs is curtailed by the fact that for every HIV-infected person that starts ARV treatment another 6 are newly infected (Fauci 2007). In South Africa it has been estimated that ~ 889000 people currently require ARV treatment and of this only ~ 350000 in the public health system have initiated treatment (South African Treatment Action Campaign website, 2008). In spite of the vast amounts of research, no form of drug therapy has to date been demonstrated to cure an HIV infection.

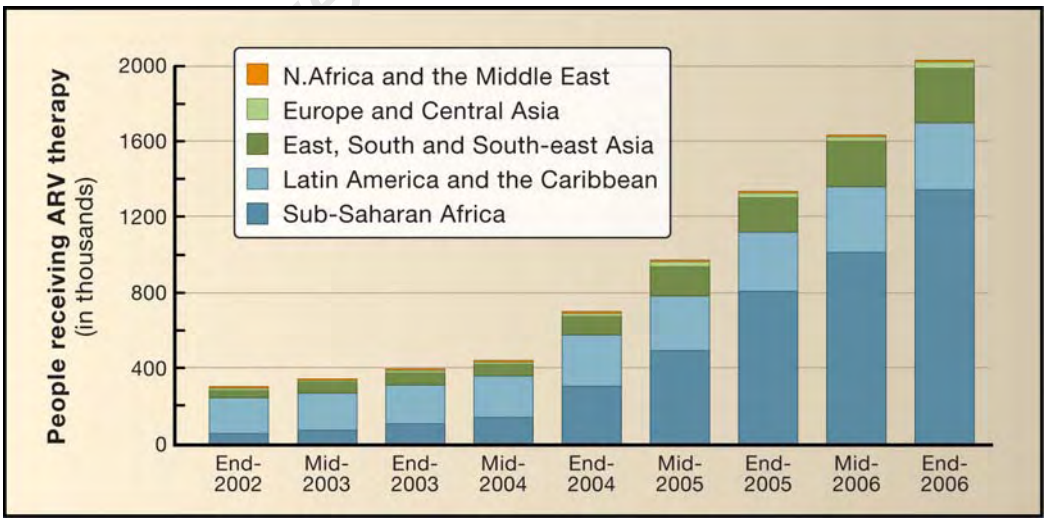


Figure 1.2 Graph illustrating ARV therapy for AIDS patients. Estimated number of HIV-infected people receiving ARV therapy in low- and middle-income countries as of December 2007. (Fauci, 2007)

1.3 HIV vaccine

Vaccines have therefore been proposed as the best means other than celibacy to prevent the virus from establishing an infection within a previously uninfected person. Thus development of an effective HIV vaccine is regarded as one of the most important future prospects with respect to vaccine design. Ideally, as with other successful vaccines, a vaccine directed against HIV should induce a neutralizing antibody (NAb) response, which would clear all detectable levels of HIV within the infected person (sterilizing immunity). Several such vaccine strategies have been proposed/tested and yet a vaccine capable of stimulating a completely sterilizing immunity remains elusive (NIH website, 2008). In the ongoing battle against HIV, numerous potential vaccine strategies have reached pre-clinical and even clinical human trial levels (HVTN website, 2008). However, these studies have generally not generated encouraging results and specific reference is made here to the recent (AIDSVAX) phase III vaccine trials by VaxGen (HIV envelope based vaccine), as well as the phase II Step and Phambili trials conducted by the NIAID-funded HIV Vaccine Trials Network (HVTN) and Merck (MRKAd5 HIV-1 gag/pol/nef trivalent vaccine, based on a weakened adenovirus (adenovirus type 5)), which appeared to offer no significant protective efficacy (Anonymous, 2003; The Step study website, 2008; NLM website, 2008; NIAID website, 2008).

1.4 HIV Vaccine development

Live attenuated vaccines have historically proven to be the most effective, inducing protective cellular and humoral immune responses (e.g.: polio, yellow fever, measles, mumps and rubella vaccines). These vaccine types have typically been effective as they present epitopes in an immunologically relevant context (particulate form), and because they replicate, have facilitated prolonged production of specific epitopes within the host from a small inoculum (see Deml *et al*., 2005 for a review). The use of live-attenuated and other vaccine types, which includes DNA and recombinant viral vaccines does however have limitations, which are particularly relevant with respect to the development of an HIV vaccine. Negative factors include the possible

reversion of the attenuated/recombinant virus to a virulent form by mutation or recombination, and viral genome insertion into host chromosomes, which could result in deleterious mutations (Redfield *et al*., 1987; Murphey-Corb *et al*., 1997; Senior. 2002; Deml *et al*., 2005 (review)). As DNA sequences produced in heterologous expression systems such as bacteria may possess foreign recognition motifes, they could be recognised as foreign bodies by the immune system (Bermann-Leitner *et al*., 2004 (review)). DNA vaccines therefore should preferably be made in mammalian cell expression systems, which are not as easy to bulk up DNA production for vaccine purposes (Bermann-Leitner *et al*., 2004 (review)). The aforementioned problems and risks make these vaccine types undesirable for HIV vaccination strategies. These vaccine types are well reviewed elsewhere and will not be discussed any further as the focus of this study is on the use of another vaccine type, subunit vaccines (Letvin *et al*., 2005; HVTN website, 2008; WHO website, 2008).

If live attenuated vaccines are regarded as the first generation of vaccines, subunit vaccines can be considered as the second generation. Most often subunit vaccines can consist of assembled viral components or individual peptides. As these often carry no nucleic acids and are unable to replicate and do not present the risks that any nucleic acid based (live attenuated, recombinant viral vector and DNA) vaccines do. The subunit group of vaccines may also include genetic vaccines,which therefore carry nucleic acids and replicate. Subunit vaccines have consisted of individual peptides, which were assumed to be immunogenic (envelope glycoproteins) (Anonymous, 2003 (AIDSVAX); Bellier *et al*., 2006; Deml *et al*., 2005 (review); WHO website, 2008). These individual peptide-based vaccines were often unable to stimulate strong immune responses and were therefore administered in very high concentrations and in several booster doses. This is most likely a result of the host's immune system being stimulated with a linear subunit peptide that bears no structural similarity to the same epitope on the assembled virus.

As a result of their weak immunogenicity, peptide-based vaccines are often co-administered with adjuvants. Adjuvants available for use in humans (aluminium phosphate/hydroxide) unfortunately have shown to bias immune systems towards Th2-type responses (Nicklas, 1992). As will be discussed at a later point, control of HIV infections is mediated by strong cellular immune responses this would therefore exclude the use of these adjuvants for HIV vaccine purposes. Other adjuvant types such as CpG oligonucleotides and cholera toxin are capable of enhancing cellular immune responses, do exhibit side effects like toxicity and inflammation and are thus not licensed for use in human vaccines (Imaoka *et al*., 1998; Dumais *et al*., 2002; Kang *et al*., 2003; Yao *et al*., 2003).

As a result of these weak immune responses, inert virus-like particles have been investigated as immunogens. Replicationally inert virus-like particles (VLP) are an ideal substitute for live attenuated and peptide based vaccines as they present epitopes in an immunologically relevant context, but lacks any replicative nucleic acid. Furthermore, VLP structural stability as well as their ability to be manipulated to present heterologous epitopes makes them attractive as vaccines. VLPs are easily produced in several heterologous expression systems and may be purified by established techniques. Several VLP vaccines have been developed for human viruses, such as Human papilloma virus (HPV) and Hepatitis B virus (HBV) (Garcea *et al*., 2004; Garland *et al*., 2007; Paavonen *et al*., 2007; Aires *et al*., 2006; Huang *et al*., 2005).

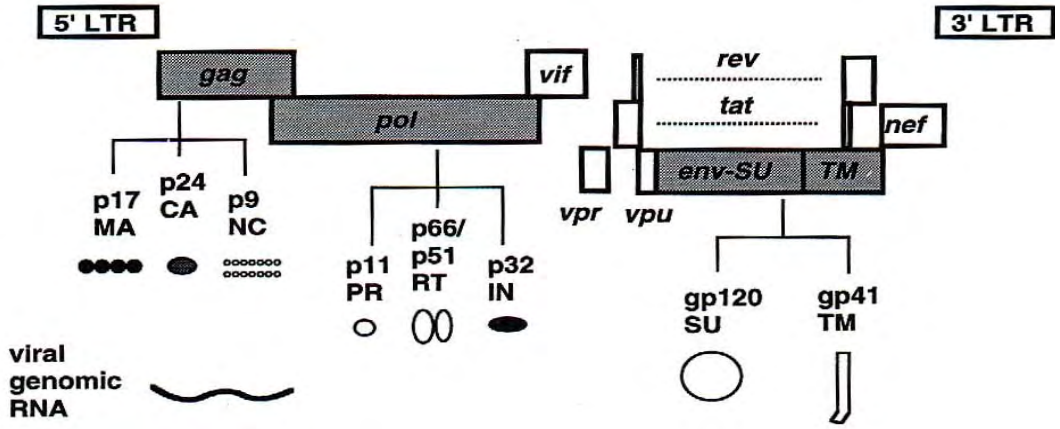
1.5 HIV structure and virion assembly

The HIV Gag (Pr55Gag) structural protein plays a key role during the completion of the HIV life cycle, which concludes with the assembly, budding and maturation of infectious viral particles. Gag is expressed as a single polyprotein composed of several domains responsible for the movement of Gag to the plasma membrane, multimerization of Gag particles and encapsidation of viral genomic RNA (Figures 1.3 and 1.4). The unprocessed Gag initially assembles into immature particles which are subsequently proteolytically cleaved by the viral protease (PR) followed by the

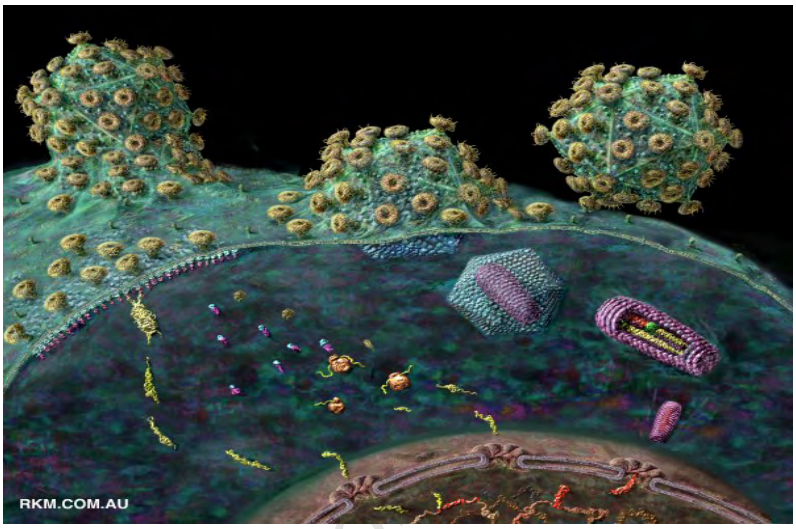
rearrangement of the individual domains to form mature infectious particles. This PR-mediated cleavage of Gag yields the individual matrix protein (MA/P17), capsid protein (CA/P24), nucleocapsid (NC/P7), P6, P1 and P2 domains in the assembled virion, which are crucial for ensuring viral infectivity (Figures 1.3 and 1.4) (Mervis *et al*., 1988; Kohl *et al*., 1988 ; Gottlinger, *et al*., 1989 ; Henderson *et al*., 1992; Kaplan *et al*., 1994; Vogt., 1996 ; Swanstrom *et al*., 1997; Garnier *et al*., 1998). Extensive research has implicated all of the above-mentioned components in infectious HIV virion production. This complex, multistep assembly process is theorised to facilitate the multiple and clearly opposing functions of the Gag proteins at different stages of the viral life cycle. The opposing functions include the fact that immature Gag enables assembly, membrane targeting and RNA binding while the processed Gag enable uncoating, disassembly and nuclear localization of the viral genome.

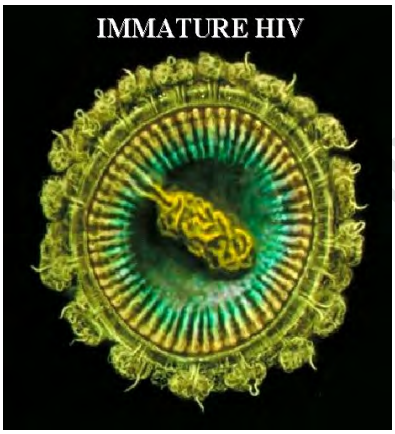
During virion assembly the unprocessed Gag-Gag and Gag-Pol complexes are shown to predominantly concentrate at regions of the cell membrane referred to as barges. Cell membrane tropism is determined by a myristylation signal located at the N-terminus of Gag (Ono *et al*., 2001; Nguyen *et al*., 2000; Lindwasser *et al*., 2001; Gottlinger *et al*., 1989). Lipid barges are densely packed lipid regions enriched with cholesterol, sphingomyelin and glycosphingolipids, which are highly resistant to disruption by non-ionic detergents (Lindwasser *et al*., 2001). Electron microscopy (EM) has revealed that during assembly of immature Gag particles, the Gag precursor proteins are radially arranged below the viral membrane with the N-terminus at the membrane and C-terminus extending inwards (Figure 1.3 (C-E)) (Wilk *et al*., 2001). The radial arrangement produces doughnut shaped immature particles presenting variable levels of electron density derived from the arrangement of the components (domains) of the Gag precursor. After proteolytic cleavage of Gag in immature viral particles a distinctive conical shape is assumed by the virion core consisting of the nucleocapsid (NC) protein and genomic RNA surrounded by the capsid (CA) protein (Figure 1.3 (E)) (Li *et al*., 2000). In this arrangement the MA protein is tightly associated with the inner surface of the viral membrane and lies above the CA protein. It is speculated that this arrangement stabilises the membrane.

A

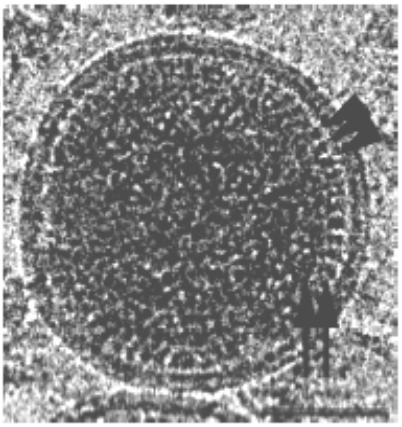


B

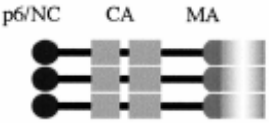


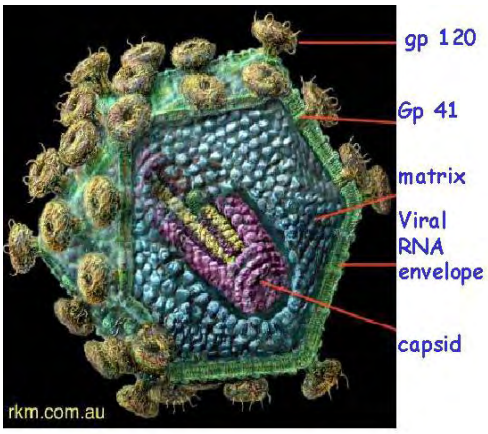


C



D





E

Figure 1.3 Illustrating HIV virion components. (A) Simplified HIV genome structure, (B) Artists representation of a typical HIV replication cycle, (C) Artist representation of an immature HIV virion prior to Gag processing, (D) Electron micrograph of an immature virion displaying the concentric Gag component layers (Wilk *et al*., 2001), (E) Artists representation of a mature HIV virion subsequent to Gag processing displaying the various layers within the virion structure. (images B, C, E are under license from Russell Kightley Media (rkm.com.au) to EP Rybicki)

1.5.1 Functions of Gag Domains: MA, CA, NC

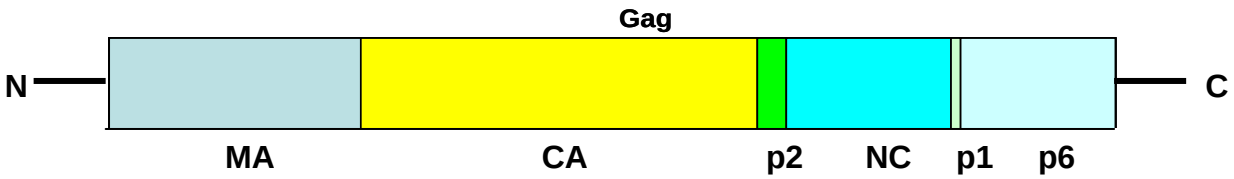


Figure 1.4 Various functional domains that constitute the unprocessed Gag polyprotein. MA: Matrix domain, CA: Capsid domain, NC: Nucleocapsid domain.

1.5.1.1 MA protein functions

In the assembled, mature HIV virion the MA protein lies beneath the viral membrane (Pepinsky *et al*., 1984; Gelderblom *et al*., 1989). This MA protein has been identified to be crucial in shuttling the Gag and Gag-Pol precursor to the plasma membrane. The N-terminus of MA in the Gag precursor serves as a myristylation site, which consequently along with other highly basic domains that form a charged patch for membrane binding, have been shown to facilitate the export to and anchorage of Gag precursors at the plasma membrane (Hill *et al*., 1996; Zhou *et al*., 1994; Gottlinger *et al*., 1989; Facke *et al*., 1993; Spearman *et al*., 1994). This protein domain has also been shown to facilitate the shuttling of the MA complex in assembled virions after infection, from the plasma membrane to the nucleus (Wang *et al*., 1998). This bidirectional transport activity has been suggested to be modulated by phosphorylation by cellular kinases incorporated into the assembled virion, whereby matrix phosphorylation induces the release of the MA complex from the viral membrane during infection (Gallay *et al*., 1995; Bukrinskaya *et al*., 1996; Jacque *et al*., 1998).

Of particular importance for the production of envelope glycoprotein-displaying (Env) virus particles is the finding that there may exist an interaction between the cytoplasmic tail of the HIV envelope glycoprotein and the N-terminal domain of MA. This is because the incorporation of Env into particles appears

to be dependent on the integrity of the MA domain (Yu *et al*., 1992; Dorfman *et al*., 1994(a)). Mutagenesis and other Env-MA interaction studies have revealed that an interaction may exist between the N-terminal region of MA (Leu12) and the C-terminal 67 amino acids of Env (Freed *et al*., 1994; 1995; 1996; Cosson., 1996). Similarly the length of the Gp41 cytoplasmic domain also plays a key role in Env-MA interaction studies as shorter domains were preferably incorporated into retroviral particles (Freed *et al*., 1995; Mammano *et al*., 1995; Haglund *et al*., 2000). A similar scenario was suggested in a study conducted by Haglund *et al*., (2000), in which HIV Gag VLPs could incorporate not only their own Gp41 (150 amino acid cytoplasmic domain) but also the shorter (29 amino acids) vesicular stomatitis virus (VSV) Gp41. Conversely, the VSV virion largely excluded the incorporation of the HIV-derived Env protein even when truncated to 10 amino acids. Although a separate study showed that incorporation was allowed when the HIV-Gp41 was conjugated with the VSV Gp41 cytoplasmic domain (Johnson *et al*., 1997). Thus length alone appears not to be the only determinant of Env incorporation and instead a study conducted by Johnson *et al*., (1998), indicated the presence of a plasma membrane localization sequence in the membrane proximal 10 amino acids of HIV Env that excludes it from sites of VSV budding.

1.5.1.2 CA protein functions

This domain is crucial for the multimerization of Gag and Gag-Pol hence contributes to virion core formation. CA is also involved in incorporating cellular factors and maturation of the virion (Luban *et al*., 1993; Smith *et al*., 1993; Srinivasakumar *et al*., 1995; Wang and Barklis., 1993; Franke *et al*., 1994; Thali *et al*., 1994). The C-terminal domain of CA (151-231) and P2 spacer peptide is functional in virion assembly (multimerization) and membrane binding while the N-terminal domain (1-145) is functional in virion maturation and incorporation of Cyclophilin A (Krausslich *et al*., 1995; Guo *et al*., 2004; 2005; Melamed *et al*., 2004; Abdurahman *et al*., 2004; Chiu *et al*., 2002; Borsetti *et al*., 1998; Huang *et al*., 1997; Chen *et al* 1997; Yoo *et al*., 1997; Gamble *et al*., 1996; Srinivasakumar *et al*., 1995). The observed

association of Gag multimerization activity with membrane binding or the simultaneous elimination of either by a simple mutation in CA has led to the proposal of a “myristyl switch” model (Hermida-Matsumoto L *et al*., 1999; Paillart *et al*., 1999; Spearman at al., 1997). This model suggests that the myristyl group attached to the 5’ terminus of MA is buried inside the Gag monomer and subsequent to multimerization the myristyl moiety is exposed, resulting in an increased affinity for the cell membrane. Cyclophilin A has been shown to facilitate virion uncoating and is predicted to do this by intercalating in between the consecutive capsid domains to loosen CA-CA interactions (Franke *et al*., 1994; Thali *et al*., 1994). The C-terminal domain of CA contains the conserved 20 amino acid major homology region (MHR). Mutations at the N-terminus of MHR prevent virus replication, while virions are still assembled and released at levels comparable to wild-type Gag (Melamed *et al*., 2004; Abdurahman *et al*., 2004; Dorfman *et al*., 1994; Mammano *et al*., 1994).

Virus assembly and release on the other hand are significantly affected by mutations that lie within or at the C-terminus of the MHR, resulting in a decrease in particle yields (Abdurahman *et al*., 2004; Chiu *et al*., 2002; Furata *et al*., 2002; Chen *et al*., 1997; Huang *et al*., 1997; Dorfman *et al*., 1994(b); Mammano *et al*., 1994; von Poblotzki *et al*., 1993). The C-terminal of MHR of CA is shown to contain 2 highly conserved sequences present in HIV-1, HIV -2 and SIV (Zhang *et al*., 1996, Myers *et al*., 1991). (sequences: ALGpGATLEE and CQGVGGpG) and mutations here have shown to inhibit virus replication by more than 90% (SU *et al*., 2000). Mutational analysis of individual amino acids occurring within these conserved regions emphasize their importance for proper virion assembly, release and maturation from transfected HeLa cells (Abdurahman *et al*., 2004). Most mutations in this experiment did not affect protein synthesis but did impair virion assembly and infectivity. The exact correlates of proper virion assembly, release and infectivity are not fully determined as results in the above-mentioned study and others appear cell-line specific (Parker and Hunter., 2000; Lee *et al*., 1998; Wang *et al*., 1998; Sakuragi *et al*., 1995).

1.5.1.3 NC functions

The nucleocapsid domain contains several structural elements crucial for the dimerization and encapsidation of viral genomic RNA. The NC domain is found to be tightly associated with viral RNA in the virion core (Aldovini *et al*., 1990; Berkowitz *et al*., 1993; Clavel *et al*., 1990; Gorelick *et al*., 1990; Zhang *et al*., 1995). As will be discussed later, the ability to coordinate RNA dimerization and encapsidation is enabled by the presence of two Zn finger-like motifs within NC, which are flanked by highly basic domains. Research has also suggested that the NC domain may play a role as a virion assembly determinant and be crucial for the release of assembled virus particles from the infected cell (Cimarelli *et al*., 2000; De Guzman *et al*., 1998; Dawson *et al*., 1998; Zhang *et al*., 1997; Krausslich *et al*., 1995; Carriere *et al*., 1995; Campbell *et al*., 1995; Wang *et al*., 1998). Data from these studies have shown that basic residues distributed throughout NC contribute to virion assembly by promoting interaction between Gag molecules.

1.5.2 RNA Encapsidation

During assembly, HIV encapsidates 2 copies of the viral genomic RNA displaying the HIV packaging signal. Encapsidation is a highly selective process facilitated primarily by the HIV Gag-NC, which selectively encapsidates viral genomic RNA over the entire pool of cellular RNAs, including spliced viral RNA and various cellular RNAs (Zhang *et al*., 1995; Aldovini *et al*., 1990; Clever *et al*., 1995; Dannull *et al*., 1994; Berkowitz *et al*., 1994; Berkowitz *et al*., 1995; Luban *et al*., 1991; Dorfman *et al*., 1993). RNA recognition involves an interaction between the NC domain of the Gag precursor with specific stem–loop domains found within the 5' LTR up to the beginning of the Gag open reading frame of the genomic RNA (Figure 1.5). As previously mentioned, the two Zn-finger like structural domains within NC along with the flanking basic amino acid residues (4-5 amino acids) are crucial for viral genomic RNA encapsidation (Dannull *et al*., 1994; Gorelick *et al*., 1993; 1996; Poon *et al*., 1996; Schmalzbauer *et al*., 1996; Zhang *et al*., 1995; Berkowitz *et al*., 1993; 1994; 1995). Deletions within the highly conserved Zn

finger-like domains and basic residues have been shown to cause defects in RNA binding activity (RNA encapsidation) while having a negligible effect on virion assembly (Ott *et al*., 2005; Alfadhli *et al*., 2005; Wang *et al*., 2003; Dawson *et al*., 1998; Cimarelli *et al*., 2000; Zhang *et al*., 1995, 1997; Dorfman *et al*., 1993; Schmalzbauer *et al*., 1996; Poon *et al*., 1996; Berkowitz *et al*., 1995; Dupraz *et al*., 1992). The basic amino acid residues flanking the first of the 2 Zn fingers were shown to play a crucial role in selective packaging of genomic viral RNA (Schmalzbauer *et al*., 1996). The aforementioned study also showed that the basic residues flanking the first Zn finger vary in their significance in terms of RNA packaging. This was noted when a single mutation (R7-N) reduced the RNA binding affinity more than any other mutation. While at the C-terminus of the first Zn finger, 2 of the 4 basic amino acid residues (R-32 and K-33) caused the most significant reduction in RNA binding affinity. The authors also noted that the identified R-7, R-32 and K-33 residues also constitute known RNA binding motifs, which resemble RxxRR or RxxRK. Other than their importance in RNA packaging, mutations of the basic residues flanking the Zn fingers of NC has resulted in several assembly defects not commonly observed in Zn finger mutants (Poon *et al*., 1996; Cimarelli *et al*., 2000; Dorfman *et al*., 1993; Bowzard *et al*., 1998; Dawson *et al*., 1998; Wang *et al*., 1998; Wang *et al*., 2002).

The *cis*-acting domain within retroviral genomic viral RNA responsible for specific viral RNA recognition and encapsidation is known as the Psi (ψ) site, which is located within the 5' long terminal repeat (Ltr) up to the beginning of the Gag open reading frame (Figure 1.5. nucleotides 230-352) (Berkowitz *et al*., 1993, 1994; McBride *et al*., 1996; Clever *et al*., 1995; Linial *et al*., 1990; Aldovini *et al*., 1990; Luban *et al*., 1994). Structural predictions reveal a system of 4 stem-loop (SL) secondary structures comprising the ψ site (SL1, SL2, SL3, SL4) (Clever *et al*., 1995; Baudin *et al*., 1993; Harrison *et al*., 1992). *In vitro* studies have shown that the stem loop secondary structures are functional in mediating the binding of synthetic RNAs (Berkowitz *et al*., 1994; Berkowitz *et al*., 1993; Clever *et al*., 1995; McBride *et al*., 1996).

Mutational analysis of individual stem loop structures only marginally affected viral genomic RNA encapsidation (McBride *et al*., 1996). An SL3 mutation on the other hand resulted in an increased encapsidation of spliced viral RNA, which may indicate a role in distinguishing between spliced and unspliced viral RNA. An SL1+SL3 double mutant (deletion or base substitution) significantly impaired viral genomic RNA encapsidation. In particular this study highlighted the presence of two important structural elements in the ψ element (SL1 and SL3), one of which needs to be present to ensure viral RNA encapsidation and suggests a co-operative relationship to enable efficient encapsidation.

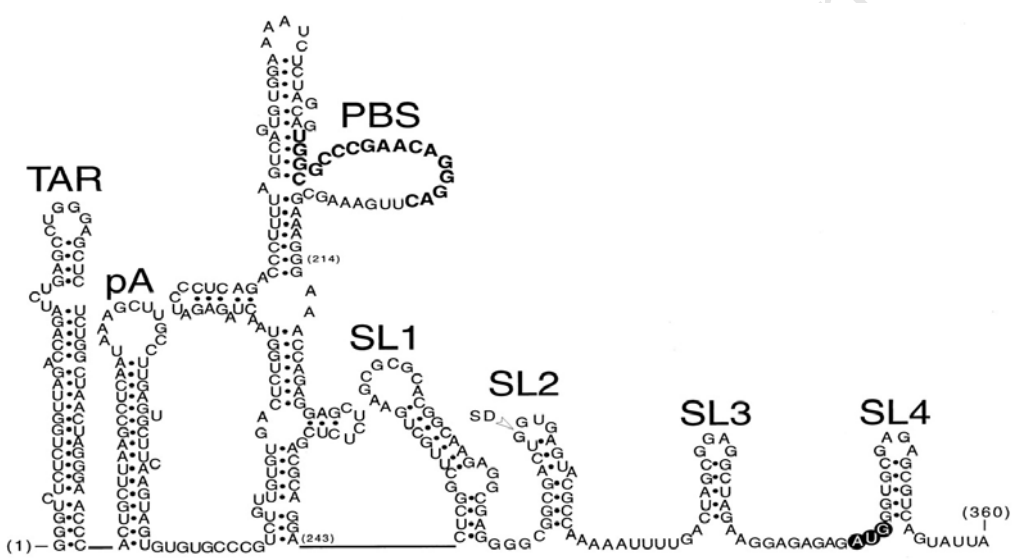


Figure 1.5 The secondary stem-looped RNA structure at the 5' end of the HIV genome – nucleotides 1 to 360. (Clever *et al*., 1999). pA: Poly(A) hairpin, PBS: Primer binding site, SL: Stem-loop.

This finding is in agreement with other studies that have also demonstrated the importance of SL3 for RNA encapsidation (Aldovini *et al*., 1990; Berkowitz *et al*., 1996; Clever *et al*., 1995; 2000; Harrison *et al*., 1998; Luban *et al*., 1994). Thus, since the major splice donor site resides within SL2 (in-between SL1 and SL3), this would explain why primarily unspliced viral genomic RNA is selectively packaged into virus particles. In spite of the severe reduction in RNA encapsidation resulting from SL1 and SL3 dual mutations, encapsidation was still observed and therefore these stem-loop structures are not considered to be solely responsible for optimal viral RNA encapsidation in wt

HIV. Additional research has implicated domains within the C-terminus domain of Gag for packaging and specificity (P1, P2 and P6 domains) of viral genomic RNA (Zhang *et al*., 1997; Luban *et al*., 1994; Parolin *et al*., 1994; Kim *et al*., 1994).

The 2 copies of the viral genome that are packaged within the assembled virion are non-covalently associated in a parallel orientation (Freed *et al*., 1998). SL1 has also been shown to be indispensable for the RNA dimerization process (Marquet *et al*., 1994; Muriaux *et al*., 1996; Russell *et al*., 2002; 2004; Paillart *et al*., 1994; Skripkin *et al*., 1994; Clever *et al*., 1996; Laughrea *et al*., 1994). This sequence is referred to as the dimerization initiation site (DIS), which via a 6-nucleotide palindromic sequence within SL1 can initiate dimerization through a kissing loop mechanism by complimentary base pairing with another viral genomic RNA (Skripkin *et al*., 1994; Clever *et al*., 1996; Laughrea *et al*., 1996; Muriaux *et al*., 1996; Paillart *et al*., 1996; Russell *et al*., 2002). More recent research though, has also implicated the involvement of the SL3 sequence as well as a downstream GA-rich sequence (nt 325-336) in RNA dimerization and encapsidation (Russell *et al*., 2003; Zhang *et al*., 1997). As observed previously the overall secondary structure rather than the primary sequence of these stem-loop structures appears paramount in maintaining functional integrity.

Researchers have suggested a relationship between the viral RNA dimerization, RNA encapsidation and virus particle assembly. It has been demonstrated that the HIV-1 UTR can assume two mutually exclusive conformations that effectively regulate RNA dimerization by varying the display of the DIS hairpin (Huthoff *et al*., 2001; Abbink *et al*., 2003; Ooms *et al*., 2004; Nikolaitchik *et al*., 2006). Specifically, the 2 conformations are the dimerization-incompetent long-distance interaction (LDI) conformation, where the DIS is masked by an upstream poly-A domain and a branched multiple hairpin (BMH) conformation which exposes the DIS hairpin to enable dimerization. These related studies revealed a direct correlation between the status of the DIS conformation with RNA dimerization and consequently virus particle assembly.

Most studies have focussed on the psi (ψ) site as the *cis*-acting functional region for packaging of viral genomic RNA. Several studies though, have shown that the process of optimal encapsidation involves several other elements that map throughout the 5'-end of the genomic RNA including the stem-loop structures within the ψ site (Berkowitz *et al*., 1995; Kaye *et al*., 1995; Helga-Maria *et al*., 1999; Kim *et al*., 1994; Luban *et al*., 1994; McBride *et al*., 1996; 1997; Richardson *et al*., 1993). Other regions identified to be crucial for RNA packaging include the HIV TAR element as well as the poly-A stem loop structure (Clever *et al*., 1999; Das *et al*., 1997; McBride *et al*., 1997) The TAR element is a 57-nucleotide sequence that forms a stem-loop structure at both ends of the viral genome and regulates transcription through its interaction with the HIV Tat protein (Dayton *et al*., 1986; Jeang *et al*., 1998; Jones *et al*., 1997). In particular, compensatory double mutations have indicated that the structure rather than specific sequence of the TAR element stem loop and the neighbouring poly-A stem loop was found to be the most crucial for RNA packaging (Clever *et al*., 1999; Helga-Maria *et al*., 1999). As expected, a series of missense mutations throughout the TAR structure resulted in striking effects on RNA packaging. The mutations that caused the most striking effects by hampering RNA packaging resided in the lower portion of the TAR stem and not in the looped region.

Therefore the packaging signal of HIV-1 genomic RNA has been shown to consist of several hairpin structures residing on either side of the multiple splice donor site. It has also been shown that the primary sequence of these sites is not as crucial as the overall secondary structure and physiochemical properties for RNA encapsidation. Helga-Maria *et al*. (1999) have suggested that each identified packaging element could be a unique domain for the binding of a host or viral factor that somehow mediates the encapsidation. An alternative suggestion is that not all elements are involved in binding. Instead they may only provide the correct structural context for RNA folding enabling the exposure of the actual packaging elements.

1.5.2.1 Encapsidation of random RNAs

As discussed, HIV incorporates several highly selective mechanisms to ensure the encapsidation of genomic RNA. It selectively encapsidates the ψ site containing viral genomic RNA. Almost exclusive encapsidation of HIV full length RNA is accomplished despite evidence that it may only constitute less than 1% of the total cellular mRNA in an infected cell (Berkowitz *et al*., 1996; Swanstrom *et al*., 1997). To elucidate a mechanism by which HIV Gag encapsidation overcomes this hurdle, researchers have proposed a *cis* packaging model. This model proposes that during Gag translation from genomic RNA, expressed Gag will encapsidate its cognate RNA and no second order function is required to bring Gag and RNA together. The *cis* packaging model was investigated by Poon *et al*. (2002) using *in vivo* packaging experiments for wild type and full length mutants that do not express Gag. It was shown that even though the mutant RNA was encapsidated, the wild type RNA was preferentially packaged even in the presence of excessive levels of competitor RNA. This study therefore verified that the *cis* packaging model for selective packaging of Gag encoding viral RNA does occur, and the linkage of this packaging mechanism with ψ site recognition by Gag NC could provide an enhanced degree of specificity.

Although the *cis* model has been verified by the above study, the authors still acknowledge that a *trans* packaging model in which RNA functions solely to produce viral proteins, or serves as genomic RNA for packaging could still occur. This study therefore also suggests that to selectively target a particular RNA for encapsidation, the process would be largely independent of the cellular levels of this RNA species. For the *cis* packaging model to be plausible it would suggest that mutant Gag unable to bind RNA, and similarly, mutant RNAs unable to express Gag, should be unable to generate infectious virus particles and would not be rescued by wt-Gag.

Several studies have, however, demonstrated several strong lines of evidence to the contrary (*Ott et al*., 2005; Nikolaitchik *et al*., 2006). These studies

findings contrasts with those of Muriaux *et al*. (2002) (discussed earlier) who analysed MuLV Gag expression from SFV-derived vectors. Here it was noted that the vector derived RNA formed the predominant species within infected cells, which despite lacking the ψ site, was encapsidated by virions. Other studies have also shown that Gag mutants possessing mutations in the NC-zinc finger, CA or MA domains were still able to produce infectious virus particles, and dual mutations that prevented assembly could be rescued in the presence of wt Gag in which recombination occurred (*Ott et al*., 2005; Nikolaitchik *et al*., 2006). The study by Nikolaitchik *et al*., (2006), mutations abolishing RNA translation by Gag AUG-mutation had no effect on the packaging of this RNA species and these RNAs frequently recombined with wt viruses. The contradictory nature of the above research results suggests that both *trans* and *cis* RNA packaging models more than likely occur.

These studies dealt with non-specific packaging and suggest that HIV VLPs produced in insect cell tissue culture expression systems should contain significant levels of insect cell-derived RNA species irrespective of the absence of the HIV ψ site. The incorporation of viral RNA (spliced or unspliced) has also been shown not to be a prerequisite for virion assembly and release as NC-deleted Gag or ψ site-deleted RNA still produce viral particles (Ott *et al*., 2005; Wang *et al*., 2003; Muriaux *et al*., 2001; Muriaux *et al*., 2002; Zhang *et al*., 1997; Aldovini *et al*., 1990; Clavel *et al*., 1990). In spite of this, *in vitro* studies have shown that Gag protein assembly occurs more efficiently in the presence of RNA and it has been suggested that RNA is required for particle assembly and may play a structural role in assembly as it may serve as a scaffold upon which multiple Gag molecules can assemble (Ott *et al*., 2005; Wang *et al*., 2002; Cimarelli *et al*., 2000; Burniston *et al*., 1999; Campbell *et al*., 1995; Gross *et al*., 1997; Campbell *et al*., 1995; 1999; Ganser *et al*., 1999; Zuber *et al*., 2000). Various studies have also shown that, other than the viral genomic RNA, HIV particles also encapsidate the various cellular RNAs (Muriaux *et al*., 2001; Berkowitz *et al*., 1996; Darlix *et al*., 1995). This non-specific RNA binding has also been shown to be mediated by the basic residues distributed throughout the NC domain of Gag (Prats *et al*., 1991; Schmalzbauer *et al*., 1996; Poon *et al*., 1996; Cimarelli *et al*., 2000)**.**

The indiscriminate packaging of RNA to enable virion assembly was further demonstrated in a study conducted by Ott *et al*,. (2005). This study showed that virion assembly occurred despite the absence of the NC domain in Gag and hence the inability to specifically package viral RNA. Double Gag mutants on the contrary, that possessed mutations in the MA RNA binding domains as well the NC were unable to package RNA and hence were unable assemble into particles. Either mutation on their own however had no effect on virion assembly and revealed a redundant role for the RNA binding domains in both NC and MA. Double mutant assembly could be rescued though in the presence of WT Gag or single MA or NC mutants. The requirement for Gag RNA binding to facilitate Gag-Gag interaction was also highlighted in this study when it was demonstrated that a leucine zipper domain that enables tetramerization, inserted into the deleted NC domain could enable virion assembly. A study conducted by Alfadhli *et al*., (2005) yielded similar results and proposed the following assembly pathway: Gag protein pairing through NC-RNA interactions enables RNA dimerization; this conformational change converts assembly-restricted domains to assembly-competent domains; final assembly occurs through a set of larger intermediates. Specifically, this study also demonstrated that the virion assembly function provided by NC RNA binding domains could be substituted by peptide domains that dimerize/multimerize.

In view of these results Muriaux *et al*. (2001, 2002) also noted that ψ-deleted retroviral particles (MuLV) not encapsidating genomic viral RNA, efficiently compensated for this by packaging increased amounts of various other cellular RNAs. It was also found that both wild-type and ψ-deleted particles encapsidated roughly similar levels of RNA be it viral-genomic or cell-derived. It is also important to note that even wild-type particles contained significant amounts of cellular RNAs although only 25-50% of what was contained by the ψ-deleted particles. This adds to the idea that RNA encapsidation by the retroviral virion retains a certain level of non-specificity. RNase treatment of either the ψ-deleted or wild type virion cores resulted in the disassembly of virions into a soluble form. Similar results have been observed in a previous

study in which CA-NC complexes treated with RNase did not assemble into virions, indicating a role for Gag-RNA (cellular or viral) interactions in maintaining particle structure (Campbell *et al*., 1995). Muriaux *et al*. (2001) therefore proposed that viral particles lacking genomic RNA use other, cell-derived RNAs as scaffolding.

A follow-up study was later conducted to determine the content and role of RNA in retroviral particles (MuLV) that lack viral genomic RNA as a result of mutations in the Gag NC domain (Muriaux *et al* 2002). Similar to the ψ element mutants, the NC mutant particles contained comparative levels of RNA to wild-type particles, but in this circumstance the viral genomic RNA was compensated for by the incorporation of rRNA, which has also been observed in other studies (Wang *et al*., 2002; Berkowitz *et al*., 1995; Poon *et al*., 1998; Zhang *et al*., 1995). NC mutant virus-like particles were found to be highly unstable, some of which were solubilized even before RNase treatment. The marked increase in VLP instability and sensitivity observed in NC mutant particles compared to ψ-deleted mutants could be accounted for by the increased encapsidation of rRNA and the corresponding decrease of mRNA incorporation into VLP. Results of this study therefore indicated that the basic regions on the N-terminal side of the Zn-finger (R16-R23) including residues on the C-terminal side of R23 of NC are critical for virion core stability and might participate in Gag-Gag or Gag-RNA interactions to maintain the structure of wild-type Gag VLP. It has also been suggested that NC may be responsible for discriminating between the incorporation of mRNA and rRNA into VLP for scaffold purposes.

The VLP instability defects resulting from mutation of the above-mentioned NC basic amino acids is corroborated in a study conducted by Wang *et al*. (2002). As expected, the mutation of 10-13 basic amino acids by replacement with alanine residues prevented viral genomic RNA packaging while not affecting Gag virion assembly at the cell membrane. Despite this, it was found that only 10% of the Gag protein found in the supernatant sedimented at the appropriate density for a retroviral particle while the other 90% sedimented as monomeric Gag. As in the Muriaux *et al*. (2001) study, mutation of the NC

positively charged residues adversely affected both specific and non-specific RNA binding and a direct correlation was observed between the percentage of Gag sedimented as particles and the amount of incorporated RNA. From this study it was concluded that RNA binding by Gag (cellular or viral) appears more critical to maintaining VLP integrity after cell membrane assembly and release, as opposed to being required on a temporal basis for assembly. RNA therefore appears to play a role more than suggested by a scaffolding label, which insinuates a temporal function and indicates that it could be removed following assembly. This is contrary to results of RNase treatment studies of VLPs.

This hypothesis was previously investigated in a study conducted by Cimarelli *et al*. (2000), who investigated the requirement for non-specific nucleic acid binding during Gag-Gag interaction and particle assembly by using a panel of HIV-1 NC mutants in which 2-10 basic amino acids were replaced with alanine. Results of this study demonstrated that NC basic residues mediate both specific and non-specific RNA recognition and encapsidation *in vitro*, which corroborates other studies (Wang *et al*., 2002; Schmalzbauer *et al*., 1996; Lapadat-Tapolsky *et al*., 1993; De Rocquigny *et al*., 1992). Also, the number of mutated basic residues correlated with the magnitude of defects in virion assembly. In contrast, mutations within the Zn finger-like domains had no effect on non-specific RNA encapsidation or virion assembly. These and other results have lead to the proposal of two arguments to elucidate the function of RNA during virion assembly. The first, postulated that RNA acted as a scaffold for Gag multimerization, where binding of Gag would drive the accumulation of Gag and thus create an environment which would drive virion assembly (Cimarelli *et al*., 2000). The other hypothesis describes the function of RNA in neutralizing the positively charged basic residues within NC, hence neutralizing repulsion between Gag monomers and thus permitting protein-protein contacts to initiate virion assembly (Burniston *et al*., 1999; Gamble *et al*., 1997).

Although these expression systems have provided invaluable details of HIV virion assembly determinants, the exact determinants (required Gag domains)

appear to differ depending on the exact expression system used (Abdurahman *et al*., 2004; Parker and Hunter., 2000; Lee *et al*., 1998; Wang *et al*., 1998; Sakuragi *et al*., 1995).

1.6 Immune responses stimulated by Gag VLP

HIV/SIV Gag VLPs have been shown to be potent stimulators of both cellular and humoral immune responses. They take advantage of the fact that the immune system responds well to particulate antigens that emulate infectious virus particles compared to their soluble component peptides. To date, several studies have documented the effectiveness of HIV Gag VLPs produced in various expression systems as vaccines (Table 1.1). These studies have investigated various aspects of vaccinology including using alternate routes and mechanisms of VLP administration and VLP formulations (adjuvants). They have also varied the VLP administration protocol (prime/boost) and evaluated the ability of VLPs to stimulate the required arms of an immune response to mount an effective defence against an HIV infection.

1.6.1 Site of vaccine administration

The mucosal surfaces that are prominent in gastrointestinal, urogenital and respiratory tracts are the most common routes of viral acquisition. The mucosal surfaces serve as the first barrier of defence against invading pathogens and include the physical barriers (mucous and epithelium), innate immune mechanisms (inflammation, complement) as well as adaptive immune responses (humoral and cellular memory immune responses). To mount an effective immune response against HIV infection it is desirable to stimulate a disseminated mucosal immune response. In addition, protective systemic (cellular/humoral) immune responses would also be desired to complete the barrier of protection against HIV infection (Table 1.1; Buonaguro *et al*., 2005; Ogg *et al*., 1998; Mascola *et al*., 1999; Koup *et al*., 1994; Bruck *et al*., 1994; Baba *et al*., 2000).

The site of vaccine administration is paramount for stimulating a particular type of immune response, be it mucosal or systemic. Parenteral (intramuscular (i.m.), intravenous (i.v.)) immunization of VLP regularly induces

Table 1.1 Summarised results of VLP based immunogenicity studies

	Immunogen	Expression system	Adjuvant	Animal model	Antibody response		Neutralisation	CTL response	Reference
					Serum	Mucosal			
1	HIV GagVLP+env (TM domain Epstein Bar virus)	Baculovirus/insect cell	N/A	New Zealand White rabbits i.m.	Strong	N/A	Strong	N/A	Deml *et al*. (1997)
			N/A	BALB/c mice i.p., s.c., i.v.	N/A	N/A	N/A	Strong (vs multiple epitopes)	
2	HIV GagVLP+env (TM domain Epstein Bar virus)	Baculovirus/insect cell	N/A	Rhesus macaques i.m.	Strong	N/A	Weak	Weak (limited cross reactivity)	Wagner *et al*. (1998)
	SIV GagVLP	N/A	Cholera toxin	Rhesus macaques i.n.	N/A	Strong (enhanced by adjuvant)	N/A	Weak	Imaoka *et al*. (1998)
	HIV GagVLP	Baculovirus/insect cell	N/A	Rhesus macaques i.m.	N/A	N/A	N/A	Strong	Paliard *et al*. (2000)
3	HIV GagVLP+env R5 primary isolate HIV-1Bx08	Vero cell transfection	QS21	Rhesus macaques i.m.	Moderate	N/A	Weak (strain specific)	Weak	Montefiori *et al*. (2001)
4	SIV GagVLP +SIV env	Baculovirus/insect cell	Cholera toxin	BALB/c mice i.p.	Strong (enhanced by adjuvant)	Weak but (enhanced by adjuvant)	Strong (enhanced by adjuvant)	Strong (enhanced by adjuvant)	Yao *et al*. (2002)
				+ i.n.	Weak (enhanced by adjuvant)	Moderate (enhanced by adjuvant)	Weak (enhanced by adjuvant)	Weak (enhanced by adjuvant)	
5	Gp120-depleted inactivated HIV particles	N/A	CpG-ODN	BALB/c mice i.n.	Strong (enhanced by adjuvant)	Strong (enhanced by adjuvant)	Strong (enhanced by adjuvant)	N/A	Dumais *et al*. (2002)
6	HIV GagVLP+VSVG env	DNA vaccine	N/A	BALB/c mice	N/A	N/A	N/A	Strong	Marsac *et al*. (2002)
7	SHIV GagVLP	Baculovirus/insect cell	Cholera toxin	BALB/c mice i.n.	Strong (enhanced by adjuvant)	Moderate to Strong (enhanced by adjuvant) when i.n. not by i.p.	Strong	Moderate- (enhanced by adjuvant by i.n but i.p better response even without adjuvant	Yao *et al*. (2003)
8	SIV GagVLP +SIV env	Baculovirus/insect cell	Rantes	BALB/c mice i.n.	Moderate (enhanced by adjuvant)	Moderate (enhanced by adjuvant)	Strong (enhanced by adjuvant)	Strong (enhanced by adjuvant)	Kang *et al*. (2003)
			CpG-ODN						
			Cholera toxin						
9	HIV GagVLP	Yeast	N/A	*in vitro* DC uptake analysis	N/A	N/A	N/A	Strong	Tsunetsuga *et al*. (2003)
10	HIV-1 Gag DNA + VLP	DNA prime/ Baculovirus VLP boost	N/A	DNA i.m. / VLP i.p.	N/A	N/A	N/A	Strong (enhanced by protein boost)	Jaffray *et al*. (2004)
11	HIV GagVLP+ HIV-1A env	Baculovirus/insect cell	N/A	BALB/c mice i.p.	Strong	Moderate	Moderate	Strong	Buonaguro *et al*. (2005)
				i.n.	Moderate	Moderate	Moderate	Moderate	
				oral	Weak	Weak	N/A	No response	
12	HIV GagVLP+ HIV-1A env	Baculovirus/insect cell	N/A	*In vitro* immature MDDC uptake analysis	N/A	N/A	N/A	Strong	Buonaguro *et al*. (2006)
13	HIV GagVLP+ VSVG env	293T-cell	N/A	Rhesus macaques i.m.	Strong	Strong	Strong	Strong	Kuate *et al*. (2006)
14	HIV GagVLP+MLV env	DNA vaccine	N/A	BALB/c mice i.v., s.c.	N/A	N/A	Strong	Strong	Bellier *et al*. (2006)
15	HIV-1 A, B, C, D, E env DNA + soluble env	DNA prime/Protein boost	N/A	Human i.m.	Strong	N/A	Moderate- weak	Moderate- strong (enhanced by protein boost)	Wang *et al*. (2008)

N/A: not applicable; i.n.: intranasal; i.m.: intramuscular; i.v.: intravenous; s.c.: subcutaneous; i.p.: intraperitoneal; MDDC: monocyte derived dendritic cell

strong systemic immunogenicity although very often no mucosal immune responses are observed. Several studies have revealed that mucosal immunization (intranasal (i.n.); oral) can effectively induce systemic as well as mucosal immune responses at sites of inoculation as well as at distal mucosal surfaces at moderate levels (Table 1.1; Kang *et al*., 2003; Yao *et al*., 2002). This is highlighted in a study conducted by Buonaguro *et al*. (2005), where HIV VLPs pseudotyped with gp120 derived from a Ugandan clade-A isolate were administered to mice using multiple routes. Both intraperitoneal (i.p.) and i.n. administration stimulated systemic as well as mucosal IgA and IgG responses. Immune sera exhibited more than 50% neutralization of autologous and heterologous primary isolates. Oral VLP administration failed to induce systemic or mucosal immune responses. While both i.p. and i.n. VLP administration stimulated specific CTL activity, i.n. administration stimulated this response at a lower efficiency. Intranasal VLP administration induced specific IgA though, which could not be accomplished by i.p. administration. This VLP based vaccine was therefore able to stimulate both arms of the immune response and results from this study serve as an ideal example and motivation for delivery of proposed vaccines by multiple routes. Noteworthy observations were also made in several studies in which nasal as opposed to oral mucosal vaccine administration was able to stimulate IgA responses in a broader range of mucosal tissues (Table 1.1: 2; 4; 5; 7; 8; 11). The precise mechanism and site of vaccine administration therefore plays an important role in priming appropriate parts of the immune system.

1.6.2 Plasmo-VLPs

The production of VLPs can be a time-consuming and expensive process, which has motivated the development of plasmo-VLP vaccines. Plasmo-VLPs are DNA vaccines that encode proteins which subsequently form VLPs in an inoculated individual. This process thus only requires DNA production (Akahata *et al*., 2005; Bellier *et al*., 2006; Young *et al*., 2006). The plasmo-VLP strategy takes advantage of endogenous production and particulate nature of the antigen in stimulating CD8+ and CD4+ T- and B-cell responses. Bellier *et al*. (2006) produced a MLV Gag VLPs pseudotyped with MLV Env from plasmid DNA, which were able to stimulate strong specific CTL

responses in mice and protected them from a lethal virus challenge. The production of VLPs from this DNA vaccine was shown to induce an immune response that was 10^6-fold more effective than the same vaccine producing non-particulate peptides.

1.6.3 Inoculation regime

Often peptide or DNA-based vaccines stimulate weak to moderate immune responses. Researchers have overcome this problem by making use of vaccine prime-boost regimes in which the animal/human model tested is initially primed with either a peptide-based/DNA vaccine/live attenuated vector and later boosted with another immunogen. This strategy has been used in several animal as well as human models and shown significantly enhanced immune responses compared to responses developed by immunogens used on their own (Table 1.1, Jaffray *et al*., 2004; Wang *et al*., 2008; Paliard *et al*., 2000; Montefiori *et al*., 2001; Meyers *et al*., 2008; Chege *et al*., 2008).

1.6.4 Adjuvants

Studies have also made use of co-stimulatory molecules/adjuvants as either part of the vaccine formulation or as an attachment to the SIV/HIV chimeric particles (Table 1.1; Skountzou *et al*., 2007; Yao *et al*., 2002; Sailaja *et al*., 2007; Kang *et al*., 2003; Dumais *et al*., 2002). These co-stimulatory factors have included bacterial enterotoxins: Cholera toxin and the *E.coli* heat-labile toxin, Rantes, CpG oligodeoxynucleotides (ODN), the dendritic cell growth factor Flt3 ligand, granulocyte-macrophage colony-stimulating factor (GM-CSF) and CD40 ligand. Co-administration of either cholera toxin or the *E.coli* heat-labile toxin mucosal adjuvants is able to enhance serum as well as mucosal antibody responses (Table 1.1: 2; 4; 7; 8). Co-administration of cholera toxin with SIV VLPs in mice enhanced immunogenicity of the VLP and improved cellular and humoral immune responses systemically and at distal mucosal surfaces and sites of application compared to VLPs delivered to mice without adjuvant (Yao *et al*., 2002).

Despite promising results in mice, due to the toxic nature of the cholera toxin, alternate adjuvants have been investigated for use in humans. The innate immune system recognises a wide range of pathogen-associated molecular patterns (PAMPs), which it perceives as danger signals rather than specific epitopes as used by the adaptive immune response. As a result the innate immune responses occur more rapidly and are believed to provide time for an effective adaptive immune response (Levy, 2001). Stimulated cellular immune responses in response to PAMP recognition is mediated by transmembrane pattern recognition receptors (PRRs), which may include surface receptors on phagocytic cells and Toll-like receptors found on DCs and macrophages. PAMP recognition by these receptors upregulates co-stimulatory molecules (CD80/86), cytokines IL-12 and IL-18 and hence promotes the induction of Th-1 biased cellular and humoral immune responses (Deml *et al*., 2005).

Researchers have successfully manipulated these innate immune responses by delivering antigen as cocktails containing molecules with PAMPs to enhance responses. Kang *et al*. (2003) demonstrated that co-administration of either RANTES or CpG-ODN (common motifs in unmethylated bacterial nucleic acid recognised as danger signals by Toll-like receptors-9 signalling pathways) in mice was more effective at stimulating neutralizing antibody titres than cholera toxin. These co-administered factors also stimulated interferon-gamma and CTL responses in spleen and lymph nodes to equivalent levels observed for cholera toxin. RANTES and CPG-ODN were also able to enhance serum and mucosal antibody responses to SIV-env at levels equivalent to cholera toxin. Chimaeric SIV VLPs that display immunostimulatory molecules such as GM-CSF or the CD40 ligand have also been investigated as vaccines (Skountzou *et al*., 2007). Incorporation of these molecules into VLPs enhanced both humoral and cellular immune responses to levels greater than those observed when administering VLPs without any GM-CSF or CD40 ligand attached or VLPs administered with soluble equivalent of these molecules.

1.6.5 Influence of expression system

Expression systems used to produce the VLPs may also have a bearing on the immune response (see Baculovirus Gp64 pseudotyping 1.7.5 and 4.1.2). In particular, the expression systems appear to have an effect on innate immune responses. Yeast-derived VLPs have previously been shown to induce DC maturation, which was accompanied by IL-12 production (Tsunetsuga-Yokota *et al*., 2003). In this study the combination of VLP structure and yeast membrane components incorporated into the VLP could bias DCs to develop strong Th-1 responses and this was found to be mediated by TLR-2 signalling.

Insect cell based baculovirus expression systems are routinely used to produce VLPs (Table 1.1) as they allow for the production of high concentrations of heterologous proteins, amongst other reasons which will be discussed at a later point. VLP purification techniques which include gradient centrifugation to isolate VLPs are fairly crude and don't easily discriminate between heterologous VLP and baculovirus particles. The contaminating baculovirus particles are speculated to act as adjuvants as their nucleic acids may possess CpG motifs similar to bacteria, which are recognised as danger signals by Toll-like receptor-9 signalling pathways (Ludwig *et al*., 2007). These adjuvant properties of contaminating baculoviruses were corroborated in a study that demonstrated that baculoviruses enhanced the immune response to VLPs by directly maturing MDDCs via IFN-γ secretion. (Hervas-Stubbs *et al*., 2005). The observed immune response to the VLPs was significantly reduced when baculovirus contaminants were removed from the inoculum. Baculovirus based insect cell produced VLPs also display significant levels of the baculovirus Gp64 envelope glycoprotein. It is speculated that these envelope glycoproteins may direct fusion of the VLPs with the membranes of antigen presenting cells and hence be a major determinant with respect to the processing and presentation of VLP epitopes (Ludwig *et al*., 2005).

1.7 Baculovirus based insect cell expression systems

The taxonomic family *Baculoviridae* comprises a family of large enveloped rod-shaped viruses that are 30-60nm in diameter and 250-300nm in length (Figure 1.6) (O'Reilly *et al*., 1994). The rod-shaped capsids are composed of the major capsid protein VP39 and enclose a double stranded DNA genome (80-180 kbp). The enveloped capsids also display envelope glycoproteins. Baculoviruses can be divided into 2 genera: *Nucleopolyhedrovirus* (NPVs) and *Granulovirus* (Fauquet *et al*., 2008 (ICTV virus taxonomy); Theilmann *et al.,* 2005; Fields *et al*., 1996). Both NPVs and granuloviruses are known to be exclusively pathogenic to arthropods.

NPVs can produce 2 virion phenotypes during infection. These include the occluded-derived virus (OV) and the budded virus (BV) (O'Reilly *et al*., 1994; Fauquet *et al*., 2008 (ICTV virus taxonomy)). The OV phenotype is adapted for stability and allows transmission between insects via oral transmission. These particles assemble within the nucleus and become enveloped when budding through the nuclear membrane (Figure 1.6). OV are often engulfed within a crystalline protein matrix of polyhedrin. BV particles on the contrary are adapted for cell to cell transmission within an infected insect and thus facilitate systemic spread within the host. These BV particles assemble at the plasma membrane and acquire their envelope as well as any membrane associated factors (glycoproteins) when budding here.

1.7.1 Baculovirus replication

In tissue culture baculoviruses undergo an infection cycle of 3 phases: early; late and very late phases. These phases correspond to the "reprogramming" the cellular machinery to create a favourable environment for viral replication; production of BV and the OV. During the very late infection phases, during OV production in the nucleus, large arrays of fibrous material begin to accumulate. The fibrous material consists of the 10 kDa polypeptide known as p10, which is suggested to aid the lysis of infected cells during the late phases of infection (+/-72 h pi). During this very late infection phase polyhedrin is also

expressed. The AcMNPV strong very late phase polyhedrin and p10 Baculovirus promoters, have been manipulated for use as insect cell based expression vectors for the large scale production of heterologous proteins, subunit vaccines and VLPs in insect cell lines.

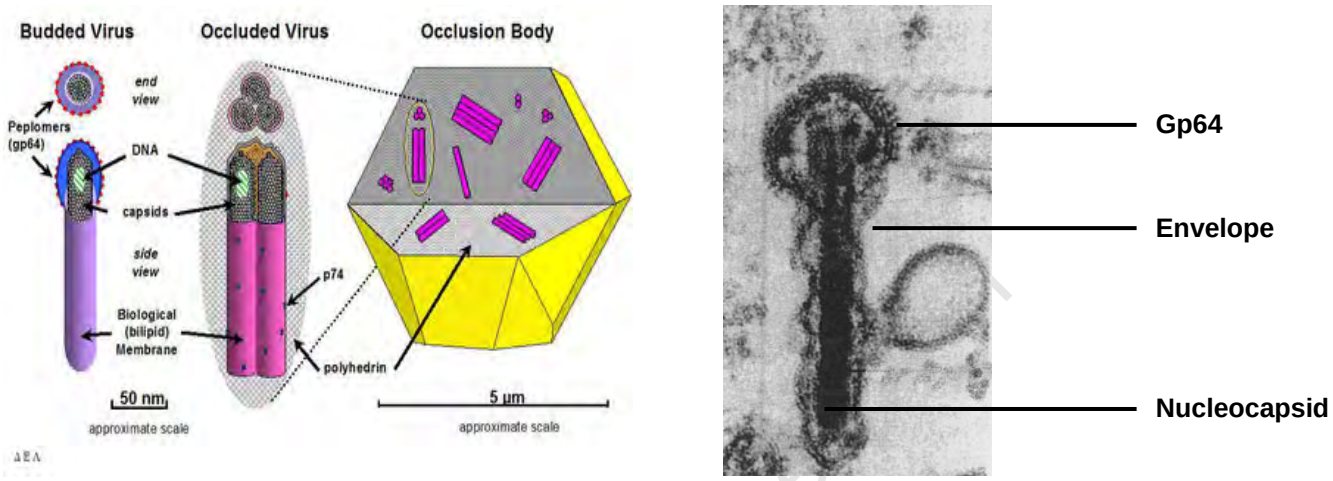


Figure 1.6 Illustrating various NPV phenotypes. (A) The virion components and sizes of OV and BV phenotypes (http://content.answers.com/main/content/wp/en/thumb/4/4a/428px-Nucleopolyhedrovirus. jpg); (B) Electron micrograph of budded form of a nuclear polyhedrosis virus. Rod shaped nucleocapsid is encapsidated by lipid bilayer derived from the host cell. The top end of the polar virion contains the Gp64 envelope glycoproteins anchored into the virion membrane (O'Reilly *et al*., 1994).

These types of expression systems have been very successful in producing foreign gene products as both polyhedrin and p10 genes are non-essential for replication in cell culture. As both polyhedrin and p10 expression occurs during the very late phase to enable occlusion, heterologous gene expression from their promoters should not interfere with BV production that occurs during the late phase. This would also therefore minimise any selective mutational pressure to delete or inactivate the transgene. Polyhedrin and p10 deletions result in an occlusion negative phenotype, and the resulting recombinant viruses are not very effective at infecting their arthropod hosts and would therefore not persist in the environment.

1.7.2 Baculovirus insect cell infection

The NPVs can be sub-divided into 2 groups. Group I NPVs such as the *Autographa californica* multiple nucleopolyhedroviruses (AcMNPV) have Gp64 as the major envelope glycoprotein. Group II NPVs lack a Gp64 homologue and instead possess unrelated fusion proteins collectively known as F-proteins (Pearson *et al*., 2000; Chen *et al*., 2001). Gp64 along with the VP39 major capsid protein comprises one of the most abundant proteins within the virion (Oomens *et al*., 1999).

Baculoviruses enter host cells by receptor mediated endocytosis, which is envelope glycoprotein dependent. Studies on AcMNPV in particular have shown that the assembled, disulphide-linked Gp64 trimers alone are sufficient to mediate cellular entry through low pH induced membrane fusion (Blissard *et al*., 1992; 1996; Oomens *et al*., 1995; Monsma *et al*., 1996; Markovic *et al*., 1998; Hefferon *et al*., 1999; Hefferon *et al*., 1999). This has been demonstrated in studies where antibodies directed against Gp64 envelope glycoproteins are capable of neutralizing BV infection (Zhou *et al*., 2006; Hohmann *et al*., 1983; Volkman *et al*., 1984; Keddie *et al*., 1985). Similarly, deletion of the AcMNPV Gp64 gene prevents the production of infectious virus (Monsma *et al*., 1996; Oomens *et al*., 1999).

Viruses utilise several entry pathways to enter target cells: these include phagocytosis, macropinocytosis, caveola-mediated endocytosis and clathrin mediated endocytosis. A study by Long *et al*. (2006) utilised various cellular entry inhibitors to identify possible paths of entry for baculovirus. The study revealed that baculoviruses chiefly enter host cells via the clathrin-mediated, pH-dependent endocytic pathway. Inhibition of caveola-mediated endocytosis on the contrary enhanced the transduction of virus particles. This observation was suggested to be a result of channelling more virus particles towards clathrin mediated endocytosis.

To mediate cellular entry, Gp64 utilises a mechanism similar to the influenza virus, which enters the cell by virion and endosome membrane fusion subsequent to endosomal acidification (Blissard *et al*., 1992; Leikina *et al*.,

1992; Monsma *et al*., 1995; Markovic *et al*., 1998; Kingsley *et al*., 1999; Plonsky *et al*., 1999; Long *et al*., 2006). Endosome acidification is crucial for mediating baculovirus cellular entry by membrane fusion as demonstrated by using inhibitors of endosome acidification (Blissard *et al*., 1992; Long *et al*., 2006). Tani *et al*. (2001) has demonstrated that an over-expression of Gp64 on the baculovirus surface has led to enhanced transduction efficiency. Group II baculoviruses that lack Gp64 were shown to be unable to transduce mammalian cells but this ability could be conferred by pseudotyping the baculovirus with Gp64 (Liang *et al*., 2005).

1.7.3 Baculovirus mammalian cell entry

Initially baculovirus transduction was assumed to be limited to insect cells. However a study by Hofmann *et al*., (1995) demonstrated first that a recombinant baculovirus carrying a reporter gene under the control of a CMV promoter was able to transduce human hepatocytes and express the reporter gene. Since then numerous studies have demonstrated the ability of recombinant baculoviruses to deliver and express foreign genes *in vivo* in mouse nasal epithelia, mouse skeletal muscle cells, rabbit endothelial cells, and *in vitro* in various mammalian cell lines including HeLa, Vero, BHK21, 293 T-cells, rat hepatocytes and human hemocytes (Boyce *et al*., 1996; Shoji *et al*., 1997; Condreay *et al*., 1999; Duisit *et al*., 1999; Airenne *et al*., 2000; Pieroni *et al*., 2001; Abe *et al*., 2003; Gao *et al*., 2007).

In spite of the efficient baculovirus transduction of mammalian cells, viral replication does not occur here and all vectors required the presence of promoters functional in mammalian cell lines to enable transgene expression. For this reason baculoviruses (AcMNPV) have recently been considered as attractive mammalian gene delivery vectors (Boyce *et al*., 1996; Delaney *et al*., 1998; Kost *et al*., 2002; Tani *et al*., 2003; Abe *et al*., 2003; Kitagawa *et al*., 2005; Changyong *et al*., 2005; Masayuki *et al*., 2006; Song *et al*., 2006; Gao *et al*., 2007). Intranasal inoculation with recombinant AcMNPV has shown to induce strong immune responses protecting mice from lethal challenges of influenza A or B virus (Abe *et al*., 2003). Related technologies have also led to the development of novel baculovirus shuttle vectors. In concert with the

Gp64 envelope glycoprotein, foreign promoters like the white spot syndrome virus (WSSV) ie1 promoter have enabled recombinant baculovirus vectors to attain high levels of reporter gene expression in both insect and mammalian cell lines (Gao *et al*., 2007). These studies have all demonstrated that Gp64 on the surface of AcMNPV is essential for transduction of mammalian cells and the baculovirus vectors here entered mammalian cells by means of the same pH-dependent entry pathway used during insect cell infection.

Not all mammalian cell lines are permissive to baculovirus transduction: these include haematopoietic and human T-cell lines. Permissivity would infer susceptibility to recombinant baculovirus uptake and gene expression. Permissive (Huh-7 and B16) and non-permissive haematopoietic (RAW 264.7 and YAC-1) mouse cell lines have been investigated to establish mechanisms by which baculovirus transduction is prevented (Schauber *et al*., 2004; Masayuki *et al*., 2006). Results from this study demonstrated that baculovirus entered both permissive and non-permissive cell lines, while only permissive cell lines were transduced (i.e. the expression cassette was expressed). Viral DNA was detectable in the permissive cell lines for up to 96 hours, while only detectable for up to 48 hours in non-permissive cell lines. This suggests that permissive cell lines take up DNA more easily or viral DNA degrades slower.

1.7.4 Pseudotyping baculovirus particles

The vesicular stomatitus virus-G envelope glycoprotein (VSV-G) is often used to pseudotype lentiviral particles used as vector delivery vehicles because of its broad tropism and the ability to confer stability and infectivity to virus particles (Burns *et al*., 1993; Yee *et al*., 1994; Kitagawa *et al*., 2005).

Several groups have reported improved transduction efficiency in a variety of mammalian and insect cell lines infected with recombinant baculoviruses expressing foreign viral envelope proteins such as VSV-G (Barsoum *et al*., 1997; Tani *et al*., 2001; 2003; Mangor *et al*., 2001; Facciabene *et al*., 2004; Kitagawa *et al*., 2005; Matilainen *et al*., 2006; Ernst *et al*., 2006; Zhou *et al*., 2008). VSV-G can also complement Gp64-null baculoviruses, which suggests that Gp64 and VSV-G are functionally analogous (Mangor *et al*., 2001; Zhou

et al., 2008). Using VSV-G to pseudotype heterologous virus particles does pose certain problems, which include the fact that it can cause cell death as a result of rapid syncytium formation (Ory *et al*., 1996). Also, because of its broad tropism it is able to transduce antigen presenting cells, which could potentially result in a deleterious immune response (Liu *et al*., 1996; DePolo *et al*., 2000). As a result of these undesirable effects when pseudotyping with VSV-G, alternate envelope glycoproteins have been investigated. Gp64 has been proposed as a potential envelope glycoprotein to pseudotype lentiviral particles (Kumar *et al*., 2003; Schauber *et al*., 2004; Oomens *et al*., 2004). Gp64 has demonstrated an ability to functionally complement heterologous virus membrane glycoproteins (HIV; MLV; HSRV; FIV) and has shown to be very effective at mediating uptake of lentiviral particles into target cells. Upon further analysis, the Gp64 mediated virus entry by the established pH-directed membrane fusion pathway, which was also prevented by anti-Gp64 antibodies.

1.7.5 Gp64 Pseudotyping heterologous viral vectors

Studies by Kumar *et al*. (2003) and Schauber *et al*. (2004) demonstrated that HIV based vectors pseudotyped with Gp64 were able to successfully transduce cells of hepatic origin, 293 T-cells, HeLa and HuH-7 human cell lines, while haematopoietic cells were not transduced. In addition, the Schauber study demonstrated efficient gene transfer potential of Gp64 *in vivo*. FIV-based vectors pseudotyped with Gp64 were shown to efficiently tranduce primary cell cultures of human airway epithelia and *in vivo* mediated gene transfer and expression in mouse nasal epithelia, which persisted for more than 11 months, while a previous study that utilised a VSV-G pseudotyped HIV-1 only persisted for 3 months (Limberis *et al*., 2002; Sinn *et al*., 2005). A study by Schauber *et al*. (2004) has also shown that Gp64 could be used to pseudotype an MLV-based oncoretroviral vector. These studies demonstrated that Gp64 conveyed similar transduction efficiency to VSV-G but had a more restricted tropism. The restricted tropism would therefore avoid the transduction of immune cells and effectively avoid vector inactivation by host sera. Tani *et al*., (2001) has suggested that this difference in tropism arises as Gp64 interacts directly with general phospholipids on the surface of mammalian cells, while VSV-G pseudotyped viruses interact with

phosphatidyl serines. It is suggested that phospholipid molecules including phosphatidylinositol and phosphatidic acid as well as proteins are possible Gp64 receptors (Chernomordik *et al*., 1995; Wang *et al*., 1997 ;Tani *et al*.,2001).

1.8 Research project outline

This literature review has discussed the importance of encapsidating RNA to enable HIV virion/VLP assembly. HIV-1 Gag utilises RNA binding as a form of molecular scaffold for the construction of virus particles. With respect to RNA encapsidation it is particularly significant that HIV genomic RNA constitutes a relatively small proportion of encapsidated RNA within assembled virions. Instead, the greater proportion of encapsidated nucleic acids consists of random cellular RNA species.

In this study we have for the first time questioned the potential for the transmission and expression of RNA species encapsidated by HIV VLPs (derived from an insect cell based baculovirus expression system). The transmission and expression of foreign-insect cell derived nucleic acids within a vaccinated individual is regarded as a problematic trait with respect to vaccine regulatory bodies, such as the United States Food and Drug Administration (US FDA). The FDA specifically requires the VLP vaccine developers to show that no live virus is produced during vaccine production (Shapiro, 2002). Similarly the FDA is also concerned with the possible generation of novel viruses and requires vaccine developers to show that VLPs do not encapsidate "specific" nucleic acid sequences from the expression system especially those encoding VLP components. The vaccine producer should also be able to demonstrate a level of control with respect that VLP consistency. This consistency might be hard to maintain as a result of the random nature of RNA packaging during VLP assembly.

Results from HIV-1 infection assays have clearly noted that in the absence of any functional envelope glycoproteins on the virion/VLP surface, the particles will be predisposed to degradation within acidified endosomal compartments. Degradation here should effectively prevent the transmission and expression of VLP contained nucleic acids (RNAs) and this would be consistent with the notion that VLPs are "inert" particles.

Insect cell based baculovirus expression systems are one of the most common expression systems used for the production of HIV-1 VLPs. As baculoviruses are pseudotyped with the Gp64 envelope glycoprotein this

enables them to transduce not only insect but mammalian cell lines as well via pH-dependent cell surface receptor mediated endocytosis. Gp64 inadvertently pseudotypes HIV-1 VLPs when budding from insect cells in baculovirus based expression systems. **The amount of Gp64 on the surface of insect cell produced HIV-1 VLPs has not been determined and hence this study also set about to determine the relative levels of Gp64 on HIV-1 VLPs.** Owing to the presence of Gp64 on the surface of VLPs a possibility exists for it to function as an RNA transmission vector. A recent study by Schauber *et al*. 2004, recently demonstrated that an infectious HIV particle produced in 293T cell lines and pseudotyped in this system with a Gp64 transgene was able to transduce mammalian cells and express a GFP gene carried by the viral genome. This study **in contrast will be conducted on a far simpler, non-replicative** HIV Gag VLP system consisting only of *gag* and *CAT* genes expressed by a recombinant baculovirus in an insect cell line. The assembled Gag VLPs were assessed for its ability to encapsidate the CAT RNAs produced in the insect cell line. **This study was therefore the first to my knowledge to analyse the potential transfer and expression of encapsidated reporter CAT RNAs from non-replicative Gag VLPs in mammalian cell lines.** These VLPs were, in comparison to the Schauber *et al*. 2004 study, not pseudotyped by any additional mechanism other than that utilised by the baculovirus expression system in the insect cell line. Thus the suspected ability of the so-called inert particles to enter mammalian cell lines and express encapsidated RNA species was assessed using cell lines previously shown to be permissive to Baculovirus transduction.

For VLP vaccine purposes, the presence of randomly encapsidated RNAs, which are random in both content and possibly concentration, could pose as an obstacle for vaccine regulatory bodies (if transmission and expression occurs). For vaccine producers to still assert a level of control on the vaccine content, they could simply rule out/neutralize the possible transmission and expression of RNA from VLPs out of the immunization equation by “inactivating” purified VLP preparations. **This was the first study to document the neutralisation of RNA transmission and expression from Gp64 pseudotyped HIV Gag VLPs.** VLPs were subjected to temperatures previously shown to neutralise infectious HIV virions without disrupting the

virion, prior to cell uptake assays. **The effect of this form of virus neutralisation on VLP immunogenicity was next questioned by inoculating the "heat-inactivated" VLPs into mice.** This allowed a comparison of cellular immune responses stimulated by heat treated VLPs to responses stimulated by non-heat treated particles. Results from this immunogenicity study will give an indication of the significance of Gp64 pseudotyping of HIV VLPs and whether a conferred ability to transduce mammalian cells contributes to VLP immunogenicity. Also it allowed us to determine if VLP heat treatment is a viable method to reduce the transmission and expression of RNA from VLPs, while retaining VLP immunogenicity.

Chapter 2

VLP Production and Gp64 quantitation

2.1 VLP Production for Vaccines

The HIV-1 Gag precursor protein (Pr55Gag) is independently capable of driving the assembly of immature HIV VLPs in a number of expression systems. These particles appear as doughnut shaped particles that are 120-150nm in diameter and are estimated to contain ~ 5000 structural Gag proteins (Figure 2.1 (C I-III)) (Briggs *et al*., 2004). Following Gag expression within the selected expression system, Gag proteins are targeted to the inner surface of the host plasma membrane by the N-terminal myristylation signal (Figure 2.1 (B I)). Assembled aggregates of Gag induce budding of particles from the host cell surface, which then acquire a host cell-derived membrane as well as membrane associated factors such as envelope glycoproteins (Figure 2.1 (B II, III)).

HIV VLPs have been produced in various expression systems in the absence of any other viral components involved in HIV virion assembly and maturation (Table 1.1; Gheysen *et al*., 1989; Haglund *et al*., 2000; Gelderblom *et al*., 1991; Karacostas *et al*., 1989; Royer *et al*., 1992, Mergener *et al*., 1992; Krausslich *et al*., 1993; Porter *et al*., 1996; Swanstrom *et al*., 1997). Results of these studies have greatly advanced our understanding of the assembly determinants of HIV particles expressed in various expression systems. These include insect cells (using a Baculovirus expression vector), yeast spheroplasts, T-cell lines, BHK cells (using a vesicular stomatitus virus expression vector), HeLa cells (Table 1.1; Abdurahman *et al*., 2004; Geysen 1989; Wagner *et al*., 1996; 1998; Haglund *et al*., 2000; Sakarugi *et al*., 2002; Deml *et al*., 2004; 2005). Other studies have also established that the entire *gag* coding sequence is not necessary for particle assembly. Wang *et al*. (1998) showed that HIV Gag mutants with 80% of MA deleted, are proficient at particle assembly. Similarly, studies conducted using C-terminally truncated forms of Gag in combination with the above-mentioned MA deletion of HIV Gag have established that the smallest Gag precursor capable of assembling into VLPs consists of a 28kDa protein made up of only a few MA amino acids with the entire CA-P2 domain (Wang *et al*., 1998; 2000).

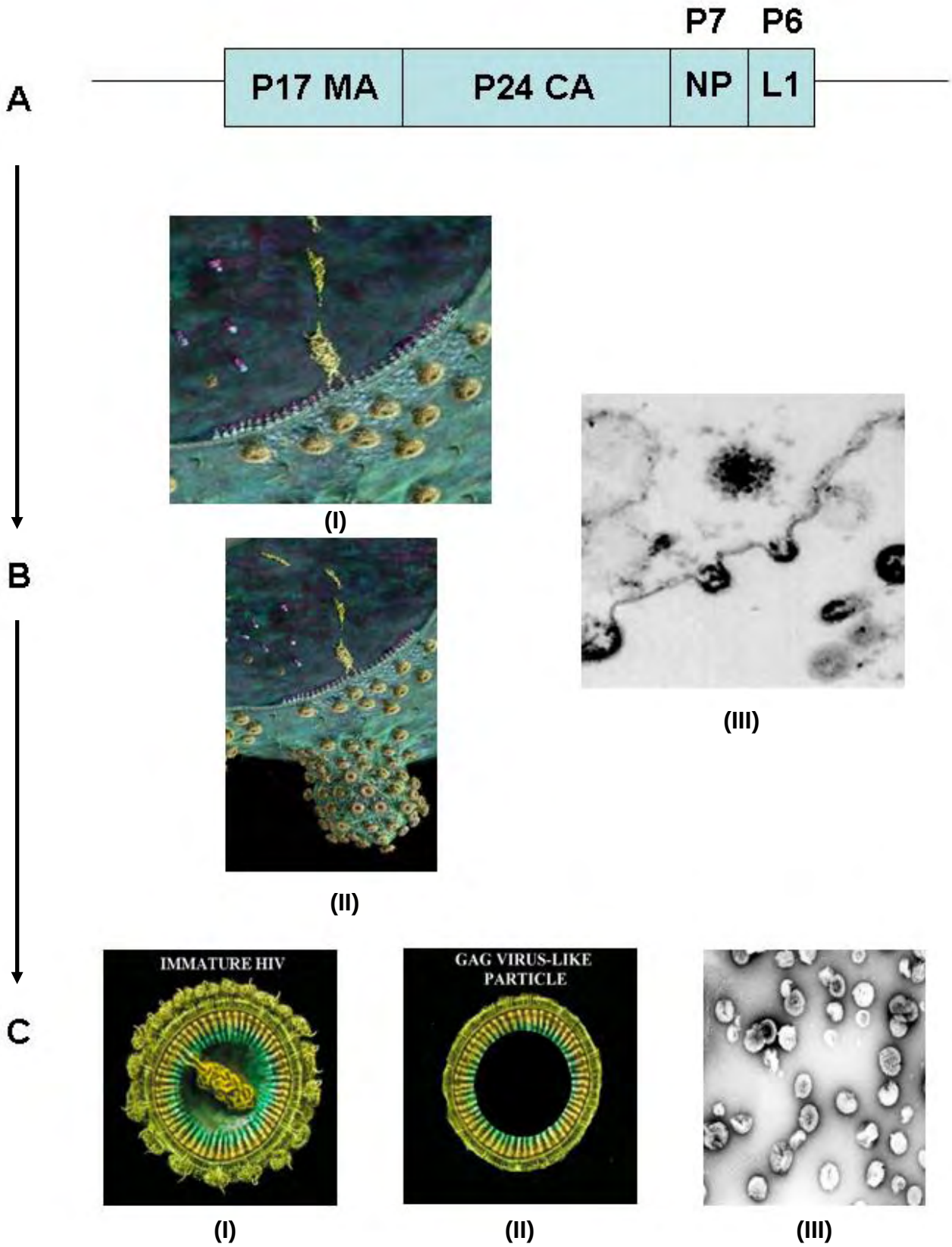


Figure 2.1 Simplified illustration of HIV VLP assembly. (A) Unprocessed Gag polyprotein. (B) Expression/production of the Gag polyprotein within cell, followed by the export, accumulation (I)+(II) of the Gag polyprotein at the plasma membrane and budding of immature HIV particles/VLP (II)+(III) (Yao *et al*., 2003). (C) Artists representation of an immature HIV virion (I), compared to an HIV VLP (II), (III) Electron micrograph of one of my insect cell-produced HIV VLP preparation. ((images B I and II, C I and II are under license from Russell Kightley Media (rkm.com.au)).

2.1.1 Manipulation of Gag VLPs

Gag VLPs can be manipulated to enhance their immunogenicity by the inclusion of heterologous epitopes in the particle. Such epitopes can be added to the VLP by the co-expression of heterologous envelope glycoproteins (type-2 VLPs) or by in-frame insertion of epitope sequences either internally or at the *gag* 3'-terminal (type-1 VLPs) (Wagner *et al*., 1996; Deml *et al*., 2005). Gag is capable of accommodating the addition of a large size range of heterologous epitopes on the 3' terminus: a study by Halsey *et al*. (2008) from this laboratory has shown that particles with C-terminal inserts in Gag as large as 778 amino acids can still be successfully produced. This study has also demonstrated that the addition of epitopes to Gag VLPs is more likely to be limited by the properties of the epitope itself as opposed to its size. As discussed in the literature review, the exact VLP assembly determinants appear to be expression system dependent. RNA encapsidation on the other hand is known to be crucial for Gag VLP assembly, where the assembling particle requires RNA to serve as a form of molecular scaffold. As discussed earlier, during particle assembly large amounts of cellular RNAs are also encapsidated by the particle.

Insect cells used with BEVS provide an ideal eukaryotic environment for the production of biologically active proteins as they enable post-translational modifications such as the proper protein folding, disulphide bond formation, oligomerization and proteolytic cleavage (O'Reilly *et al*., 1994). As a result, post-translational modifications in the insect cell expression systems are most often identical to proteins produced in insect and mammalian cells. As indicated, BEVS have been very successful at producing high concentrations of heterologous proteins through the manipulation of the very late infection p10 and PH gene promoters (Table 1.1). Deletion of these p10 and PH open reading frames has no effect on virus replication and provides an additional advantage of not producing the OV phenotype (O'Reilly *et al*., 1994). These recombinant vectors are therefore very unlikely to be transmissible to their insect hosts.

As discussed earlier the NPV envelope glycoprotein, Gp64 is one of the most abundant proteins within the BV, and its inclusion on BV is crucial for infectivity in insect and mammalian cells. Gp64 envelope glycoproteins are presented in high concentrations on the insect cell surface. As a result, any protein bodies produced within baculovirus infected insect cells that bud through this membrane (BV, HIV VLPs) will simultaneously collect high concentrations of Gp64. Pseudotyping of the inert Gag VLPs here may subsequently confer the Gp64-mediated cell entry ability to the VLP that it confers to baculovirus.

2.1.2 Aims:

This study required the use of BEVS-produced Gag-VLPs that had encapsidated CAT RNA as a reporter RNA species. As discussed at the end of chapter 1, these VLPs could serve as a tool to determine whether the RNAs encapsidated by VLPs during assembly can enter the cells of an inoculated host and be expressed.

This chapter describes the cloning steps taken for the manipulation/construction of a dual BEVS that co-expressed both an HIV *gag* as well as a *CAT* ORF. Gene products of these co-expressed ORFs should assemble into Gag-VLPs that may have encapsidated a CAT encoding RNA species within infected insect cell lines at relatively high concentrations. The expression of the Gag ORF and subsequent assembly into VLPs was assessed by means of Gag-specific western blotting and electron microscopy of infected insect cell supernatant. The expression of the CAT open reading frame was confirmed by means of CAT ELISA analysis of infected insect cell supernatant.

The Gp64 content of produced Gag VLPs was also assessed on purified Gag VLPs. It was surmised that a quantitative analysis of Gp64 on the surface of Gag VLPs would therefore give an indication of whether sufficient Gp64 is present to potentially mediate the aforementioned Gp64 entry into mammalian cell lines.

2.2 Materials and Methods

2.2.1 Vectors used:

The pFastBac™ Dual (pFBD) baculovirus insect cell expression vector (Invitrogen) was used for the co-expression of a chloramphenicol acetyltransferase (CAT) reporter open reading frame (ORF) along with a human codon optimized Pr55 *gag* (hm*gag*) ORF (Appendix B (2, 4)).

2.2.2 DNA Sequences used:

The hmGag ORF used for cloning was derived from the Du_{422} clinical isolate identified by Williamson *et al*. (2003) and was human codon optimized by Operon Technologies, USA. This hmGag ORF was kindly provided by Mr. Richard Halsey (MCB, UCT) as a clone in a pGEM®-T Easy cloning/sequencing vector (Promega). The CAT reporter ORF was obtained from the pcDNA3.1/Zeo/CAT Cloning vector (Invitrogen) (Appendix B (3)). The hmGag and CAT ORFs were cloned into the pFBD vector multiple cloning sites under the p10 (*NcoI*/*NsiI*) and polyhedrin (PH) (*NotI*/*XbaI*) promoters, respectively.

2.2.3 Construction of baculovirus expression vector (BEV) clones

Initially both the pFBD vector and the pGEM®-T easy vectors containing hm*gag* ORF were subjected to an *NcoI*/*NsiI* (Fermentas) double restriction enzyme digest using 1 unit of enzyme/ug of DNA (Figure 2.2 (A and B)). Digest reactions were set up as per suppliers protocol using supplier provided buffers and bovine serum albumin (BSA). Digests were incubated at 37^{o}C for 2 hours before analysis. The *NcoI*/*Nsi*I excised hmGag gene and pFBD vector were gel-purified as described in Appendix A (4). The hmGag ORF insert was subsequently ligated to the *NcoI*/*NsiI* restriction sites of the pFBD vector under the control of the p10 promoter (Figure 2.2 (C)). Ligations were conducted in 10μl reaction volumes using 1 unit of T4-DNA ligase (Roche) at a 10:1 insert to vector ratio.

Competent *E. coli* DH5α cells were then transformed with this ligation product (Appendix A (5)) and resulting recombinant plasmids were isolated using a small-scale plasmid DNA extraction protocol (Appendix A (2.1)). The sequences of recombinant pFBD+ Gag clones were verified by sequencing using P10 forward and reverse primers (Appendix A (6)). A selected pFBD+Gag clone was isolated and plasmid DNA was purified by means of a large-scale plasmid DNA extraction method (Appendix A (2.2)).

Both pFBD+Gag and the pcDNA3.1/Zeo/CAT cloning vectors were subjected to *XbaI*/*NotI* double digests using 1 unit of enzyme/ug of DNA as before (Figure 2.2 (D and E)). The pcDNA3.1/Zeo/CAT cloning vectors contained the relevant CAT ORF with either no attached ψ-encoding sequence or with a ψ-encoding sequence at either the 5' or 3' end of the CAT ORF. The contained CAT ORF was kindly provided by Dr Ann Meyers (Appendix B (8)). The *XbaI*/*NotI* digested pFBD+Gag vector and excised CAT ORF, 5'ψ-CAT or 3'ψ-CAT were gel purified as described in Appendix A (4). The CAT ORF insert was ligated to the *XbaI*/*NotI* restriction sites of the pFBD+Gag vector under the control of the PH promoter using the same reaction conditions as for the pFBD+hmGag ligation (Figure 2.2 (F)). Competent *E. coli* DH5α cells were then transformed with this ligation product (Appendix A (5)) and resulting recombinant plasmids were isolated using a small-scale plasmid DNA extraction protocol (Appendix A (2a)). The sequences of several recombinant pFBD+Gag+CAT, pFBD+Gag+5'ψ-CAT and pFBD+Gag+3'ψ-CAT clones were verified by sequencing the final vector product using PH forward and reverse primers and one was selected for further use (Appendix A (6)).

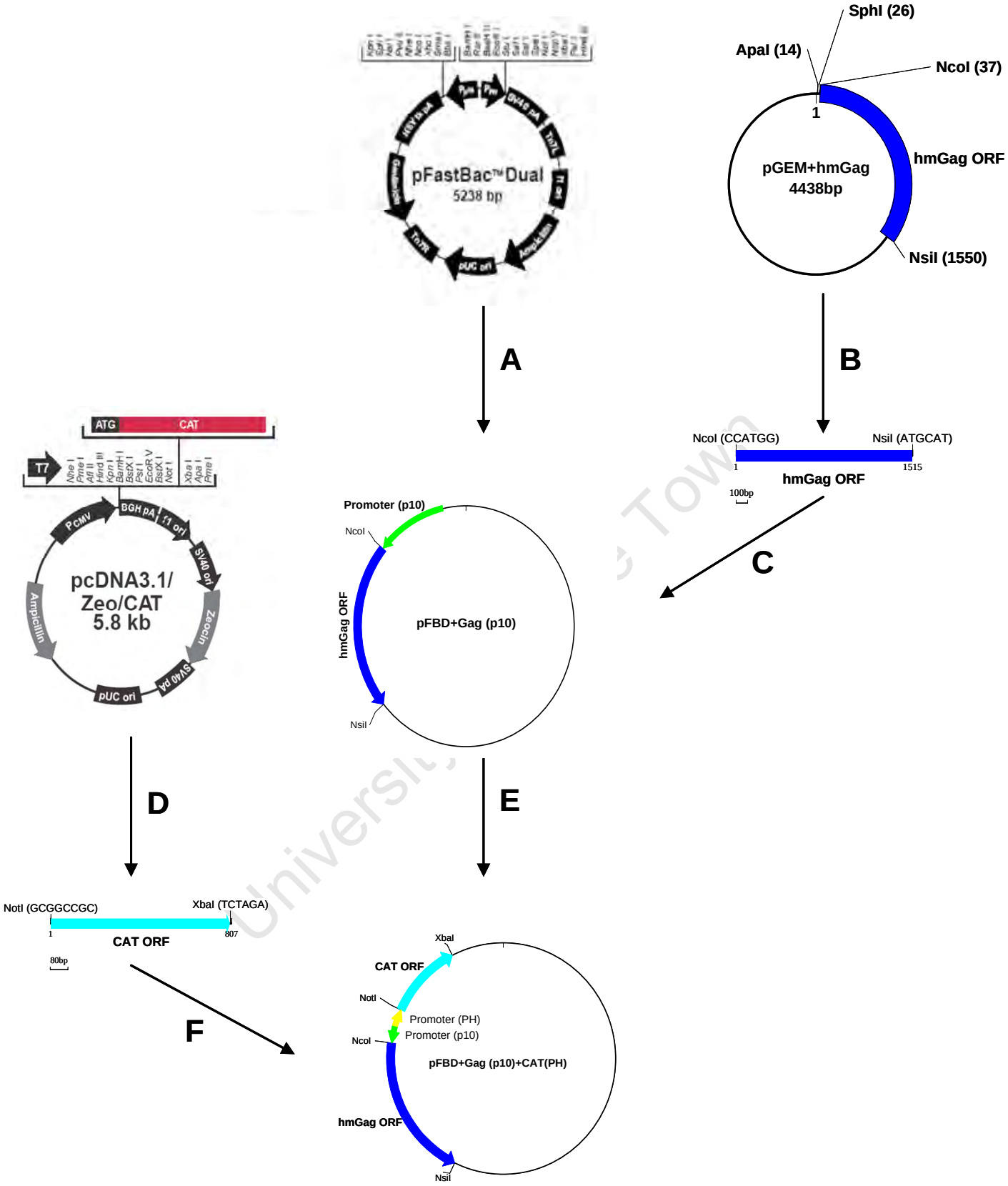


Figure 2.2 Cloning steps used to produce the baculovirus based dual expression construct that co-expressed both the hmGag ORF and CAT ORF in infected insect cell lines (pFBD+Gag+CAT). (A and B) Initial NcoI/NsiI digestion of the pFBD cloning vector and pGEM cloning vector containing the hmGag ORF to (C) enable insertion/ligation of the hmGag ORF into the pFBD vector (pFBD+Gag(p10)). (D and E) XbaI/NotI digestion of pFBD+Gag vector and pcDNA-vector containing CAT ORF to (F) enable insertion/ligation of the CAT ORF into the pFBD+Gag vector (pFBD+Gag(p10)+CAT(PH)).

2.2.4 Production and Isolation of recombinant bacmid DNA

2.2.4.1 Transposition of pFBD+Gag+CAT into a bacmid containing *E.coli* strain (DH10Bac™ Invitrogen)

Competent DH10Bac cells (Invitrogen) were thawed on ice followed by the addition of 8ng of pFBD+Gag+CAT plasmid DNA (Appendix B (6 and 7)). This transformation protocol deviated from that described in Appendix A (5), by requiring a 45 minute initial incubation on ice, a heat shock for 45 seconds (37^{o}C) and incubating the DNA/competent cell mixture at 37^{o}C for 4 hours. At this point 200µl of the transformation mix was plated onto Luria agar plates (Appendix A (1)) supplemented with 50µg/ml kanamycin, 7µg/ml gentamycin, 10µg/ml tetracycline, 40µg/ml X-gal and 40µg/ml IPTG and incubated for 24-48 hours at 37^{o}C. Positive bacmid clones produced large white colonies.

2.2.4.2 Isolation of Recombinant Bacmid DNA

A single bacmid colony was inoculated into 2ml Luria broth (Appendix A (1)) supplemented with 50µg/ml kanamycin, 7µg/ml gentamycin and 10µg/ml tetracycline and incubated overnight at 37^{o}C with agitation. Bacmid DNA was extracted using a protocol based on the alkaline hydrolysis technique of Sambrook *et al*., (1998). Bacmid DNA was handled with care and samples were not subjected to vigorous pipetting or agitation to minimise shearing of the ~130kb bacmid construct.

2.2.5 Insect Cell Culture

All insect cell culture was carried out under sterile conditions in a biological safety cabinet (Baker SterilGARD® III Advance). The *Spodoptera frugiperda* (*SF*21) insect cell line (Invitrogen) was used in all VLP production procedures. Cells were cultured in supplemented TC-100 insect cell growth medium (TC-100, Sigma®). The growth medium was supplemented with 10% (v/v) foetal calf serum (FCS, containing up to 50mg/ml serum proteins) (Gibco), 50µg/ml

neomycin, 69.2μg/ml penicillin G and 100μg/ml streptomycin (supplemented/ complete medium). Cells were grown as monolayers in sterile Nunclon cell culture flasks (Nunc™) at a constant temperature of 27^{o}C. All steps during transfection were carried out using preheated (27^{o}C) cell culture medium. Continuous SF-21 cultures were maintained by seeding mid-log cells at 5x10^{5} cells/ml every 48-72 hours. Cell viability was determined by staining cells with trypan blue stain and counting cells in a Neubauer counting chamber.

2.2.5.1 Transfection of *SF*21 insect cells with recombinant bacmid DNA/ Production of recombinant baculovirus infectant

A 35mm Falcon tissue culture dish (Becton Dickinson) was seeded with 1x10^{6} SF-21 cells in 2ml supplemented TC-100 and the cells allowed to settle for 1-2 hours. The following solutions (A and B) were prepared in separate polycarbonate tubes: Solution A – approximately 250ng recombinant bacmid DNA (5μl) was combined with 100μl of unsupplemented TC-100; Solution B – 6μl cellfectin transfection reagent (Invitrogen) was combined with 100μl unsupplemented TC-100. Solutions A and B were mixed together and incubated at room temperature for 45 minutes to allow micelle formation. Unsupplemented TC-100 (800μl) was gently added to the cellfectin-DNA mixture and mixed. The seeded SF-21 cell monolayer was washed twice with 2ml unsupplemented TC-100 and overlayed with the above cellfectin-DNA mix (~1ml) and incubated at 27^{o}C for 5 hours. Supplemented TC-100 (1ml) was added to the overlayed cells and incubated at 27^{o}C for 72 hours in a humid environment. The first virus-containing supernatant (SN1) (2ml) was harvested from the cells and replaced with another 2ml of supplemented TC-100, which was incubated in the same manner for another 72 hours before the harvesting of the second virus-containing supernatant (SN2). Following the removal of each supernatant at time points 1 and 2 (SN1 and SN2) they were clarified by centrifugation at 4000g for 5 min and stored in the dark at 4^{o}C.

2.2.6 Insect cell infection for the production of HIV-1 Gag VLPs:

2.2.6.1 Infection:

Four 500ml Nunclon cell culture plates (Nunc™) were seeded with $1x10^6$ SF-21 cells in 25ml supplemented TC-100 growth medium and cells were allowed to settle for 1-2 hours. Cells were infected by the addition of SN2 (infectious second supernatant above) and incubated for 72 hours at 27^oC.

2.2.6.2 VLP purification:

One hundred ml of cell culture medium was harvested from the 4 plates and clarified by centrifugation at 1000g for 15 minutes. The cell culture medium was further clarified by filtering through a 0.45µm syringe filter (Acrodisc® Syringe filters). VLPs in the clarified cell culture medium was next concentrated by means of cross-flow filtration. For this technique the MidGee™ cross-flow filter unit (GE Healthcare) with a 300kDa cut off MidGee™ cross-flow filter cartridge was used to concentrate the 100ml cell culture supernatant to 40ml according to the manufacturers instructions. Cross-flow filter cartridges were flushed with 100ml of pre-heated (50^oC) ultra-pure water (MilliQ) prior to each filtration and were stored in 30% ethanol. VLPs were next isolated from the concentrated 40ml cell culture supernatant by centrifuging the sample at 83000g in an Optima™ L-100 XP ultracentrifuge (Beckman Coulter). The VLP pellets were resuspended in 300µl of 1x sterile Dulbecco's phosphate buffered saline (PBS, Sigma®). VLPs were treated with 7.5u RNase A and 1.33u RNase T1 for 2 hours at 37^oC to remove any residual RNA (Ott *et al*., 2005). VLPs were purified from RNase by centrifugation through a 20% sucrose cushion at 83000g as before. VLP pellets were resuspended in 300µl of Dulbecco's PBS and stored as a 15% trehalose solution at -70^oC.

2.2.7 Electron microscopy

Transmission electron microscopy (TEM) was used to confirm the formation of HIV-1 Gag VLPs as follows. Carbon coated copper grids (200 mesh) were floated carbon coated side down onto a 15μl volume of undiluted VLP extract for 60 minutes. Grids were then washed twice with distilled water. Excess moisture was blotted from the grid between each wash. Grids were next stained with 2% uranyl acetate for 2 minutes, before excess stain was blotted from the grid. Grids were allowed to dry and stored in a grid holder. Sample grids were analysed using a LEO 912 OMEGA Energy Filter Transmission Electron Microscope.

2.2.8 Gag detection and quantitation

HIV-1 Gag was detected and quantified by means of anti-Gag western blots. The basic protocol for the polyacrylamide gel electrophoresis (PAGE) and subsequent western blotting are in Appendix A (7) and (8). Gag blots were probed with a 1/5000 dilution of anti-p24 polyclonal antiserum (ARP432) (Dr G Reid, Programme EVA, centre for AIDS reagents, NIBSC). Subsequently an anti-rabbit IgG (whole molecule) - alkaline phosphatase conjugated secondary antibody produced in goat (Sigma®) and was used at a 1/5000 dilution to probe the blot.

2.2.8.1 Gag Quantitation:

For Gag VLP quantitation an HIV-1 p17/24 C clade protein standard (ARP695.2) was used to generate a standard curve (FIT Biotech, Programme EVA, centre for AIDS reagents, NIBSC). This p17/p24 (Gag) standard was diluted to concentrations of 2.4, 7.2, 12 and 24ng dilutions on the PAGE along with a 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} VLP dilution series. The amount of Gag in samples on the blot was determined using densitometric analysis. For this analysis the Gene Genius Bio imaging system was used (Syngene) along with Gene tools program (Syngene) to quantitate Gag using a standard curve generated by using graduated amounts of the p17/p24 standard.

2.2.8.2 Gp64 detection and quantitation on VLPs

(a) The Gp64 content of VLPs was determined by means of western blotting (Appendix A (7), (8)). VLP samples blotted onto nitrocellulose membranes were probed with a purified anti-Baculovirus Gp64 envelope glycoprotein mouse primary antibody AcV5 (eBiosciences). The secondary antibody used for these blots was an anti-mouse IgG (whole molecule) - alkaline phosphatase conjugated antibody produced in goat (Sigma®) and was used at a 1/5000 dilution.

(b) Gp64 was quantified by means of quantitive PAGE. PAGE was carried out as indicated in Appendix A (7) The amount of Gp64 loaded on the gel was determined by using a BSA standard curve in which BSA was loaded as 100, 200, 500, 1000ng concentrations. Densitometric analysis was again used to estimate protein concentration as used above.

2.2.9 CAT expression analysis

CAT ELISA kit (Roche) was used for this assay according to the method described in Appendix A (9). Transfected insect cell lines were lysed using CAT ELISA kit lysis buffer and lysates were diluted in provided sample buffer before being used for CAT ELISA.

2.3 Results

2.3.1 Manipulation of the Baculovirus Expression Vector System (BEVS)

The human codon optimised Gag ORF (hmGag) as well as the CAT ORF were successfully cloned into the pFBD BEVS under the control of the p10 and polyhedrin (PH) promoters, to yield the clone pFBD+Gag+CAT, respectively. Sequencing of putative clones confirmed the presence of both genes under appropriate promoter control (Appendix B (6 and 7)). The cloning procedure is detailed in Figure 2.2.

2.3.2 Expression of Gag and CAT ORFs in Sf-21 insect cell line

The pFBD+Gag+CAT construct was subsequently transformed into the DH10Bac™ cell line to facilitate the transposition of the relevant Gag and CAT ORFs and their respective promoter elements into the bacmid. Recombinant bacmids were then assessed for their ability to express Gag and CAT in transfected *Sf-21* insect cell lines as follows.

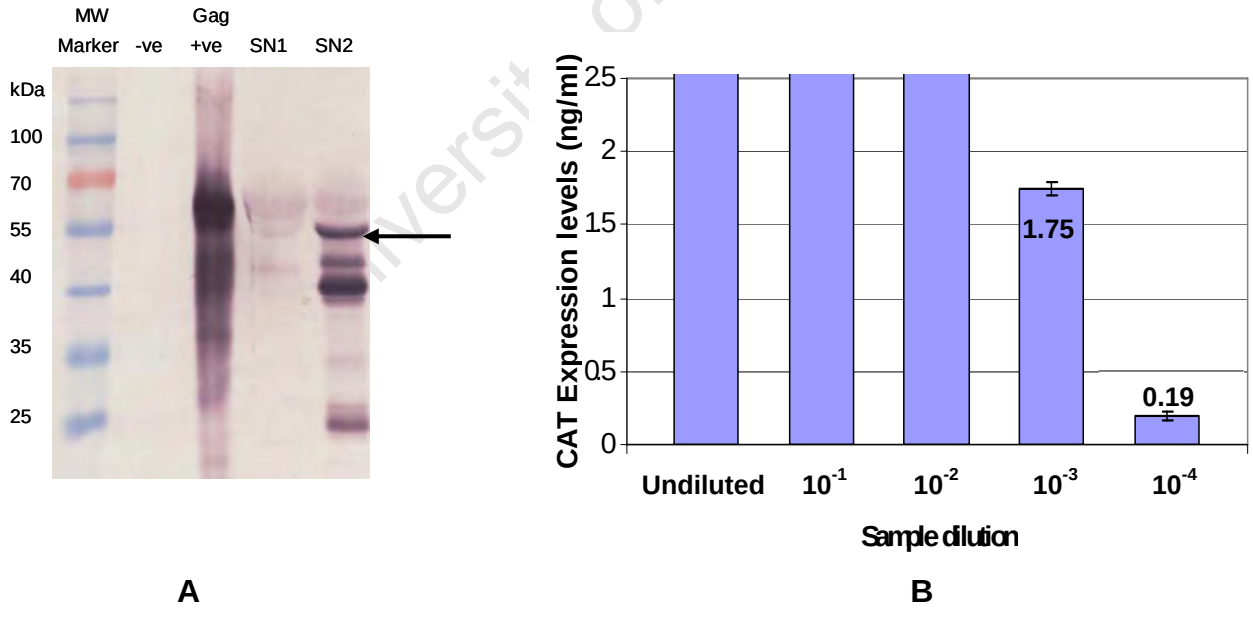


Figure 2.3 Expression analysis of Bacmid clone. Anti-Gag western blot of infectious supernatants derived from 2ml *Sf*-21 transfections at time points 1 and 2 (SN1: 76 hours, SN2: 152 hours) arrow identifies the p55 Gag band . Bar graph illustrating CAT ELISA results of transfected Sf-21 cell lysates at dilutions 0-10^{-4}. Expression levels of above 600ng/ml were beyond the maximum detection limits of the assay. Error bars display the variance between 3 separate experiments

Equivalent amounts of the first and second cell culture supernatants (SN1 and SN2) derived from the 2ml *Sf*-21 transfections were analyzed for the expression of the hmGag ORF. An anti-p24 western blot confirmed the expression of the 55 kDa Gag protein in the transfected *Sf*-21 cell line, illustrated by the arrow (Figure 2.3 A). The supernatants as well as the Gag positive control developed several bands on the anti-Gag western blot, which are most likely a result of Gag degradation products. The 55 kDa Gag band for both SN1 and SN2 appeared slightly distorted/curved on the blot as these crude SN samples still contained BSA. The BSA is visible on the blot as a non-specific smear/blob above the 55 kDA Gag band in both SN1 and SN2 samples. It was also noted that Gag expression levels from the SN isolated at time-point 2 (SN2-152 hours) was higher in concentration than SN1 (76 hours), which is congruent with the understanding that by the second time-point post transfection the concentrations of recombinant, infectious baculovirus is significantly higher.

As the ultimate function of the CAT ORF in this clone was to produce a reporter RNA species for encapsidation by assembling VLPs, I needed to confirm whether this RNA species was produced by the BEVS. This was indirectly accomplished by monitoring for the production of the CAT enzyme in transfected cell lines. Cells transfected with the recombinant bacmid clone containing the Gag and CAT ORFs were lysed and lysates were used in a CAT ELISA (Figure 2.3 B). ELISA results showed that the CAT ORF was expressed at high concentrations. Expression levels of the undiluted, 10^{-1} and 10^{-2} samples were beyond the detection limits of this assay and as such only 10^{-3} and 10^{-4} dilutions were quantified to be 1.75ng/ml and 0.19ng/ml respectively.

2.3.3 VLP assembly

Transmission electron microscopy was used to confirm whether the expressed pFBD+Gag+CAT produced Gag VLPs using the purified VLP sample derived from a 100ml SF-21 infection (Figure 2.4).

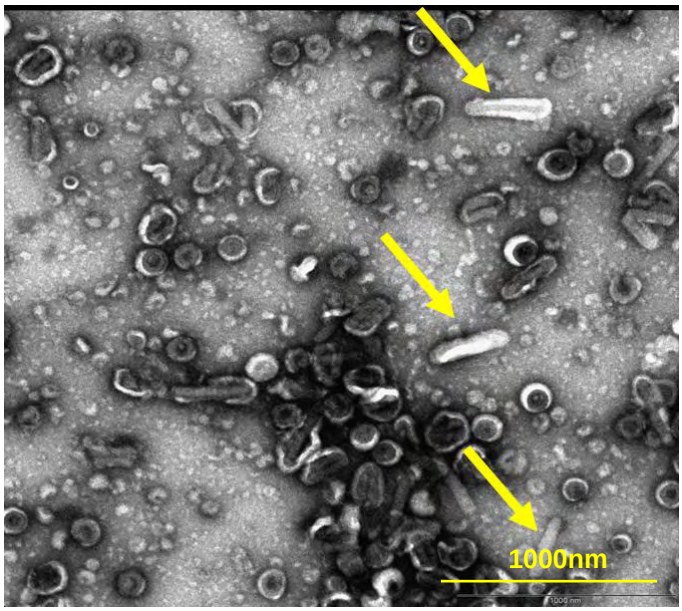


Figure 2.4 Transmission electron micrograph of purified VLP sample. Arrows indicate the presence of contaminating baculovirus particles.

The electron micrograph seen in figure 2.4 demonstrates the presence of numerous doughnut-shaped particles 120-150nm in diameter, which indicates that Gag VLPs are produced by the recombinant BEVS. From this electron micrograph it is also evident that the baculovirus content of the VLP sample has been significantly reduced by means of cross-flow filtration (Figure 2.4, yellow arrows). VLPs purified without cross-flow filtration very often contain high concentrations of contaminating baculovirus.

2.3.4 Gp64 Quantitation

The Gp64 envelope glycoprotein plays a crucial role in the baculovirus life cycle in mediating virus entry into the host cell and is one of the most abundant proteins in baculovirus virions. Gp64 envelope glycoproteins are displayed in high concentrations on the infected cell's surface and are thus included in the membrane of budded Gag VLPs. To confirm the presence of baculovirus Gp64 envelope glycoprotein on Gag VLPs produced by pFBD+Gag+CAT, it was detected by means of qualitative anti-Gp64 Gag western blots (figure 2.5). For this method 2 SDS PAGE gels were loaded with identical sample sets consisting of 3 VLP preparations and used for western blotting. One blot was probed with an anti-Gag primary antibody while the

other was probed with an anti-Gp64 primary antibody to confirm the co-purification of significant levels of Gp64 with Gag VLPs (Figure 2.5).

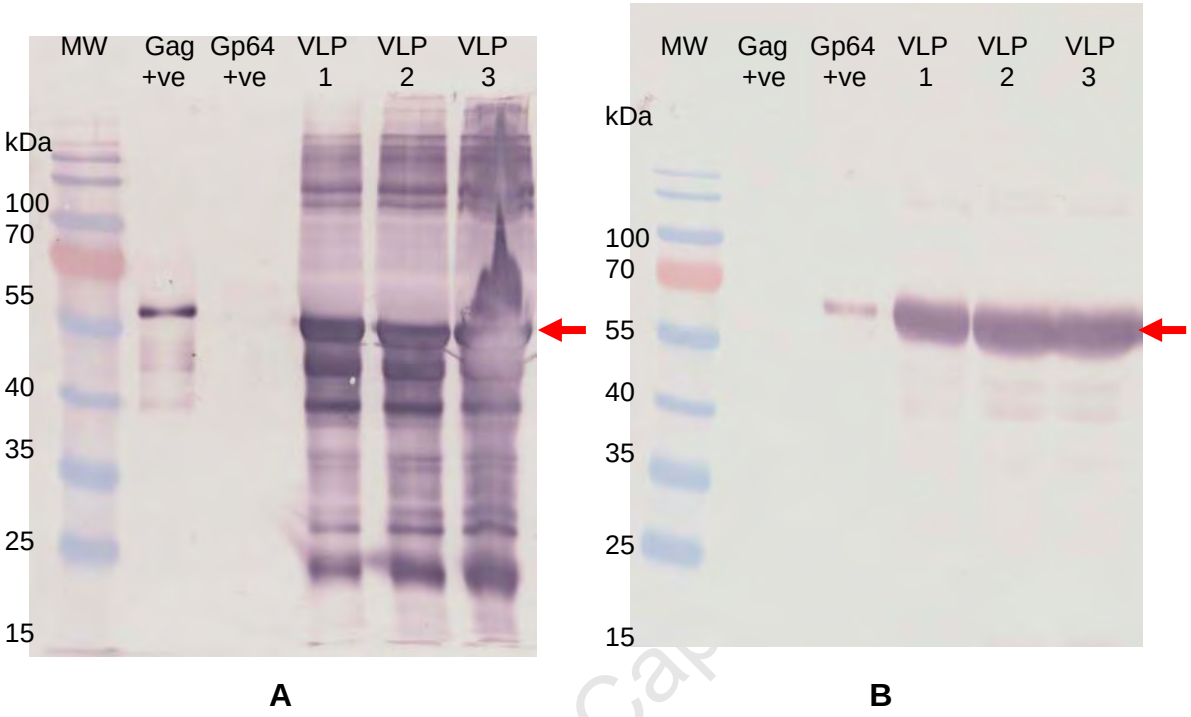


Figure 2.5 Comparative western blots of purified VLP extracts. (A) Probed with anti-Gag primary antibody, (B) Probed with anti-Gp64 primary antibody. VLP 1, 2, 3 denotes 3 separate VLP preparations. Red arrows indicate the position of 55KDa HIV Gag and 64KDa VSV Gp64

The anti-Gag western blot shown in Figure 2.5 (A) confirmed the presence of 55kDa Gag protein in each of the 3 VLP extracts. This anti-Gag western blot also displayed several bands per VLP sample to suggest the presence of Gag degradation products. The identical blot shown in Figure 2.5 (B), probed with the anti-Gp64 primary antibody displayed a broad 64kDa band for each of the 3 VLP extracts. This therefore confirmed the incorporation of large amounts of Gp64 in Gag VLPs

Following this, the relative amount of Gp64 incorporated into the Gag VLPs per microgram of VLP Gag protein was determined. As no quantified Gp64 standard was available to allow quantitive western blotting or ELISA, the VLP Gp64 concentration was determined by means of a quantitive PAGE, which used BSA as the quantified protein standard for densitometric analysis (Figure

2.6). To ensure that the correct band on the PAGE was quantified an anti-Gp64 quantitive western blot was run in parallel with an identical set of VLP sample dilutions (Figure 2.6(A and B)).

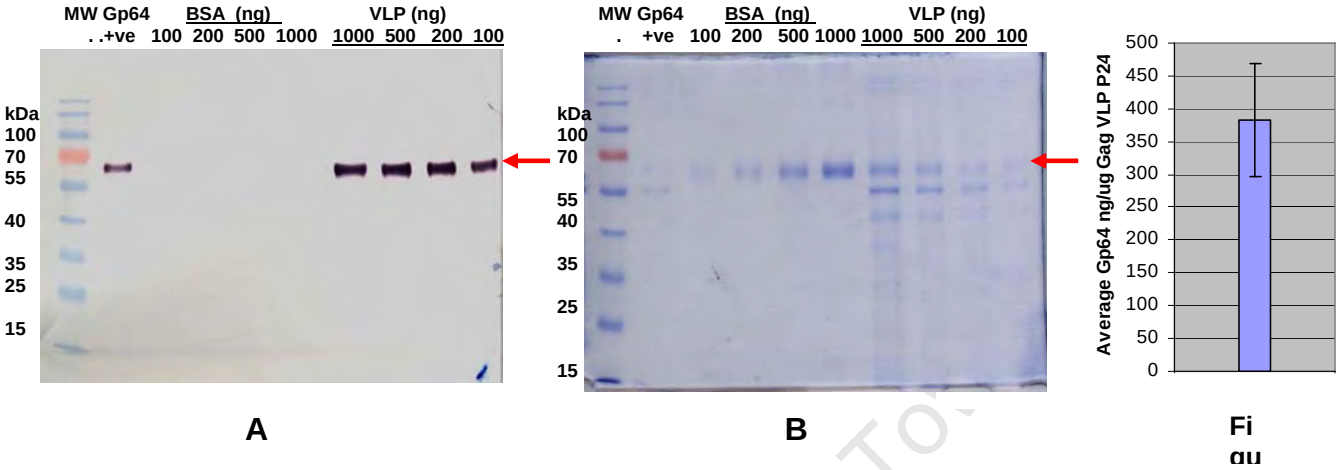


Figure 2.6 Gp64 quantitation analysis, arrows indicate the position of quantified bands. Typical illustration of (A) Anti-Gp64 western blot of VLP extracts (1µg Gag), (B) corresponding quantitive PAGE gel of VLP extracts (1µg Gag). Red arrows indicate the position of the quantified Gp64 band. (C) Bar graph illustrating the estimated amount of Gp64 in 1µg of Gag VLP. Error bars display the variance between 4 separate VLP preparations

The VLP sample dilutions run on the quantitive PAGEs yielded a 64kDa bands that aligned with 64kDa Gp64 bands on the identical western blots (Figure 2.6 (A and B)). Results from repeated quantitive PAGEs of several VLP extracts estimated an average of 382.94ng Gp64/ug of Gag in the Gag VLPs with a variance of 85.75 (Figure 2.6 C).

2.4 Discussion

A recombinant BEVS was made which enabled the successful co-expression of Gag and CAT proteins in transfected SF-21 cell lines. Production of Gag was confirmed by means of anti-Gag western blotting. Electron microscopy of VLP preparations confirmed the production of ~120-150nm doughnut shaped particles (VLPs) in this system. Transcription of the CAT ORF and hence the production of CAT RNA species was indirectly confirmed by assaying for the production of the CAT enzyme using the CAT ELISA. High levels of CAT expression were measured.

As indicated previously, the baculovirus Gp64 envelope glycoprotein is abundantly produced and presented on the plasma membrane of cell lines infected with this virus. VLPs produced using the BEVS appeared to contain very high levels of this Gp64 envelope glycoprotein. Despite previous studies documenting the presence of Gp64 on the VLP surface, this is the first study to make quantitive estimates of the amount of Gp64 on the VLP surface. Quantitive analysis of the amount of Gp64 incorporated into Gag VLPs determined that ~ 383ng of Gp64 was detected per 1µg of Gag VLP sample. From sequence analysis I established that the relative molecular mass of hmGag and Gp64 are 55kDa and 64kDa respectively. A study by Briggs *et al*. (2004) has found that an average HIV Gag VLP/immature particle of 120-150nm contains ~5000 structural Gag proteins, which would therefore mean the mass of a VLP is ~275000kDa. In the analysed 1µg of Gag VLP, the total molecular mass is assumed to be 6.022173643 x 10^{14}kDa, which equates to Gp64 in ~2.2x10^{9} Gag VLPs (1µg). In this 1µg of Gag VLPs I detected ~380ng of Gp64, corresponding to ~2.3x10^{14}kDa, and therefore contained ~3.6x10^{12} Gp64 molecules (64kDa). This data therefore suggests that each VLP contains ~ 1600 Gp64s, which is roughly 1 Gp64 envelope glycoprotein for every 3 Gag proteins in each VLP. This value may be distorted by the presence of contaminating baculoviruses, which carry their own Gp64: however it has been observed in our laboratory that any baculovirus contamination during VLP extraction by means of the cross-flow filtration technique is minimal, which would limit the influence on Gp64 quantitation.

Initial estimates by means of EM have estimated the presence of approximately 72 envelope spikes on infectious HIV virions (Gelderblom *et al*., 1991), while more recent studies have indicated that this number may be as low as 2 to 14 trimers (expressed trimers) (Chertova *et al*., 2002; Zhu *et al*., 2003; 2006). All together these results indicate that the baculovirus Gp64 is presented in high densities on the surface of BEVS produced Gag VLPs, greatly exceeding that of HIV Gp120 on infectious HIV virions. Properties that this high density of Gp64 confers on the VLP surface is investigated in the following chapter.

Chapter 3

VLP Cellular Entry and RNA Expression

3.1 HIV cellular entry

Enveloped viruses gain entry to their target cells by means of membrane fusion. This fusion can occur either at the cell surface or from within acidified intracellular vesicles. Until recently the primary mode of productive HIV cellular entry was assumed to proceed via pH-independent cell surface receptor mediated endocytosis exclusively at the plasma membrane (Stein *et al*., 1987; Dimitrov *et al*., 2001). The HIV genome encodes its envelope glycoproteins as a single 160 kDa precursor (Gp160), which is subsequently cleaved into 120 kDa (Gp120) and 41 kDa (Gp41) functional glycoproteins by a cellular protease. The 550 amino acid gp120 envelope glycoprotein is displayed at the exterior surface of the virion membrane (Figure 1.3 (E)) (Fields *et al.,* 1996). This surface envelope glycoprotein is non-covalently associated with the transmembrane protein Gp41 (Figure. 1.3 (E)) and contains a binding domain for CD4 receptors and chemokine co-receptors (CCR5/CXCR4) found on the surface of T-lymphocytes, monocytes, macrophages, mature B-cells and dendritic cells. These Gp120/41 associations form clusters of tri-molecular complexes on the virion membrane surface (Lu *et al.,* 1995; Chan *et al.,* 1997; Farzan *et al.,* 1998). Gp120 interaction with target CD4 receptors and chemokine co-receptors (CCR5/CXCR4) plays an important role during virus infection by initiating the fusion of the virion membrane to the host cell membrane, and thus allowing entry of the virus into the host cell (Dalgleish *et al.,* 1991; Deng *et al.,* 1996; Wyatt *et al.,* 1998 A).

During cellular infection, if Gp120 interaction with the CD4 receptors and chemokine co-receptors is considered as the initiation step, the activity of the Gp41 transmembrane protein is viewed as the functional unit that facilitates actual membrane fusion as follows (Chan *et al*., 1998). Free virions are assumed to be in a native or non-fusogenic conformation, in which the Gp41 fusion peptide is buried inside the trimeric glycoprotein complex. Upon CD4 and chemokine receptor binding by Gp120, structural rearrangement alters the Gp120/41 interaction and triggers Gp41 to undergo its own structural rearrangement. This rearrangement results in the exposure of hydrophobic epitopes at the N-terminus of the Gp41 fusion peptide, which are then inserted

into the target cell membrane. Further structural rearrangement within the Gp41 trimeric-coiled coil results in membrane apposition and allows fusion of the virion and cell membranes (Dimitrov *et al*., 2005; Wyss *et al*., 2005).

3.1.1 Alternate routes of HIV cellular entry

In spite of the above highly detailed pathway that leads to productive cellular entry, other studies have documented viral entry into cells via **receptor independent** endocytosis, and shown the presence of HIV particles contained within acidified endocytic vesicles (Fredericksen *et al*., 2002; Marēchal *et al*., 1998; Marechal *et al*., 2001; Pauza *et al*., 1988; Schaeffer *et al*., 2004; Fackler *et al*., 2000). This path of cellular entry frequently leads to HIV inactivation/ degradation in these acidified compartments (Grewe *et al*., 1990; Marechal *et al*., 1998; Schaeffer *et al*., 2001; Stein *et al*., 1987; Schaeffer *et al*., 2004). Despite this, several studies have also demonstrated that endocytic entry can lead to productive infection if under the appropriate conditions (Fackler *et al*., 2000; Fredericksen *et al*., 2002; Marechal *et al*., 2001; Schaeffer *et al*., 2004). Optimal infection of endocytosed virions was shown to require target cell line treatment with pharmacological agents that raise the endosomal/lysosomal pH (Fredericksen *et al*., 2002; Marēchal *et al*., 1998; Pauza *et al*., 1988; Schaeffer *et al*., 2004; Marechal *et al*., 2001; Fackler *et al*., 2000).

Marechal *et al*. (1998) have shown that even in the presence of receptor mediated HIV infection/uptake in various CD4-displaying cell lines, the cytosolic fraction of p24 only represented 10-40% of intracellular p24. This suggests that the endocytic/degradative pathway of cellular entry represents the primary route of entry. Thus, even with appropriate envelope glycoprotein-receptor interactions, 50-90% of viral material has been shown to enter the cell by the endosomal pathway. These results have been corroborated in several other studies (Fackler *et al*., 2000; Fredericksen *et al*., 2002; Schaeffer *et al*., 2004). Schaeffer *et al*. (2004) have demonstrated a “compensatory” relationship between HIV-1 fusion and endocytosis, whereby inhibiting fusion enhances endocytic uptake and inhibiting endosome acidification or trafficking enhances the fusion pathway. Unlike most other studies of this nature, that utilised HeLa cells for the assays (Fredericksen *et*

al., 2002; Marechal *et al*., 1998, Fackler *et al*., 2000), this study utilised SupT1 T-cells or PHA-activated lymphoblasts. With these cell lines, a dependence on CD4 was established to enable both fusion and endocytosis of HIV virions. Thus, after the initial envelope-receptor interaction a competition ensued between the endocytic and fusion pathways, determined by secondary co-receptor interactions. A similar compensatory relationship between virion fusion and endocytosis was revealed in a study conducted by Fackler *et al*., (2000), which also demonstrated the presence of an HIV strain (HIV-1_{SF2}) that has an enhanced infectivity when entering the Target 293T cells via the endocytic route. Researchers also suggested here that the excessive use of therapies that block CD4 and chemokine receptor binding could select for those HIV variants that preferentially utilise the endocytic pathway.

Cells of the immune system have evolved several methods to enable the uptake of exogenous materials. These include clathrin mediated endocytosis; non-clathrin mediated endocytosis (caveolae); phagocytosis and macropinocytosis. Different viruses have also evolved mechanisms to utilise these distinct cellular structures to enter target cells. Macropinocytosis in particular is a key mechanism used in antigen uptake (Lanzavecchia *et al*., 1996; Yewdell *et al*., 1999), which is cell type specific (constitutive in dendritic cells). It is a receptor-independent process of extracellular antigen capture using macropinosomes which are large vesicles 0.2-3 μm in size. Virus particle uptake studies have demonstrated that HIV uptake by macropinocytosis is a receptor and pH-independent process, as macropinosomes were shown to internalize non-infectious HIV lacking Env as well as virions displaying the envelope glycoproteins (Marechal *et al*., 2001; Schaeffer *et al*., 2004). Despite this, only the Env-displaying virions, which could enable vesicular membrane fusion, accessed the cytosol and resulted in a productive infection by engaging the CD4 receptors and chemokine co-receptors on the internal surface of the macropinosome.

The contribution of clathrin and caveola-mediated endocytic uptake pathways during productive HIV infection has been evaluated in CD4-positive HeLa cell lines (Daecke *et al*., 2005). The cell lines used in this study contained

mutations that prevented the development of endocytic vesicles from the inner surface of the cell membrane to effectively disrupt these uptake pathways. Clathrin dependent endosomal entry in this study was shown to contribute significantly to productive HIV entry in this cell line. Mutations interfering with this uptake pathway led to a 40-90% (experimental condition-dependent) reduction in the number of cells productively infected with HIV-1. The caveolin mutant, which inhibited caveola mediated entry had no significant effect on productive HIV-infection. Daecke *et al*. (2005) have also suggested that entry via the endocytic route may have advantages over plasma membrane fusion. Entry via the endocytic pathway sees the HIV particle delivered directly to the cytosol and avoids the sub-cortical actin cytoskeleton, which is regarded as a difficult barrier to overcome.

The general consensus of the aforementioned studies is therefore, that HIV endocytosis can lead to a productive infection if provided with proper conditions. Also, an important prerequisite in enhancing infectivity from endosomal compartments is the inhibition/arrest of endosomal acidification. A common trend emphasized in all the above studies strongly suggests that, irrespective of the path of entry into the target cell, productive HIV infection is dependent on receptor/co-receptor interactions at the cell surface or within vesicles. Hence, the gradual decrease of pH in endocytic vesicles may permit escape to the cytoplasm for some HIV particles before they are disrupted by low pH. The inactivation of HIV particles by the disruption of their envelope glycoproteins should therefore prevent escape from endosomes before acidification and hence prevent infection by this uptake pathway.

3.1.2 Virus inactivation

Several chemical and physical methods have been assessed for the inactivation of putative live virus vaccines. Whole inactivated vaccines have been produced using these inactivation methods for several retroviruses, which have included feline immunodeficiency virus (FIV) (Yamamoto *et al*., 1993; 1991); SIV (Stott *et al*., 1990; Cranage *et al*., 1992; Johnson *et al*., 1992) and HIV (Race *et al*., 1995 (A) and (B); Grovit-Ferbas *et al*., 2000; Poon

et al., 2005 (A) and (B)) all of which have shown promise as potential vaccines.

Chemical agents for virus inactivation have included formalin, beta-propiolactone, formaldehyde and binary ethylenimines (Race *et al*., 1995; Grovit Ferbas *et al*., 2000; Poon *et al*., 2005). In general these chemical agents function as “fixing agents” by interacting with organic groups on virus particles. Interactions with these groups lead to cross linking of proteins and often render them non-functional. The use of these chemical agents for virus inactivation do have limitations as they often produce toxic by-products, which are unacceptable for vaccine formulations.

Thermal inactivation has been tested as an alternate means of inactivating retroviruses and has shown great success (McDougal *et al*., 1985; Grovit Ferbas *et al*., 2000; Poon *et al*., 2005; Piszkiewicz *et al*., 1986; Quinnan *et al*., 1986). Thermal inactivation overcomes the problem of production of toxic by-products, which can reduce the effect of potential vaccines. It has been shown with HIV virion isolation, that thermal inactivation results in further shedding of Gp120 from the virion surface. This has a detrimental effect on the type of immune response stimulated. Poon *et al*. (2005) have demonstrated, however, that a large proportion of Gp120 can be retained on the virion surface by treating virions with low concentrations of formaldehyde prior to heat inactivation. This stabilises Gp120 on the virion surface by facilitating cross linking with reactive groups (amino) on the virion surface.

These physical and chemical means of virus inactivation predominantly function by disrupting the virion structure including surface glycoproteins. This in turn affects the eventual immunogenicity of the virion. Short wavelength (254nm) ultraviolet (UV) radiation serves as a more “delicate” method for virus inactivation. Virus inactivation by UV has been shown to be highly effective for a range of different DNA and RNA viruses including tobacco mosaic virus, feline calcivirus, canine calcivirus; adenovirus 41 and poliovirus 1 where dose dependent UV radiation decreased viral infectivity by several log values (Evans *et al*., 1969; de Roda Husman *et al*., 2004; Ko *et al*., 2005; Simonet *et al*., 2006). Virus inactivation by UV radiation functions primarily by alterations

to the viral genome (Simonet *et al*., 2006; Lytle *et al*., 2005). Short wavelength UV is absorbed by the nucleic acids (DNA and RNA) of micro organisms/viruses and results in the development of pyrimidine: thymine dimers on the same strand, pyrimidine hydrates and cyclobutane, which, if not repaired obstruct replication/expression. Generally pyrimidines are more sensitive than purines and thymine and uridine are the most sensitive bases in DNA and RNA respectively (Cadet *et al*., 1991; Durbeej *et al*., 2003; Harm *et al*., 1980; Kuluncsics *et al*., 1999). UV radiation produces limited toxic by-products compared to chemical virus inactivation treatments and has a minimal effect on virion structure (Haider *et al*., 2002).

3.1.3 Aims:

The studies above suggest that HIV-1 Gag VLPs that lack functional envelope glycoproteins may be predisposed to degradation within acidified endosomal compartments as endocytic uptake would be the only mechanism of cellular entry. This reflects the “inert particle” title given to non-pseudotyped Gag VLPs but does not take into account the presence of baculovirus Gp64 on the surface of BEVS-produced Gag VLPs. The Gp64 envelope glycoproteins enable baculovirus as well as other heterologous live viruses like VSV to successfully enter mammalian cell lines by means of receptor mediated pH-dependent endosomal fusion. Results in Chapter 2 showed that there are high concentrations of Gp64 on the surface of BEVS-produced Gag VLPs. The aim of the investigation described in this chapter is to determine whether these envelope glycoproteins confer the mammalian cell entry ability to otherwise inert Gag VLPs. This was determined by conducting cell uptake assays with several mammalian cell lines previously shown to be receptive to baculovirus entry. As the Gag VLPs encapsidated quantified concentrations of a CAT reporter RNA, successful cellular entry was confirmed by conducting CAT ELISAs on cell lysates 24 hours after VLP uptake. Subsequently, control of VLP cellular entry and RNA expression was assayed following virus inactivation techniques.

This would be the first instance that this type of analysis would be done on a non-replicative (inert) VLP system as previous studies have all

been conducted on more complex live virus models that carried reporter genes as part of the viral genome (Schauber *et al*., 2004; Kumar *et al*., 2003). This system, on the contrary, represents an actual vaccine candidate that lacks any replicative nucleic acids and hence the reporter RNA is representative of the random nucleic acids encapsidated by VLPs during assembly.

3.2 Materials and Methods

3.2.1 *In vitro* transcription

Poly-A tailed and capped RNA transcripts of the CAT ORF were generated as positive controls for RT-PCR and cell uptake assays using the Ribomax™ Large Scale RNA Production System – T7 kit (Promega). Transcription reactions were carried out according to the manufacturer's instructions using 5μg of template pcDNA3.1/Zeo/CAT vector DNA per 50μl reaction. Prior to the transcription reaction, DNA templates were treated with 1u/μg DNA of human placental RNase inhibitor (Roche) for 1 hour at 37° C. After synthesis RNA samples were stored at -70° C. RNA product derived from the *in vitro* transcription was quantified using the NanoDrop® ND-1000 spectrophotometer.

3.2.2 Reverse transcriptase-PCR (RT-PCR)

RT-PCR to detect the encapsidation of CAT RNA transcript by Gag VLPs was carried out using the Access RT-PCR System (Promega), which provided all enzymes, buffers and reaction constituents. RT-PCRs were run on the Mastercycler Gradient (Eppendorf) PCR machine. All RT-PCRs were made-up in 50μl reaction volumes using 1mM $MgCl_2$ and 50pmol of each primer, which amplified a ~350bp DNA fragment. As this was a qualitative assay, each reaction was made up using 10^{-2} VLP preparation dilution, while *in-vitro* transcribed CAT RNA was used as a positive control.

RT-PCR Primers

(a) CAT Forward primer: 5' GCA ATG AAA GAC GGT GAG C 3'

(b) CAT Reverse primer: 5' ATG AAC CTG AAT CGC CAG C 3'

RT-PCR reaction profile:			
	45° C	45 min	RT
	94° C	2 min	RT inactivation
	94° C	30 sec	x25 cycles
	57° C	30 sec	
	72° C	1 min	

3.2.3 RNA Extraction

Total RNA was extracted from Gag VLPs using the RTP Virus DNA-RNA Isolation Kit (Invitek). Extractions were carried out according to manufacturer's protocol and each RNA extraction was carried out on 5µg of quantified Gag VLPs and final RNA extracts were eluted into 50µl of the extraction kit elution buffer.

3.2.4 Real-time RT-PCR kit

The CAT RNA content of the total VLP RNA extracts were analysed using the SensiMix One-Step real-time RT-PCR kit (Quantace), which through the use of SYBR® green enabled RNA quantitation in a single reaction setup (source: 470nm/detector: 510nm). Real-time RT-PCRs were run on the Rotor-Gene RG-3000A real-time PCR machine (Corbett Research). All reactions were setup according to manufacturer's protocol in 25µl reaction volumes using 50mM $MgCl_2$ and 50pmol of each primer, which amplified a ~150bp DNA fragment. As this was a quantitive assay RNA derived from the equivalent of 1µg of Gag VLPs was used per reaction and *in-vitro* transcribed CAT RNA dilutions was used as a quantified positive control.

Primers

(a) Real time CAT Forward primer: 5' AGA TGT GGC GTG TTA CGG T 3'

(b) Real time CAT Reverse primer: 5' ATG AAC CTG AAT CGC CAG C 3'

Real-time RT-PCR reaction Profile:

49° C	45 min	RT	
95° C	10 min	RT inactivation	
95° C	15 sec	x 30 cycles	
57° C	15 sec		
72° C	15 sec		

Following the above PCR profile a melt curve was also run to confirm homogeneity of the quantified PCR product. Melt curve reaction profile: Ramp from 57° C to 95° C, raising temperature by 1° C each step. Waited for 45 sec on first step and 5 sec on each step afterwards. All real-time RT-PCR data

was analysed using the Rotor-gene 6, Version 6.0 (Build 27) software (Corbett Research).

3.2.5 Mammalian Cell Culture

3.2.5.1 Cell lines and growth conditions

All mammalian cell culture was done under sterile conditions in a biological safety cabinet (Labomark Lab Series 1800mm Class II). Mammalian cell uptake assays were conducted using the Murine RAW 264.7 macrophage (RAW 264.7), Baby Hamster kidney (BHK-21), Human Embryonic Kidney (HEK 293) and HeLa cell lines. All cell lines were maintained as continuous cultures and were incubated in a H_2O jacketed, CO_2 incubator at 37^o C at 5% CO_2 (Forma Scientific). Continuous RAW 264.7, BHK-21, HEK 293 and HeLa cell cultures were maintained by seeding mid-log phase cells at 1-2 x 10^6 cells/ml every 48-72 hours. Cell viability was determined by staining cells with Trypan blue stain and counting cells in a Neubauer counting chamber.

3.2.5.2 Growth medium

The RAW 264.7 cells were cultured in supplemented Roswell Park Memorial Institute (RPMI) medium (RPMI 1640+GlutaMAX™-1, Gibco®), while the BHK-21, HEK 293 and HeLa cells were cultured in supplemented Dulbecco's modified eagles medium, DMEM (DMEM+GlutaMAX™-1, Gibco®). Cell growth media were supplemented with 10% (v/v) foetal calf serum (FCS, containing up to 50mg/ml serum proteins) (Gibco), 1% penicillin G / streptomycin and 1% Fungin (supplemented/ complete medium). Continuous culture cell monolayers were grown in medium-sized (200ml) sterile Nunclon cell culture flasks (Nunc™) in 20ml culture volumes at a constant temperature of 37^oC. All steps during transfection and Gag VLP uptake were carried out using preheated (37^oC) PBS or cell culture medium.

3.2.6 Transfection of mammalian cells/ Uptake assays

3.2.6.1 Seeding of cell lines

For RNA transfection/ Gag VLP uptake assays the RAW 264.7, BHK-21and HeLa cell cultures were seeded in sterile 6 well plates (Nunc™) in 2ml complete/supplemented at a concentration of 0.5 x 10^5 cells/ml per well, while HEK 293 cells were seeded as 0.2 x 10^5 cells/ml per well 24 hours prior to the assay.

3.2.6.2 RNA transfection

Quantitated *in vitro*-transcribed CAT RNA transfection into the above cell lines was carried out using a non lipid cationic reagent, Transmessenger™ Transfection Reagent (Qiagen). This transfection reagent is used specifically for the transfection of eukaryotic cells with RNA/siRNA. The transfection assay was carried out using kit components according to the manufacturer's protocol. Cells seeded in the 6-well plates were washed twice with 2ml unsupplemented cell culture medium to remove all traces of FCS prior to the addition of the transfection reagent/ specific [CAT RNA] mixture. Each well was then topped up to 1ml with unsupplemented cell culture medium and plates were incubated at 37^o C for 4 hours to allow for transfection. The cell culture medium was then removed from the wells and replaced with 2ml of complete medium and plates incubated for a further 24 hours at 37^o C.

3.2.6.3 VLP uptake

Gag VLP cell uptake assays were carried out using 5µg of quantified Gag VLPs that contained a known amount of CAT RNA. Cells seeded in the 6-well plates were washed twice with 2ml PBS to remove all traces of FCS prior to the addition of Gag VLPs containing the CAT RNA. Well volumes were then topped up to 1ml with unsupplemented cell culture medium and plates were

incubated at 37^{o} C for 2 hours to allow VLP uptake. The cell culture medium was then removed from the wells and replaced with 2ml of complete medium and plates were incubated for a further 24 hours at 37^{o} C.

3.2.6.4 Analysis of RNA transfection/cell uptake assays

A CAT ELISA kit (Roche) was used for the analysis of the RNA transfection/cell uptake assays according to the method described in Appendix A (9). Mammalian cell lines were lysed using the CAT ELISA kit lysis buffer and lysates were diluted in the provided sample buffer before assaying. Assay negative controls were not displayed in results as they fell within the background reading range.

3.2.6.5 RNA/VLP treatment:

Prior to RNA transfection or Gag VLP uptake in mammalian cells, RNA/VLP samples were treated with short wavelength (260nm) ultraviolet radiation or heat treatment at 62^{o} C.

For UV treatment, CAT RNA/Gag VLP samples were irradiated with a UV source that delivered 1J/sec/cm^{3} for 1-2 minutes during which samples were removed periodically for the transfection/uptake assays.

Heat treatment of CAT RNA/Gag VLP samples was based on the method derived from Poon *et al*., (2005) used for inactivating live HIV. CAT RNA/Gag VLP samples were incubated at 62^{o}C for 10 or 20 minute periods prior to their use in the transfection/uptake assays.

3.3 Results

3.3.1 CAT RNA quantitation:

Gag-VLPs produced by the co-expression of the hm*gag* ORF and a CAT ORF described in the previous chapter were analysed for the encapsidation of the CAT-encoding RNA species.

A preliminary qualitative reverse transcriptase (RT)-PCR was carried out on RNase-treated VLP extracts using CAT-specific primers to detect the encapsidation of CAT RNAs. As this was simply a qualitative test to detect CAT encapsidation, 10^{-2} diluted VLP extracts were added directly to the RT-PCR. Positive RT-PCR results were identified by the amplification of a 350bp band as seen for the positive control, which was generated by utilising *in vitro* transcribed CAT RNA (Figure 3.1). The negative control derived from uninfected insect cell supernatant correspondingly did not amplify RNA in the RT-PCR. RT-PCRs of 3 individual VLP preparations (5' psi, 3' psi, no psi) each amplified a 350bp band, which confirmed the encapsidation of the CAT RNA species by Gag VLPs.

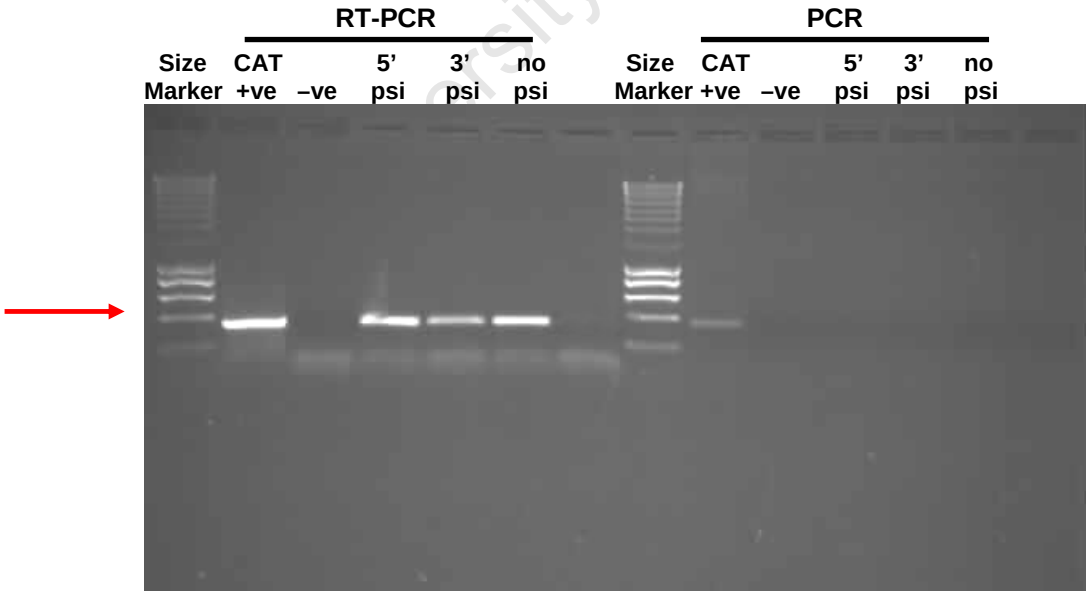


Figure 3.1 Agarose gel displaying CAT-specific PCR and RT-PCR products made from VLP extracts. 5'psi and 3'psi denotes CAT RNA species that contained the HIV psi-site at the 5' and 3' ends of the CAT ORF respectively, while no-psi refers to a CAT RNA species that lacks any psi-tag. Positive controls for RT-PCR and PCR consisted of *in vitro* transcribed CAT RNA and CAT ORF respectively. Red arrow indicates the position of the 400bp reaction product.

The observed positive result could have arisen due to the presence of contaminating recombinant baculovirus that carried CAT encoding DNA in the VLP preparation. To confirm that the positive results observed on the gel was a result of CAT RNA encapsidation by VLPs, the VLP preparations were also subjected to a CAT-specific PCR using the same primer set (Figure 3.1). This PCR would amplify any CAT containing DNA present in the VLP preparation, which could only be present in the form of a baculovirus contamination. All VLP preparations (5' psi, 3' psi, no psi) subjected to this PCR failed to amplify a 350bp band, which confirmed the absence of CAT DNA. Negative PCR results from this reaction thus confirm the absence or undetectable levels of contaminating baculovirus or encapsidated baculovirus DNA.

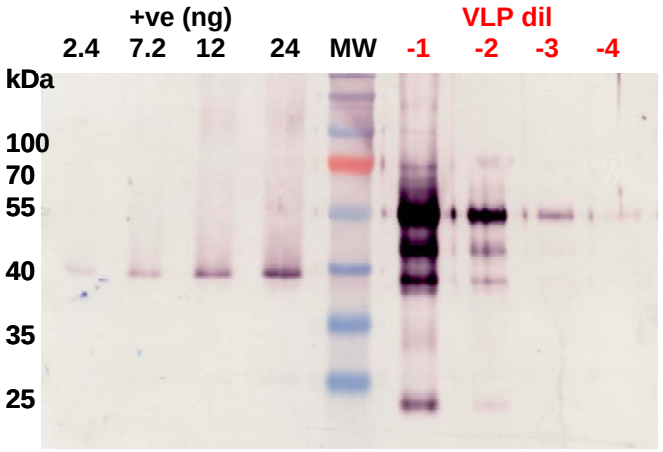
Initially, attempts were made to enhance CAT RNA encapsidation through the addition of the described HIV-RNA packaging signal (psi-site) to either the 3' or 5' end of the CAT encoding RNA species. Results from the above qualitative analysis (RT-PCR) and the following quantitive analysis (real-time RT-PCR) show that there is no particular encapsidation bias conferred to psi-tagged CAT RNA during VLP assembly (results not shown). The addition of the ψ-sequence to the CAT sequence did not only not lead to an enhanced CAT RNA encapsidation, it also conferred no particular level of control to CAT RNA encapsidation. This variable level of CAT RNA encapsidation exhibited by the 5' or 3' ψ-tagged CAT RNA was similar to that exhibited by untagged CAT RNA. High levels of CAT expression from the very strong PH baculovirus promoters coupled with the fairly indiscriminate nature of RNA encapsidation could account for the inability of the psi-tagged RNA to be encapsidated at higher concentrations than non psi-tagged CAT RNA. As a result, further experiments in these studies only utilised non-psi tagged CAT RNA.

Real-time RT-PCR was used to determine the amounts of CAT RNA encapsidated by the Gag-VLPs. As this was a quantitive assay, VLP preparations were first quantified by means of quantitive western blot (Figure 3.2 (A)), which enabled RNA extraction from specifically determined concentrations (5µg) of Gag/VLP. Real-time RT-PCR was therefore conducted on VLP extracted RNA samples derived from an equivalent of 1µg of Gag VLPs (Figure 3.2 (B)). All VLP-RNA extractions were done in replicate

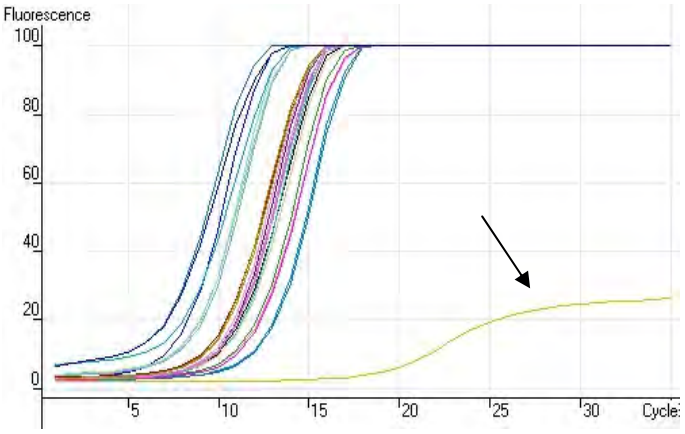
(duplicate or triplicate) and each of these was quantified in triplicate. The positive control for this reaction consisted of an *in vitro*-transcribed CAT RNA species. A dilution series of this positive control was used to form a standard curve to enable VLP-CAT RNA quantitation. Following each real-time RT-PCR, the homogeneity of the quantified reaction product was confirmed with a melt curve (Figure 3.2 (C)). The melt curve from these reactions displayed a single peak for all analysed RNA extracts as well as the *in vitro*-transcribed CAT RNA positive control. This indicated that all quantified, amplified product from the reaction was from the CAT RNA species. The negative control displayed a distinct melting curve peak as indicated by the arrow (Figure 3.2 (C)). The negative control very often produces this separate peak as a result of primer dimer formation.

The real-time RT-PCRs were successful at determining the amount of CAT RNA encapsidated per µg of Gag/VLP and each reaction typically displayed a reaction efficiency of 0.94 (94%) based on standard curve accuracy data (Figure 3.2 (B)). A compilation of the real-time RT-PCR results for 9 separate VLP extractions is displayed in figure 3.2 (D). There was no common trend with respect to the amount of encapsidated CAT RNA/µg Gag VLPs. The encapsidated CAT RNA content was observed to vary from anything between 0.1ng to as high as 8ng/ug of Gag VLP. This variation of the encapsidated CAT RNA content was observed between VLP preparations despite infecting cells with the baculovirus infectant at the same multiplicity of infection.

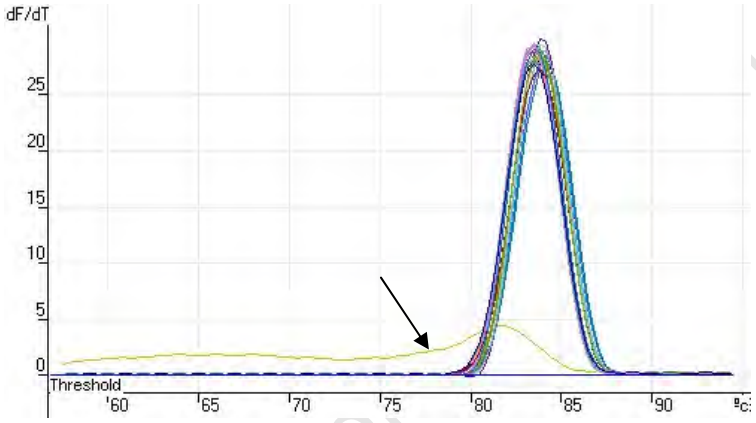
Multiple RNA extractions form the same VLP extract in contrast encapsidated similar amounts of CAT RNA/µg Gag VLP. An inverse trend was noted between the yield of Gag in the VLP extraction and the amount of encapsidated CAT RNA. As this observed trend was beyond the scope of this study, it was not further investigated.



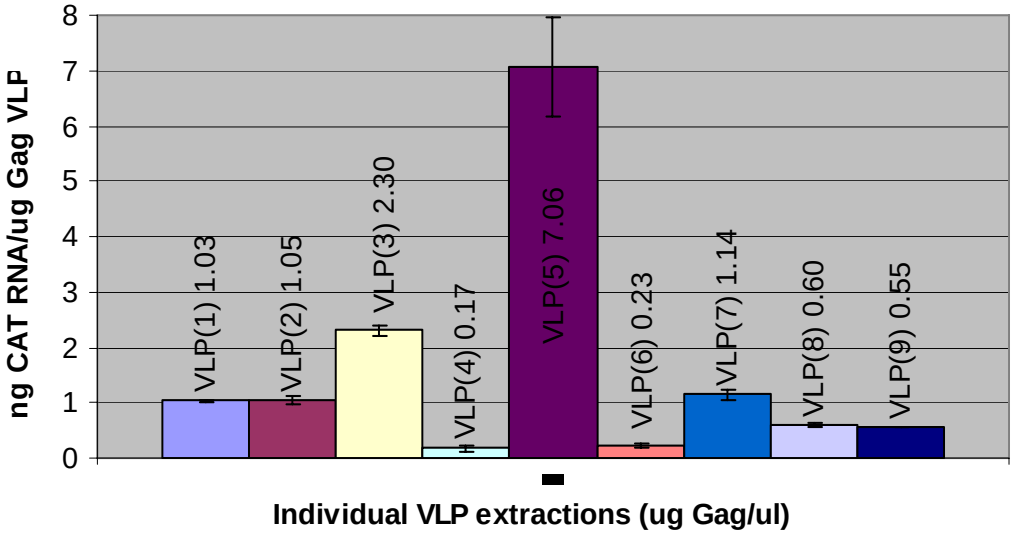
A



B



C



D

Figure 3.2 VLP encapsidated CAT RNA quantitation. (A) Typical quantitive Gag western blot used to determine Gag concentration of VLP extracts. (B) Typical real-time RT-PCR quantitation curves used to determine CAT RNA concentration, arrow illustrates negative control. (C) Typical melt curve to determine real-time RT-PCR homogeneity, arrow illustrates negative control. (D) Bar graph illustrating a compilation of real-time RT-PCR results of multiple RNA extractions from 9 individual VLP extracts. Error bars display the variance between 3 separate real-time RT-PCRs

3.3.2 Cell uptake assays:

Once I had confirmed and quantified encapsidation of CAT RNA by Gag-VLPs, I proceeded with mammalian cell uptake assays to determine whether the Gp64 pseudotyping of Gag VLPs facilitates Gag VLP cellular entry and expression of the encapsidated CAT RNA. The mammalian cell lines selected for these assays are known to be receptive to transduction by baculoviruses (BHK-21, HEK 293 and HeLa cells) (Boyce *et al*., 1996; Shoji *et al*., 1997; Condreay *et al*., 1999; Duisit *et al*., 1999; Airenne *et al*., 2000; Pieroni *et al*., 2001; Abe *et al*., 2003; Gao *et al*., 2007). It was thought that due to the presence of the Gp64 envelope glycoprotein on the VLP surface, the selected cell lines would be ideal to assess whether Gp64 confers the ability to enter these cell lines and express the contained CAT RNA. As a cell uptake control to confirm whether the encapsidated RNA can be expressed within a mammalian cell line, an *in vitro*-transcribed CAT RNA species was transfected into the BHK-21 cell line at various concentrations (50ng-2000ng).

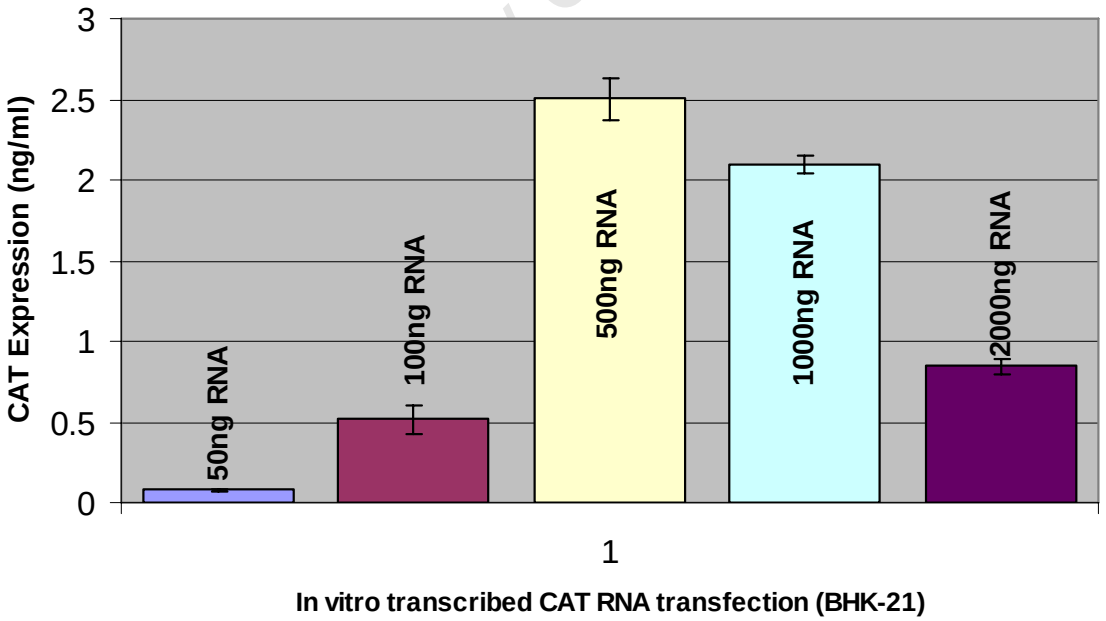


Figure 3.3 Bar graph illustrating CAT expression levels in cell lysates 24 hours after transfection of BHK-21 cells with various amounts of *in vitro* transcribed RNA. Error bars display the variance between 3 separate experiments

Twenty four hours post transfection, the cells were lysed and lysates were analysed for CAT expression by means of a CAT ELISA. RNA transfection results shown in figure 3.3 demonstrate that the VLP encapsidated CAT RNA species can be translated/expressed within mammalian cells. It was observed that 50 to 500ng of transfected CAT RNA exhibited a linear relationship between the amount of transfected RNA and CAT expression levels. Optimal transfected CAT expression levels were observed for the 500ng CAT RNA transfection. When more than 1000ng of CAT RNA was transfected into cell lines, CAT expression levels were observed to decrease. This is most likely due to a toxic effect caused by transfection of high RNA concentrations.

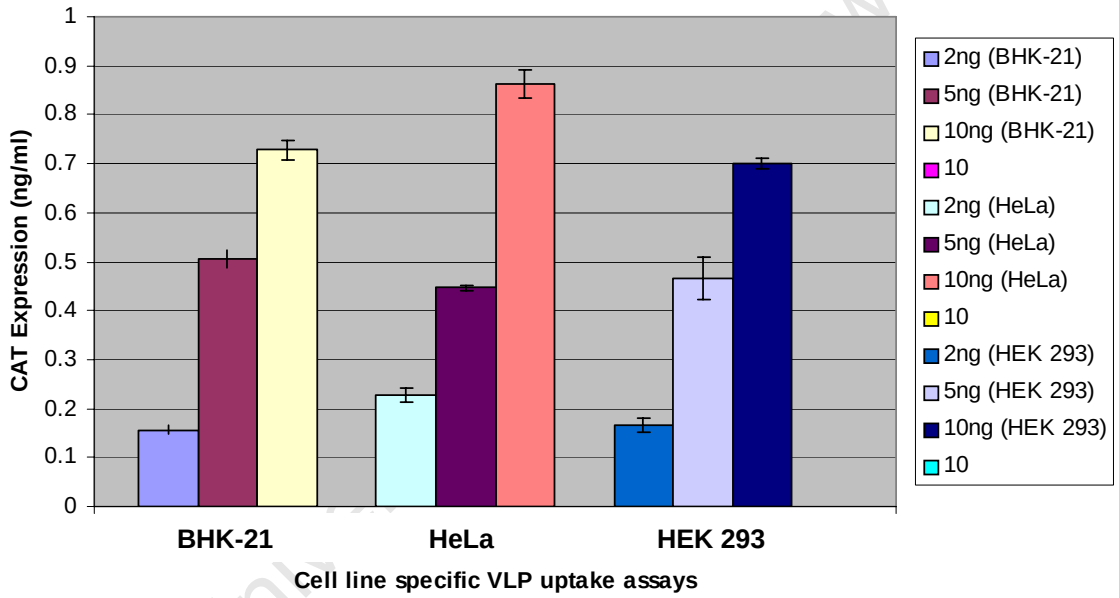
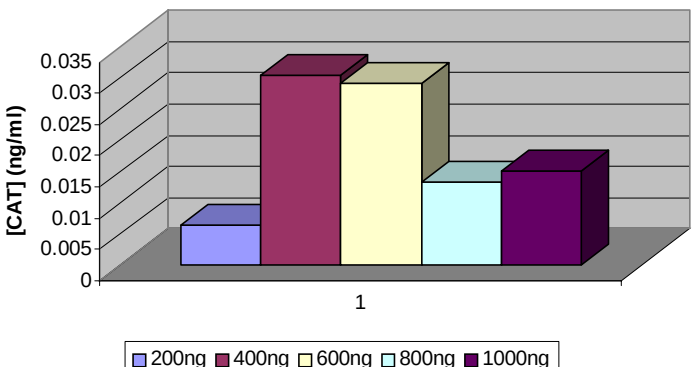


Figure 3.4 Bar graph illustrating CAT expression levels of BHK-21, HeLa and HEK 293 cell lysates 24 hours after VLP uptake assay. Figure key indicates the amount of CAT RNA within the VLP preparation used in each uptake assay. Error bars display the variance between 3 separate experiments

Once it was established that the CAT RNA species could be expressed in mammalian cell lines, we proceeded with the VLP-cell uptake assays. Gag VLPs that contained known concentrations of CAT RNA were subsequently analysed for their ability to enter mammalian cells and express the foreign CAT RNA species. The selected BHK-21, HeLa and HEK-293 cell lines were grown as 2ml culture volume monolayers in 6 well plates. These cell monolayers were exposed to Gag VLP concentrations that contained a total of 2ng, 5ng and 10ng of CAT RNA for 2 hours to allow Gp64-mediated VLP

uptake before washing cells and incubating them for a further 24 hours (to allow for CAT expression). CAT ELISA results of the cell lysates shown in figure 3.4, indicate that CAT was produced in all cell lines. Expression levels between the 3 different cell lines appeared similar ranging from 0.15 – 0.22; 0.44 – 0.5; 0.7 - 0.86ng/ml of CAT, when transfected with 2, 5 and 10ng of VLP CAT RNA respectively and thus appeared directly proportional to the amount of VLP/encapsidated CAT RNA used in the uptake assays. Thus baculovirus-derived, and hence Gp64 pseudotyped Gag VLPs that encapsidate foreign RNA species are capable of entering mammalian cell lines and expressing the encapsidated RNAs.

These CAT-containing Gag VLPs were also used to conduct a cell uptake assay with a cell line previously shown not to be receptive to baculovirus-Gp64 mediated transfection (macrophage RAW 264.7) (Schauber *et al*., 2004; Masayuki *et al*., 2006). CAT-specific ELISA of the RAW 264.7 cell lysates did not yield any quantifiable results that were above background levels and therefore failed to detect any CAT expression (not shown). This cell line appeared to be equally resistant to the expression of RNA carried by the Gp64-pseudotyped Gag VLPs as well. As a control in this instance to determine whether the CAT RNA can be expressed in this cell line, CAT RNA was also transfected into the RAW 264.7 cell line. Cell lysates were analysed by CAT-specific ELISA and CAT expression was readily detected (Figure 3.5).



It was here established that the productive transmission of a foreign nucleic acid from a so-called “inert” vaccine is possible and as previously indicated this is regarded as a problematic trait. As the transmission and expression of the RNA content encapsidated by the Gag VLPs pose a problem for vaccine regulatory bodies, attempts were made to “neutralize” transmission/ expression of CAT RNA from VLPs by means of virus inactivation.

It was initially decided to expose the VLPs to various doses of short wavelength (254nm) UV radiation previously shown to be sufficient to inactivate RNA viruses. Gag VLPs carrying 23ng CAT RNA/cell uptake assay was irradiated with specific amounts (0 – 60 Joules/cm^2) of short wave UV light prior to the cell uptake assay in BHK-21 cells. The BHK-21 cell line was randomly selected as the test mammalian cell line. Results of individual cell uptake assays using the UV irradiated VLPs are shown in Figure 3.6. The UV irradiation of Gag VLPs evidently reduced CAT expression to almost 50% of the non-UV treated (0 Joules) Gag VLPs. It must be noted though that despite the approximately 50% decrease in expression levels, CAT expression still remained quite high. The levels to which the expression of CAT has been reduced appears to be independent of the amount of UV exposure as CAT expression remained constant for the entire UV exposure range (0-60 Joules cm^2), which was previously shown to be more than sufficient to neutralize RNA viruses. Frequently, UV exposure doses that fall into the 100-1000 millijoule range have decreased virus activity by several log values (Simonet *et al*., 2006; Evans *et al*., 1969; Ko *et al*., 2005).

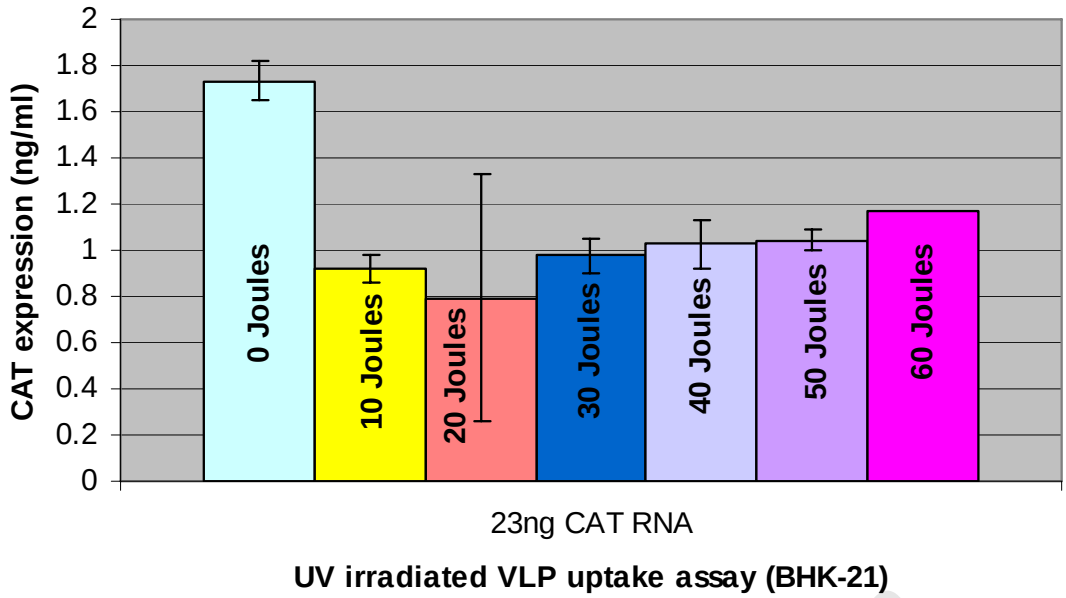


Figure 3.6 Bar graph illustrating CAT expression levels of BHK-21 cell lysates 24 hours after VLP uptake assay with UV (254nm) irradiated Gag VLPs. Each bar represents a separate uptake assay with VLP irradiated with 0, 10, 20, 30, 40, 50 or 60 Joules/cm^2. Error bars display the variance between 3 separate experiments

To determine if the observed decrease in CAT expression resulted from RNA damage caused by UV, *in vitro*-transcribed CAT RNA was also subjected to UV irradiation. In this control experiment *in vitro*-transcribed CAT RNA samples (1000ng) were irradiated with a larger 0-120 Joules/cm^2 UV exposure range before transfection into BHK-21 cells (Figure 3.7). UV irradiation resulted in CAT expression levels decreasing to ~20% of the expression levels resulting from unexposed CAT RNA transfection. RNA samples appeared to be more sensitive to UV than encapsidated RNA, as determined by CAT expression levels, which exhibited an approximate 50% expression level following UV exposure. Results from this control assay, therefore, suggested that the decreased CAT expression could have resulted from RNA damage. However, as with the previous assay, expression was still detectable after 120 Joules/cm^2 of exposure and CAT expression levels also appeared to be independent of the amount of UV exposure in this range.

The combined results from these 2 VLP/RNA – UV exposure cell uptake assays suggest that the diminished ability of the VLP to express the encapsidated CAT RNA arises through RNA damage caused by UV exposure.

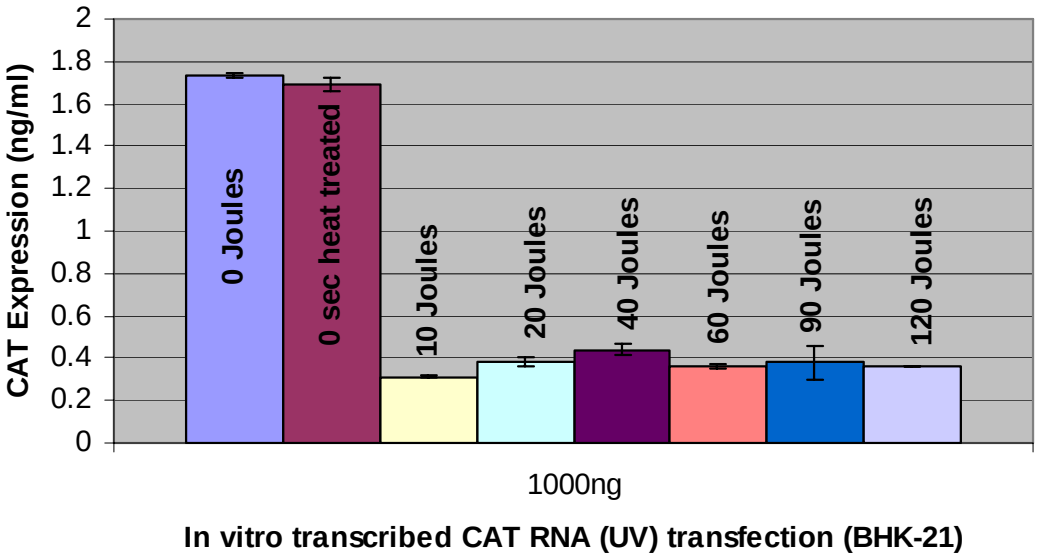


Figure 3.7 Bar graph illustrating CAT expression levels of BHK-21 cell lysates 24 hours after control *in vitro* transcribed RNA transfections. RNA was irradiated with UV (254nm) Each bar represents a separate RNA transfection with RNA irradiated with 0, 10, 20, 40, 60, 90 or 120 Joules. RNA was also exposed to 62^{o}C for 20 minutes prior to transfection. Error bars display the variance between 3 separate experiments

UV irradiation of VLPs was therefore unable to completely neutralize CAT expression. Additionally, it was determined that encapsidated RNA is less UV sensitive than naked RNA, suggesting an effective shielding provided by the capsid. An alternative HIV heat-based inactivation technique derived from Poon *et al*. (2005), was therefore used. The original technique required formaldehyde treatment of live virus particles followed by heat inactivation. As described, formaldehyde is used as a cross-linking agent and serves to stabilize or prevent the shedding of Gp120. As the VLPs currently under investigation did not possess any Gp120 that needed to be retained we therefore overlooked this step. Furthermore, formaldehyde treatment of VLPs could pose a complication with respect to toxic effects caused by residual formaldehyde in the final VLP sample. Heat was required to "disrupt" the surface particle structure, after which a heat inactivated Gp64 could be tested for its ability to mediate entry and CAT expression in mammalian cell lines. Gag VLPs carrying 2, 5 and 10ng of CAT RNA were incubated at 62^{o} C for 10 or 20 minutes periods prior to cell uptake in BHK-21, HEK 293 and HeLa cell lines. Twenty four hours following cell uptake, cell lysates were analysed for CAT expression by CAT ELISA (Figure 3.8).

VLPs not exposed to heat exhibited high CAT expression levels in the transfected BHK-21; HeLa and HEK-293 cell lines. CAT expression levels in the BHK-21 cell line were: 0.15, 0.50 and 0.73ng/ml; the HeLa cell line exhibited expression levels at 0.20, 0.43 and 0.87ng/ml and the HEK-293 cell line exhibited expression levels at 0.15, 0.45 and 0.70ng/ml when transfected with Gag VLPs containing 2, 5 and 10ng of CAT RNA respectively (Figure 3.8 (A), (B) and (C)). Heat treatment of VLPs for either 10 or 20 minutes in both BHK-21 and HeLa cell lines resulted in a significant reduction in CAT expression levels.

CAT expression levels in the BHK-21 cell line appeared strongly restricted by the VLP heat treatment as it exhibited CAT expression levels that approached practically undetectable levels. The HeLa cell line exhibited slightly higher expression levels for each concentration of heat treated VLPs used in the uptake assay in comparison to the BHK-21 cell line. These results show that heat - treatment of VLPs prior to exposure to cell BHK21 and HeLa cell lines can cause a significant reduction in CAT/RNA expression levels.

Cell uptake assays using heat treated VLPs with the HEK-293 cell line, on the contrary, appeared to have very little effect on VLP uptake and CAT RNA expression for higher VLP/encapsidated RNA concentrations. This may suggest that the HEK-293 cell line employs an alternate uptake mechanism to the proposed Gp64-mediated uptake pathway that may be used by the BHK-21 and HeLa cells lines.

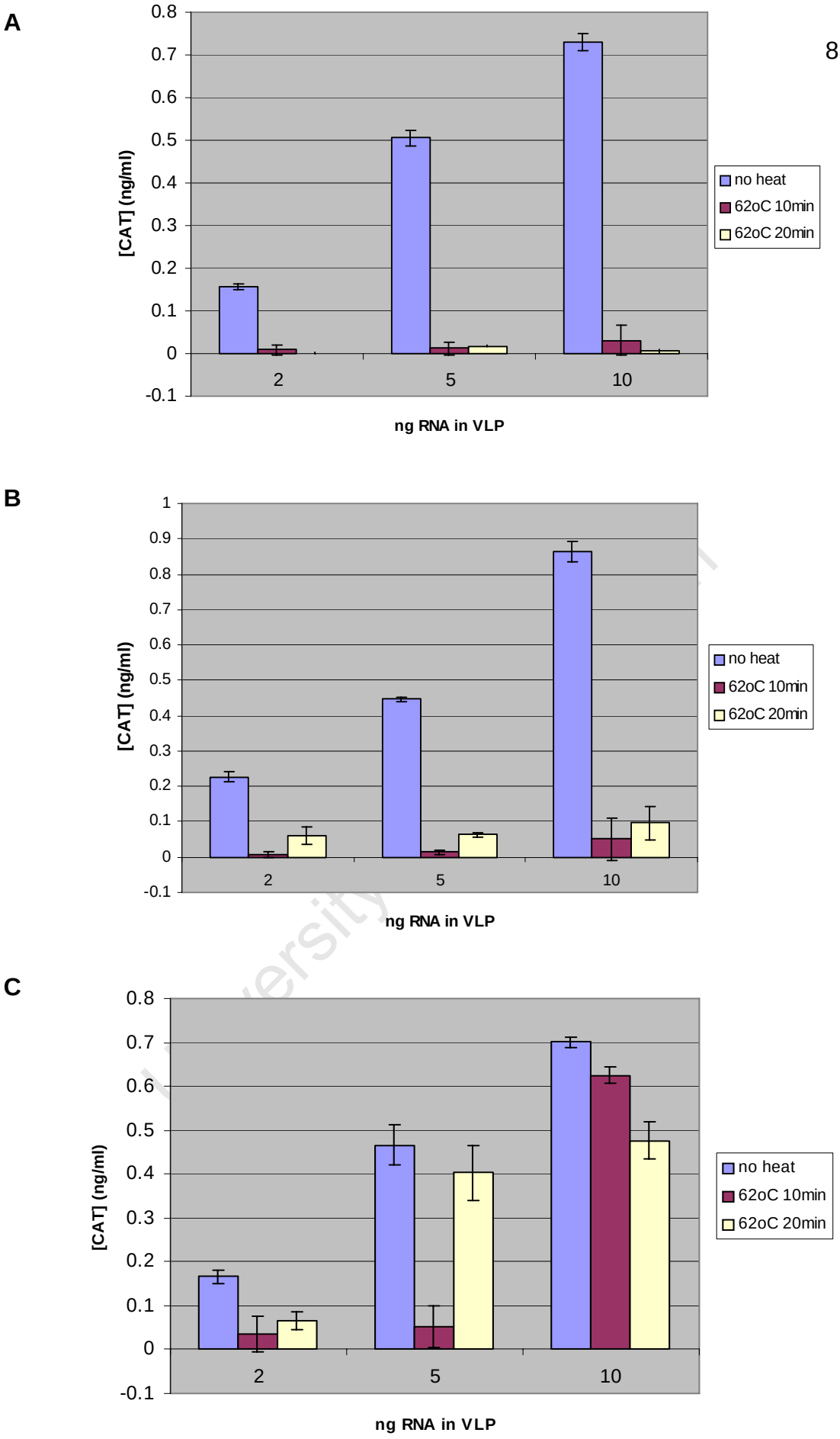


Figure 3.8 Bar graphs illustrating CAT expression levels of (A) BHK-21, (B) HeLa and (C) HEK 293 cell lysates 24 hours after a heat treated VLP uptake assay. Gag VLP containing 2, 5 and 10ng of CAT RNA were used for the cell uptake assays in each cell line and prior to each uptake, VLP were not exposed to heat, incubated for 10 min at 62^{o}C or for 20min at 62^{o}C. Error bars display the variance between 3 separate experiments

To determine whether the reduction of CAT expression caused by heat treatment of VLPs is a result of RNA damage or possible Gp64 damage, *in vitro*-transcribed RNA was also incubated at 62^{o}C for 20 min prior to transfection into BHK-21 cell lines. Cell lysates were analysed by CAT ELISA 24 hours later and results shown in figure 3.7 demonstrate that heated CAT RNA express almost identical levels of CAT when compared to non-heated CAT RNA. Results suggest that as heat has no effect on CAT-expressing RNA, the reduced levels of CAT expression is most likely due to Gp64 damage on the VLP surface.

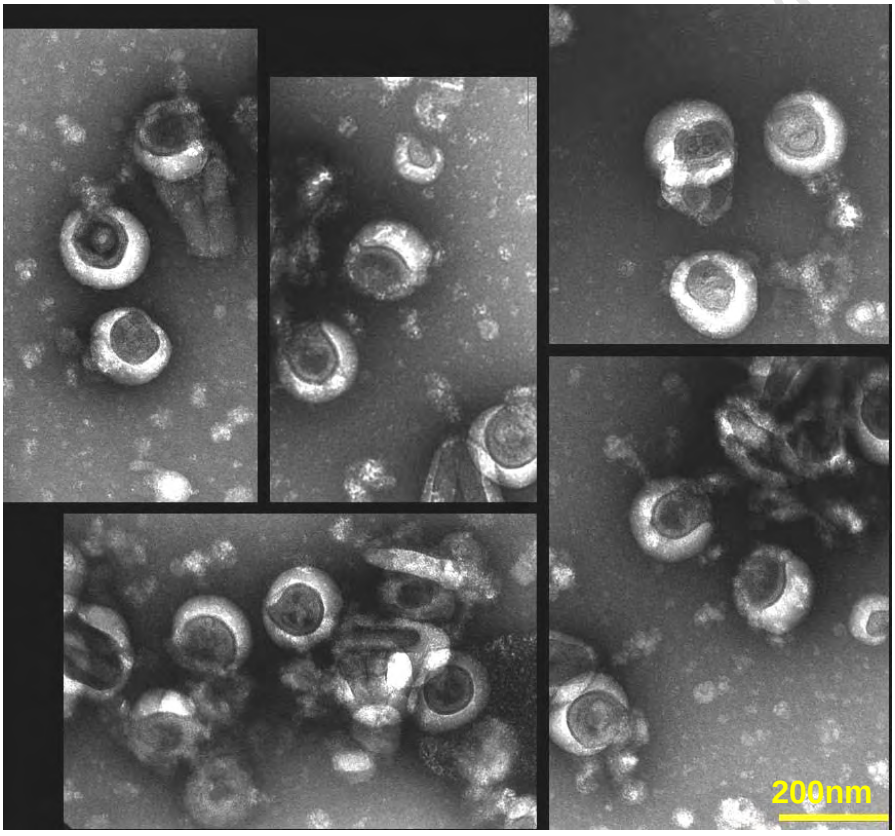


Figure 3.9 Transmission electron micrographs of Gag VLPs heat treated at 62^{o}C for 20 minutes

The significant reduction of CAT expression levels in BHK-21 and HeLa cell lines is more than likely a product of disrupted Gp64 envelope glycoprotein but it could also have resulted from VLP disruption/lysis during heat treatment. A counter argument was that if any significant disruption to the heat-treated VLPs had occurred, the non-heat affected expression levels in the HEK 293

cell line would not have been observed. To nevertheless visually determine whether the heat treated VLPs were structurally damaged or lysed by the heat treatment, these VLPs were visualized by TEM. Figure 3.9 displays a series VLP electron micrographs of VLP heated to 62^{o}C for 20 minutes. The images here display VLPs as intact doughnut shaped particles similar to the non-heated particles displayed in figure 2.4. The frequency of these intact particles when visualized by TEM also appeared unaffected. This suggests that heat treatment of Gag VLPs, as with similar live HIV inactivation techniques, does not appear to disrupt the particles.

3.4 Discussion:

The results presented in this chapter comprehensively show that BEVS-produced, Gp64 pseudotyped Gag VLPs are able to enter mammalian cell lines and deliver non-specifically encapsidated RNA for expression.

3.4.1 RNA encapsidation:

Encapsidation of the reporter CAT RNA was initially confirmed by means of RT-PCR, where all VLP preparations that were produced during CAT RNA co-expression in the BEVS encapsidated this RNA species. On the contrary, CAT ORF-specific PCR of the same VLP preparations failed to detect any CAT-encoding DNA in the VLP preparation, which could only be derived from recombinant baculoviruses. The absence of CAT-encoding DNA suggests that baculovirus contamination of the VLP preparation was either absent or had been kept to an undetectable minimum. This lack of baculovirus contamination could be attributed to the cross-flow filtration technique used during the isolation of VLPs. The negative PCR result obtained here also suggests that the Gag VLP-Gp64 quantitation analysis performed in Chapter 2 was either minimally or completely unaffected by the presence of contaminating baculoviruses.

Real time RT-PCR quantitation analyses of RNA extracts from VLP preparations indicated that encapsidated CAT RNA levels varied significantly (0.1 – 8ng CAT RNA/µg Gag VLPs) between individual VLP preparations. If we accept that 1ug of Gag VLPs is made up of ~2.2 x 10^9 VLPs (Refer to **2.4**), this would indicate that ~2.2 x 10^9 VLPs would encapsidate between 0.1 and 8ng of CAT RNA. If we also accept that a CAT-encoding RNA has a total relative molecular mass of 212 kDa, which is 3.5 x 10^{-10}ng, this suggests that 1µg of Gag VLPs encapsidates between 2.8 x 10^8 and 2.3 x 10^{10} copies of CAT RNA which also equates to between 0.1 and 11 copies of CAT RNA per Gag VLP.

Attempts were made to control the amount of encapsidated RNA in Gag VLPs through the addition of the HIV RNA encapsidation signal (ψ-site) to either the

5’ or 3’ ends of the CAT RNA. Data not presented in this thesis indicated that the incorporation of this encapsidation signal was unable to confer any level of consistency with respect to the amount of CAT RNA encapsidated. As suggested, the reason for this was most likely a result of the strong baculovirus promoters used in the BEVS to express CAT, along with the fairly indiscriminate nature of RNA encapsidation which occurs during VLP assembly. In other words, the CAT mRNA concentration was probably so high that whether or not it had the ψ-site, it would be the predominant species encapsidated. As a control to detect any ψ-site directed packaging I could express the reporter, ψ-tagged RNA from a weaker promoter and monitor encapsidation and compare encapsidation levels in NC deleted and WT Gag

3.4.2 Uptake assays:

Research discussed earlier (refer to **3.1.1**) demonstrated that HIV particles that lack envelope glycoproteins are most likely to be degraded in endocytic vesicles. The presence of high concentrations of Gp64 on the VLPs were therefore tested to determine if they enabled Gag VLP entry and encapsidated CAT RNA expression in mammalian cell lines. Cell lines tested in the uptake assays (BHK-21, HEK 293, HeLa) were all previously shown to be permissive to recombinant baculovirus uptake and expression of their nucleic acids. These cell lines, when used in VLP uptake assays, expressed encapsidated CAT RNAs at similar levels. In these assays, expression of recombinant baculovirus CAT DNA is unlikely to contribute to the overall CAT expression levels as expression in mammalian cell lines requires mammalian promoter sequences. These encapsidated CAT RNAs are representative of other RNAs normally present in the VLP expression system, which are encapsidated during assembly.

Results in this study therefore for the first time demonstrate that there is a strong probability for the expression of randomly encapsidated, expression system produced CAT RNAs in mammalian cell lines following VLP inoculation. The mouse macrophage RAW 264.7 cell line, which is known to be non-permissive to recombinant baculovirus entry and

gene expression, was similarly unable to express CAT RNA carried by Gp64 pseudotyped Gag VLPs (data not shown). Results therefore suggest that Gag VLP entry into the tested cell lines is mediated by baculovirus Gp64. This notion is supported by the VLP inactivation experiments conducted. The VLP heat inactivation experiments showed that incubation of VLPs at 62^{o}C could reduce CAT expression in certain cell lines (BHK-21 and HeLa) to almost undetectable levels. Heat treatment was also shown to have no effect on the virion CAT RNA content over a time course of 20 minutes, while it also did not detectably disrupt/ lyse the VLPs. Inhibition of CAT expression is therefore assumed to be a result of Gp64 damage on the VLP surface.

CAT expression levels at higher uptake concentration in the HEK 293 cell line on the contrary appeared unaffected by VLP heat treatment. This may suggest that HEK-293 cell lines employ an additional/alternate VLP cell uptake pathway to that used by the BHK-21 and HeLa cell line. This unknown pathway would thus be biased for VLP entry upon VLP Gp64 disruption. As the delineation of the exact VLP uptake pathways that lead to RNA expression was beyond the scope of this study, these were not further investigated. Future studies could investigate these uptake pathways through the use of specific cell pathway uptake inhibitors or antibodies specific for functional receptors on the virion surface such as Gp64. Similarly it may therefore be more effective to heat treat as well as UV irradiate VLPs prior to inoculation to prevent encapsidated RNA expression. Alternately formaldehyde treatment of the VLPs could also have been used in combination with the heat treatment. As formaldehyde is a cross linking reagent, it could function by simultaneously inactivating any receptors used for alternate VLP uptake pathways used by the HEK-293 cell line.

CAT RNA transfection control experiments were also conducted to determine if the RNA could be expressed in mammalian cell lines as well as to determine the effect of heat and UV on the CAT RNA. It was clear that when CAT RNA was encapsidated by Gag VLPs, very low concentrations (2, 5, 10ng) produced relatively high expression levels in the tested cell lines. Transfection of *in vitro*-transcribed CAT RNAs, on the contrary, required much higher RNA concentrations (20-fold in some instances) to attain similar expression levels.

This may suggest that if the concentration of the Gag VLPs encapsidated RNA could be modulated, they could serve as highly efficient gene delivery/transient expression vectors.

Heat treatment of VLPs (at 62^{o}C for 10 – 20 minutes) effectively reduced transmission and expression of RNA carried by Gag VLPs in BHK-21 and HeLa cell lines through the disruption of Gp64. Functional Gp64 is suggested to play an important role in stimulating cellular immune responses to BEVS-produced Gag VLPs by enabling cellular entry (refer to **1.6.5**; **4.1.2**). Disruption of Gp64 after heat treatment possibly does not alone contribute to the reduced CAT expression levels and the reduced CAT expression could result form the disruption of any of several unexplored membrane factors affected during heat treatment. Alternatively, the separate pathway utilized by the HEK-293 cell lines may still enable cell entry and hence cellular immune responses despite Gp64 disruption. Whether heat treatment/inactivation of VLPs therefore has any bearing on VLP stimulated cellular immune responses is assessed in the following chapter.

Chapter 4

Heat-treated VLP Immunogenicity

4.1 HIV-1 Cellular immune responses:

The precise correlates of protection for an adequate/protective immune response against HIV remain unclear. Data presented in numerous immunology studies, though, have aided towards achieving an understanding and towards the decrypting of the requirements for a protective immune response.

Several studies have documented the importance of stimulating effective cellular immune responses in combination with a neutralizing humoral immune response to control/prevent an HIV infection. Effective host anti-HIV immune responses do not exhibit the classical protective immune response paradigm, where humoral immune responses would prevent viral infection. Instead, cellular immune responses reduce viraemia in HIV-infected individuals and prevent the development of disease in long-term-non-progressors (LTNPs) (see references below).

Several convincing lines of evidence highlight the importance of cellular immune responses. Protective cytotoxic T-cell responses have been demonstrated in human LTNPs before the development of symptoms and parallel a decrease in plasma viral RNA (Pantaleo *et al*., 2004; Rowland–Jones *et al*., 1998; Kaul *et al*., 2001; Harari *et al* 2003). Furthermore, the maintenance of HIV-1 specific T cell responses following the disruption of antiviral therapy in HIV-1 infected individuals is associated with viraemia control (Rosenberg *et al*., 2000). Vaccine-induced, cell-mediated immune responses in monkeys have successfully controlled challenges by the SHIV89.6 AIDS virus model (Amara *et al*., 2002; Barouch *et al*., 2000; Shiver *et al*., 2002; Belyakov *et al*., 2001). The role of CD8 T cells has been highlighted in studies where depletion of CD8 T cells in infected monkeys resulted in a loss of virus control, but virus levels were subsequently controlled following the restoration of CD8 T cell responses (Jin *et al*., 1999; Schmitz *et al*., 1999; Table 1.1, Rowland-Jones *et al*., 1997; Borrow *et al*., 1994; Koup *et al*., 1994; Ogg *et al*., 1998).

4.1.1 VLP-stimulated immune responses

Distinct antigen processing pathways have been established for the presentation of foreign antigens. Endogenous antigens derived from viral/bacterial infections and DNA vaccines, typically enter the CD8, MHC-1-driven CTL immune response. The CTL response plays a key role in the adaptive/memory immune response by eliminating cells infected with intracellular pathogens. Several studies have demonstrated the importance of strong specific CTL responses during acute HIV infections (see references in next paragraph).

Non-infectious HIV VLPs are like “inactivated viruses” in that they are considered exogenous antigens. They induce the MHC-2-restricted cellular and humoral immune responses and should typically be unable to induce a MHC-1-driven CTL response. HIV/SIV-derived VLPs have been found, nevertheless, to efficiently stimulate the MHC-1-driven CTL responses as well as humoral immune responses in spite of the absence of cellular infection or intracellular replication (Table 1.1, Jaffray *et al*., 2004; Marsac *et al*., 2002; Bachmann *et al*., 1996; Buonaguro *et al*., 2002; 2006; Ruedl *et al*., 2002; 2005, Kuate *et al*., 2006; Deml *et al*., 1997; Paliard *et al*., 2000; Deml *et al* 2005).

4.1.2 Pseudotyped VLPs

Pseudotyping VLPs has often been used to manipulate particle fusogenicity and hence their immunogenicity. This is achieved by the presentation of modified/stabilised HIV or heterologous (MLV, VSV, Baculovirus) envelope glycoproteins on the VLP surface to facilitate their entry into antigen presenting cells. This, in turn, enhanced Gag and Env-directed antibody as well as CTL responses (Table 1.1; Guo *et al*., 2003; Bellier *et al*. 2006; Marsac *et al*., 2002; Kuate *et al*., 2006; Granelli-Piperno *et al*., 2000; Schauber *et al*., 2004). In a study conducted by Buonaguro *et al*. (2006), HIV VLPs displaying HIV-gp120 derived from a Ugandan HIV isolate were efficiently taken up by monocyte-derived dendritic cells (MDDC). Uptake occurred primarily by actin dependent macropinocytosis and endocytosis,

which induced maturation and activation of MDDC (Buonaguro *et al*., 2006). These activated MDDC showed enhanced Th1- and Th2-cytokine production and were able to activate autologous naïve CD4 T-cells and drive them towards a Th-1 response. In a previous study these VLPs were shown to successfully stimulate strong CD4 and CD8 T-cell responses in mice and generated cross clade neutralizing antibody responses in the absence of adjuvant (Buonaguro *et al*., 2002; 2005).

The vesicular stomatitis virus G-envelope glycoprotein (VSV-G) has also commonly been used to pseudotype HIV/SIV VLPs to enhance immune responses, by allowing entry into a broad range of cells including dendritic cells (Bellier *et al*. 2006; Marsac *et al*., 2002; Granelli-Piperno *et al*., 2000; Kuate *et al*., 2006; 2003). Immunization of monkeys with VSV-G pseudotyped SIV particles generated SIV-specific humoral and cellular immune responses, which significantly reduced peak viraemia levels of a challenge virus (Kuate *et al*., 2003; 2006). Peak viraemia dropped to almost 20-fold lower in VLP-inoculated monkeys in the 2006 study, which in general remained below detection limits for 120 weeks post infection. In mice, HIV VSV G-pseudotyped VLPs stimulated Gag-specific IgG and IgG1 antibody titres that were ~100 fold higher than non-pseudotyped VLPs. As a control, a fusion defective VSVG envelope was also displayed on the HIV VLPs and was not able to enhance the VLP immune response. These researchers speculate that the fusion competent VSV G enabled cytoplasmic delivery of VLP components, which resulted in enhanced MHC presentation of epitopes. Due to the broad target cell range of the VSV G envelope glycoprotein pseudotyped particles, toxic effects have been observed. Alternate envelope glycoproteins have been investigated that could confer the same enhanced immunogenicity to the pseudotyped particle but with a limited target cell range.

Enhanced immunogenicity results have been corroborated in several other studies, which have pseudotyped HIV VLPs with influenza HA, MLV env, VSV-G Env and Baculovirus Gp64, where the presence of fusion competent envelope glycoproteins enabled both MHC-1 and -2 Gag protein processing pathways (Guo *et al*., 2003; Bellier *et al*., 2006; Marsac *et al*., 2002; Schauber

et al., 2004). The previously described vaccine study by Bellier *et al*., (2006) that utilised MLV+Env based plasmo-VLPs has also partly attributed the successful stimulation of strong T-cell responses to the presence of the MLV Env. In relation to this study, Wong *et al*., (2005) showed that VLPs that did not display Env were unable to induce immune responses that were stronger than a DNA vaccine that produced soluble polypeptides unable to assemble into particles.

Pseudotyping Gag VLPs with functional/fusion competent envelope glycoproteins thus appears to be of paramount importance in stimulating both MHC-1 and MHC-2 Gag processing pathways. The envelope glycoproteins mediate an enhanced immune response by enabling cellular entry, in a manner which does not result in VLP lysosomal degradation.

Results shown in chapter 3 demonstrated, though, that the entry of Gp64 pseudotyped Gag VLPs into several mammalian cell lines is accompanied by the expression of reporter CAT RNAs carried by the VLPs. As expression of the foreign encapsidated nucleic acids is not a desired VLP trait, data in chapter 3 indicated that disruption of Gp64 envelope glycoprotein by heat treatment could prevent uptake in several cell lines. Heat inactivation of the Gp64 envelope glycoprotein significantly reduced expression in the tested BHK-21 and HeLa cell lines. CAT expression levels within the HEK 293 cell line on the contrary appeared unaffected by VLP heat treatment. This may suggest an alternate route of VLP cellular entry in this cell line that, as with Gp64-mediated uptake, also does not result in VLP lysosomal degradation. This alternate pathway would suggest that VLP heat treatment and hence reduced non-degradative cellular entry in some cell lines may not completely terminate MHC-1 driven cellular immune response.

Several other studies have been conducted to further characterize protective cellular immune responses. These studies have shown that the co-production of immune cytokines interferon-γ (IFN-γ) and interleukin-2 (IL-2) are necessary (but not sufficient) for protective cellular immune responses (Harari *et al*., 2003; Sadagopal *et al*., 2005; Kannanganat *et al*., 2007(A); (B); Boaz

et al., 2003; Boaz *et al*., 2002; Iyasere *et al*., 2003; Younes *et al*., 2003; Robinson *et al*., 2007).

4.1.3 Aims:

Heated/inactivated Gag VLPs were thus inoculated into mice to determine whether these had a reduced capacity to stimulate the MHC-1 and MHC-2 arm of the immune response. Heated VLPs stimulated cellular immune responses in mice were gauged by assaying for IFN-γ and IL-2 production in mouse spleens. **This immunogenicity analysis is the first instance where the viability of heat treatment and hence the restriction of foreign RNA expression in an inoculated host was carried out to determine its effect on VLP immunogenicity.** Also, since VLPs have shown to be potent immunogens when administered as boosts, mice that were initially primed with a DNA vaccine, received a heated VLP boost. This prime boost inoculation regimen allowed us to determine if there are any differences between the immunogenicity profiles of heated VLPs when used as single inoculations or in prime-boost regimens.

4.2 Materials and Methods

4.2.1 Inoculation of mice

All animal procedures were approved by the University of Cape Town, Faculty of Health Sciences Animal Ethics Committee. Female H-2^d BALB/c mice were purchased from South Africa Vaccine producers Pty Ltd (Johannesburg, South Africa) and housed at the University of Cape Town Animal Unit. They were allowed to acclimatize for a minimum of 10 days prior to vaccination.

Gag VLPs used for inoculation into mice was prepared and quantified as described in **2.2.6** and **2.2.8** materials and methods. VLP dilutions were heated prior to inoculation as described in **3.2.6.4** materials and methods. The pTHGagC DNA vaccine, which expresses the Du_{422} HIV-1 C *gag* gene (the same isolate that the VLP Pr55Gag amino acid sequence was derived from), was used either as a positive control or as a DNA prime in the mouse inoculation schedule aimed at assessing cellular immune responses (Table 4.1). The Du_{422} HIV-1 C *gag* gene was previously selected for this DNA vaccine as it had closest amino acid similarity (98.2%) to a derived South African subtype C consensus sequence and would thus maximize the number of potential epitopes in common between the vaccine and circulating viruses (van Harmelen *et al*., 2003). The *gag* gene from Du422 was human codon optimised by Operon Technologies Inc., USA. The myristylation site was mutated from MGA to MAA, which prevented virions budding and hence VLP assembly. This DNA vaccine was manufactured by Aldevron and supplied at DNA concentrations of 2mg/ml PBS.

As shown in the mouse inoculation schedule, 14 groups (n = 3) of mice were either primed with the pTHgagC DNA vaccine (100µg) and boosted with varying concentrations of heated or unheated VLPs or given an inoculation of these VLPs (Table 4.1). VLP and pTHgagC inocula were made up in 100µl total volumes made up in sterile Dulbecco's PBS (Sigma®). Mice were inoculated intramuscularly with 50µl per tibialis muscle. At 12 days post VLP inoculation, mice were sacrificed and spleens were removed to analyse cellular immune responses induced by the various vaccine procedures.

Table 4.1. Mouse inoculation schedule. (pTHgag: DNA vaccine, GagRNA: unheated Gag VLP, GagRNAHI: heated Gag VLP)

Inoculation schedule				
Mouse Group With 3mice per group	**Day 0**	**Day 12**	**Day 28**	**Day 40**
1	pTHgag 100ug (**DNA**)	Sacrifice	-	-
2	pTHgag 100ug (**DNA**)	-	pTHGag 100ug (**DNA**)	**Sacrifice**
3	pTHgag 100ug (**DNA**)	-	GagRNA 100ng (**VLP 1**)	**Sacrifice**
4	pTHgag 100ug (**DNA**)	-	GagRNA 200ng (**VLP 2**)	**Sacrifice**
5	pTHgag 100ug (**DNA**)	-	GagRNA 400ng (**VLP 4**)	**Sacrifice**
6	pTHgag 100ug (**DNA**)	-	GagRNAHI 100ng (**VLP H1**)	**Sacrifice**
7	pTHgag 100ug (**DNA**)	-	GagRNAHI 200 ng (**VLP H2**)	**Sacrifice**
8	pTHgag 100ug (**DNA**)	-	GagRNAHI 400ng (**VLP H4**)	**Sacrifice**
9	GagRNA 100ng (**VLP 1**)	**Sacrifice**	-	-
10	GagRNA 200ng (**VLP 2**)	**Sacrifice**	-	-
11	GagRNA 400ng (**VLP 4**)	**Sacrifice**	-	-
12	GagRNAHI 100ng (**VLP H1**)	**Sacrifice**	-	-
13	GagRNAHI 200 ng (**VLP H2**)	**Sacrifice**	-	-
14	GagRNAHI 400ng (**VLP H4**)	**Sacrifice**	-	-

4.2.2 Preparation of mouse splenocytes:

Mouse spleens from each group were pooled from the three mice in each group in 10ml supplemented RPMI (refer to **3.2.5.2**) and broken up into single cells by mashing them with a rubber stopper through a metal grid (Sigma®) placed in a Petri dish. The cell suspension was made up to 50ml with RPMI

(supplemented, **3.2.5.2**) and transferred to a 50ml centrifuge tube. The cell suspension was centrifuged for 5 minutes at 450g. Cell pellets were resuspended in 50ml RPMI and centrifuged as before. After each cell pellet wash, extracellular matter was removed from the suspension using a Pasteur pipette. Cells were next resuspended in 50ml RPMI (supplemented) and viability was determined by staining cells with trypan blue stain and counting them in a Neubauer counting chamber. Erythrocytes were lysed by suspending $50x10^6$ cells in 1ml of RBC lysis buffer (1ml/$50x10^6$ cells/ml) (5mM Tris-HCL, 140mM NH_4CL, pH 7.3, Sigma) for 2 minutes. The cell suspension was centrifuged at 400 g for 7 minutes and the lysed erythrocytes in suspension was discarded. Splenocytes were resuspended in 5ml RPMI (supplemented) and samples of this suspension were stained with trypan blue and counted in a Neubauer chamber. Cells were then prepared to a final concentration of 5 x 10^6 cells/ml in supplemented RPMI medium. The frequencies of antigen (Gag)-specific CD4 or CD8 T cells secreting cytokines in the spleen of inoculated mice were determined by means of interferon-gamma (IFN-γ) and interleukin-2 (IL-2) ELISPOT assays.

4.2.3 ELISPOT assay:

ELISPOT assays were conducted to detect Gag-specific IFN-γ and IL-2 secreting splenocytes using the BD Biosciences kit according to manufacturer's recommendations, which provided all reagents for the assay. Ninety six well ELISPOT plates were coated with IFN-γ or IL-2 capture antibody (5µg/ml in PBS), sealed and incubated at 4^oC for 16 hours. Wells were washed once with 200µl/well blocking solution (R10 medium: RPMI; 1% penicillin G/streptomycin; 10% FCS; 0.1% 2-mercaptoethanol). Blocking solution (200µl/well) was added to the wells and plates were incubated for 2 hours at ambient temperature. The Blocking solution was discarded and Gag as well as control peptide stimulants were added (100µl) in triplicate to the wells in the ELISPOT plate.

Amino acid sequence of Gag-derived peptides used for stimulating cytokine expression in splenocytes corresponded to BALB/c mouse epitopes in Du_{422} HIV-1 Gag. An irrelevant peptide (negative control), concanavalin A (Con A)

(non-specific assay positive control) or RPMI medium (peptide-free culture medium) (background control) (Table 4.2) was included in the assay. Each of the peptide stimulants were added to the wells in triplicate at a final concentration of 2µg/ml (100µl/well). Con A (Sigma®) (positive non-specific stimulant) was added at a concentration of 0.5µg/ml (100µl/well).

Table 4.2 Cytokine stimulants used in ELISPOT assays.

Stimulant/ Control	Description	Amino acid sequence of peptides	Working concentration
Medium	Supplemented RPMI growth medium (Background control)	N/A	
Con A	Assay positive control	N/A	10µg/ml (100µl/well)
irrelevant peptide	H-2K^d binding peptide (CD8 peptide)	TYSTVASSL	2µg/ml (100µl/well)
GagCD8	H-2^d –restricted class I peptide (CD8 peptide)	AMQMLKDTI	2µg/ml (100µl/well)
GagCD4(13)	H-2^d -restricted class II peptide (CD4 peptide)	NPPIPVGDIYKRWIILGLNK	2µg/ml (100µl/well)
GagCD4(17)	H-2^d -restricted class II peptide (CD4 peptide)	FRDYVDRFFKTLRAEQATQE	4µg/ml (100µl/well)

Splenocytes (500 000 cells/100µl) were plated in triplicate (for each stimulant) and incubated for 24 hours, at 37^{o}C in a 5% CO_2 incubator. Splenocyte suspensions were discarded and wells were washed twice with double-distilled water, followed by 3 washes with wash buffer (1x PBS-0.05 tween 20, pH7.4 (Sigma®)). The detection antibody, biotinylated anti-IFN-γ or -IL-2 was diluted in dilution buffer (2µg/ml) (PBS with 10% FCS) and 100µl was added to each well. Plates were sealed and incubated at ambient temperature for 2 hours. The detection antibody solution was then discarded and wells were washed 3 times with wash buffer. Avidin-horseradish peroxidase (avidin-HRP) was diluted (5µg/ml) in dilution buffer and 100µl was added to the wells and plates were then incubated at ambient temperature for 1 hour. The avidin-

HRP solution was discarded from plates, which were then washed 3 times with wash buffer followed by another 3 washes with PBS (Sigma®). The Nova Red Substrate solution (Southern Cross) was prepared by adding 3 1`drops of reagent I, 2 drops of reagent II, 2 drops of reagent III and 2 drops of H_2O_2 to 15ml of H2O. This Nova Red solution (100µl/well) was added to the ELISPOT plates and spots were allowed to develop in the dark and reactions were stopped by rinsing plates with H_2O. ELISPOT plates were air-dried at ambient temperature overnight in the dark. Plates were analysed by scanning the ELISPOT plate wells using the ELISPOT CTL Analyser (Series 3B). Spots were counted using the ImmunoSpot Image Analyzer (Cellular Technology Ltd, Cleveland Ohio) with ImmunoSpot Version 3.2 software. The mean number of spots in triplicate wells was calculated and background subtracted. The values were expressed as net antigen-specific IFN-γ or IL-2 spot forming units (SFUs) per million splenocytes. Positive responses were determined by setting a cut-off value, whereby any values falling below this cut-off were considered as negative. The cut-off value was set at the number of SFU/10^6 splenocytes stimulated by the no peptide (culture medium) assay control added to its corresponding standard deviation. Values displayed on the graphs are positive responses above the determined cut off value.

4.3 Results:

4.3.1 Summary of ELISPOT results:

Immunogenicity comparisons of unheated and heated Gag VLPs were investigated in BALB/c mice (3 per group) as VLP only inoculations (100, 200 or 400ng VLP), or in a DNA prime/ VLP boost regimen. BALB/c mice were therefore inoculated with varying concentrations of heated and unheated VLPs to establish differences to their cellular immunogenicity profiles with and without prior priming with the DNA vaccine pTHGagC. A DNA vaccine prime was included as VLPs are typically effective as boosts and therefore allowed us to determine if heated VLPs are also effective as boosts. VLPs used in this study only stimulated weak to moderate cellular immune responses in prior studies. Cellular immune responses and hence the frequencies of Gag-specific CD4 and CD8 T cells secreting IFN-γ and IL-2 after inoculation were determined using IFN-γ and IL-2 ELISPOT assays. For this splenocytes pooled from each set of inoculated mice were stimulated in the ELISPOT assay with two Gag CD4 Du_{422} peptides and one Gag CD8 derived peptide.

Splenocytes stimulated with the non-specific positive control, Con A stimulated high spot counts (~1400 SFU/10^6 splenocytes) for all sets of mouse spleens (not shown). A general overview of all responses including background responses and responses to irrelevant peptide for the IFN-γ and IL-2 ELISPOT assay is displayed in figure 4.1 (A) and (C), respectively. The IFN-γ ELISPOT background controls, generally stimulated low spot counts for most splenocyte sets (≤ 25 SFU/10^6 splenocytes) (Figure 4.1 (A)). However, splenocytes isolated from mice given 400ng of unheated VLPs only or a DNA prime followed by boosting with 200ng of VLPs or 400ng of heated VLPs, yielded relatively high background spot counts (~150 SFU/10^6 splenocytes). In these instances, though, background counts were negligible compared to the total spot counts for that particular group of splenocytes stimulated with the CD4 and CD8 epitope peptides. These combined results enabled one to distinguish between positive and negative responses; therefore, compilation of positive responses is displayed in Figure 4.1 (B). The positive result cut-off was set as the number of SFU/10^6 splenocytes stimulated in the peptide-

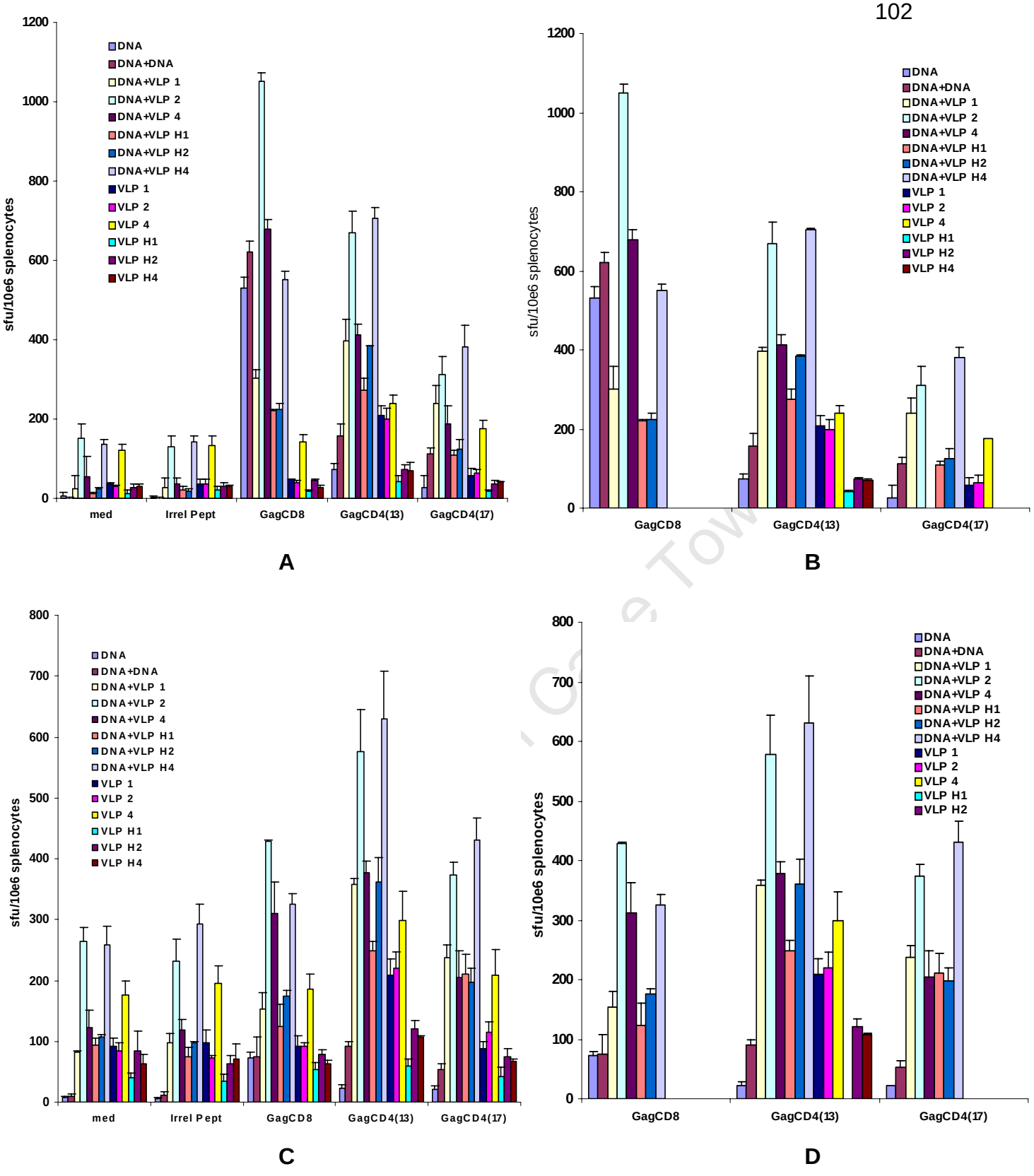


Figure 4.1 General overview of the magnitude of HIV-1 Gag-specific CD8 and CD4 T cell responses as measured by (A and B) IFN-γ and (C and D) IL-2 ELISPOT assays. Splenocytes used in assay were derived from mice inoculated with the pTHgagC DNA vaccine (DNA), with (100; 200 or 400ng) heated or unheated Gag VLP (VLP 1, 2, 4 and VLP H1, H2, H4 respectively). Splenocytes were stimulated with the gagCD8, gagCD4(13) or gagCD4(17) peptides. Assay controls included an analysis of splenocytes stimulated with cell culture medium (med) as well as an irrelevant peptide (Irrel pept). (A and C) Represents All IFN-γ and IL-2 ELISPOT results respectively, while (B and D) represents positive results above cut off for a positive response from the corresponding ELISPOTs. Error bars are the standard deviation of mean triplicates in the ELISPOT assay.

free culture medium control added to its corresponding standard deviation.
IL-2 ELISPOT background responses were approximately four-fold higher than the majority of background responses observed in the IFN-γ ELISPOT. In this ELISPOT, the same sets of splenocytes observed in the IFN-γ-specific reaction displayed high background spot counts (~250 SFU/10^6 splenocytes). The higher background spot counts therefore made the criteria for qualifying as positive IL-2 responses more stringent (Figure 4.2 (D)). Superficially, the frequency of IFN-γ responses appears greater than the frequency of IL-2 responses. This observation has no particular bearing on the actual immune response as it does not take into account the potency and immunologic relevance of the concentration of either cytokine required to carry out its immunologic function.

4.3.2 VLP-only inoculated mouse ELISPOT results

Positive IFN-γ and IL-2 ELISPOT data for DNA or VLP-only inoculated mice are displayed as cumulative responses for both CD4 and the one CD8 peptide in Figure 4.2 (A) and (B) respectively. The IFN-γ ELISPOT results shown in Figure 4.2 (A) allowed direct comparisons of the overall immunogenicity of heated, unheated or DNA only vaccine inoculations. From this graph it is apparent that the DNA vaccine has cumulatively stimulated IFN-γ responses, which are stronger in magnitude and broader than VLP-only inoculations. Stronger cumulative responses are noted by a higher overall spot count for peptides tested (~620 SFU/10^6 splenocytes). Broader responses are noted as the DNA vaccine has stimulated responses to all Gag peptides tested, while VLPs display a more limited immunogenicity profile. In particular, it is noted that the strongest response (520 SFU/10^6 splenocytes) stimulated by the DNA vaccine was towards the Gag CD8 peptide epitope. Based on studies discussed earlier (refer to **4.1.1**) this was an expected result, as DNA vaccines effectively produce internal antigens and generally induce CD8 T cell responses. VLPs (Gp64 pseudotyped, unheated) on the contrary, based on earlier discussions (refer to **4.1.2**), should also have stimulated responses to the CD8 peptide. Instead, both heated and unheated VLPs failed to stimulate any CD8 responses which could be distinguished above the observed

background values, and VLP stimulated responses were confined to the CD4 peptide epitopes.

The unheated VLPs elicit responses to both CD4 peptides, which was consistent with the notion that VLPs are exogenous antigens. Cellular responses to the CD4(13) Gag peptide epitope (~ 200 SFU/10^6 splenocytes) appeared independent of the inoculated VLP (unheated) dose as spot counts remained consistent over the 100-400ng VLP inoculum range. Cellular responses to the CD4(17) peptide epitope were amplified 3 fold (60-180 SFU/10^6 splenocytes) when the VLP (unheated) inoculum was increased from 100ng to 400ng. The strongest cumulative cellular response stimulated by unheated VLPs was thus stimulated by the 400ng VLP inoculum.

Comparisons of cumulative responses to heated and unheated VLPs showed a distinct reduction of the INF-γ response for heated VLPs. On average, responses to heat treated VLPs amounted to ~ 60 SFU/10^6 splenocytes for each of the three tested concentrations. It was first noted that the range of CD4 responses was limited to only the CD4(13) peptide epitope. Cumulatively, heat treatment of VLPs on average resulted in a 70 – 80% reduction in the stimulated response when compared to unheated VLPs. Cellular responses to heated VLPs also appeared independent of the size of the VLP inoculum (100, 200 or 400ng).

The IL-2 ELISPOT results shown in figure 4.2 (B) allowed direct comparisons of the immunogenicity of VLPs (heated, unheated) and DNA-only vaccine inoculations. From this graph it is apparent that the splenocytes derived from mice following a DNA-only vaccination were able to elicit a broad IL-2 response to all 3 peptides (two CD4 and one CD8) used to stimulate splenocytes. In this instance, stimulation by the CD8 peptide again appeared strongest. Despite the broad response to all 3 peptides (130 SFU/10^6 splenocytes), cumulative responses from the DNA vaccine were overall weaker than those elicited by the unheated VLP inocula as well as the 200ng and 400ng heated VLP inocula.

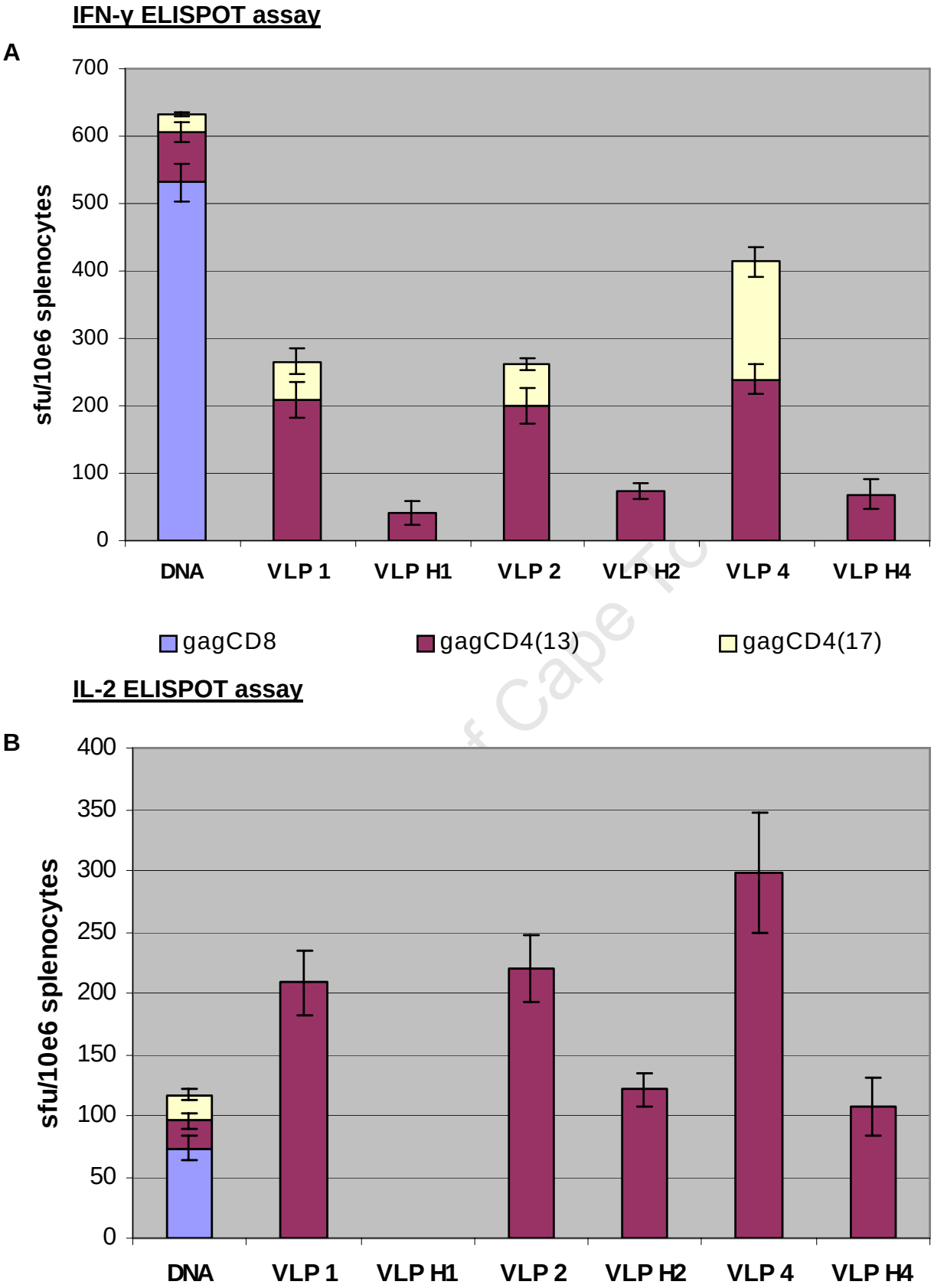


Figure 4.2 Comparative view of heated and unheated Gag VLP stimulated positive CD8 and CD4 T cell responses as measured by (A) IFN-γ and (B) IL-2 ELISPOT assays. Splenocytes used in assay were derived from mice inoculated with 100; 200 or 400ng of heated or unheated Gag VLP (VLP 1, 2, 4 and VLP H1, H2, H4 respectively). Splenocytes were stimulated with the gagCD8, gagCD4(13) or gagCD4(17 peptides). Error bars are the standard deviation of mean triplicates in the ELISPOT assay.

Stimulated IL-2 responses for both heated and unheated VLPs were observed to be more narrow, as splenocytes only responded to the CD4(13) peptide, while other peptides failed to produce any results which could be distinguished from ELISPOT background. Responses stimulated by the CD4(13) peptide remained consistent (220-300 SFU/10^6 splenocytes) over the entire range of unheated VLP inoculum doses (100, 200 and 400ng) and thus appeared VLP concentration-independent. When stimulated responses of heated and unheated VLPs were compared, again a clear depreciation in the IL-2 response was observed for the heated VLPs, (~ 100%; 50% and 60% decrease between the 100ng; 200ng and 400ng unheated and heated VLP inocula respectively). The difference between heated and unheated VLPs was most evident for mice inoculated with 100ng of VLPs. In this instance, the heated VLPs failed to enable the stimulation of any positive responses which could be distinguished above background levels.

The heated 200ng and 400ng VLP inocula displayed a ~ 50-60% reduction in IL-2 stimulation when compared to unheated VLP-stimulated responses. From these results, with the exception of the 100ng heated VLP inoculum, it appeared that cellular responses to heated VLPs also appeared independent of the VLP inoculum/dose (200ng or 400ng), which was similar to the IFN-γ ELISPOT. As with the IFN-γ ELISPOT, the 400ng unheated VLP inoculum resulted in the strongest cellular response.

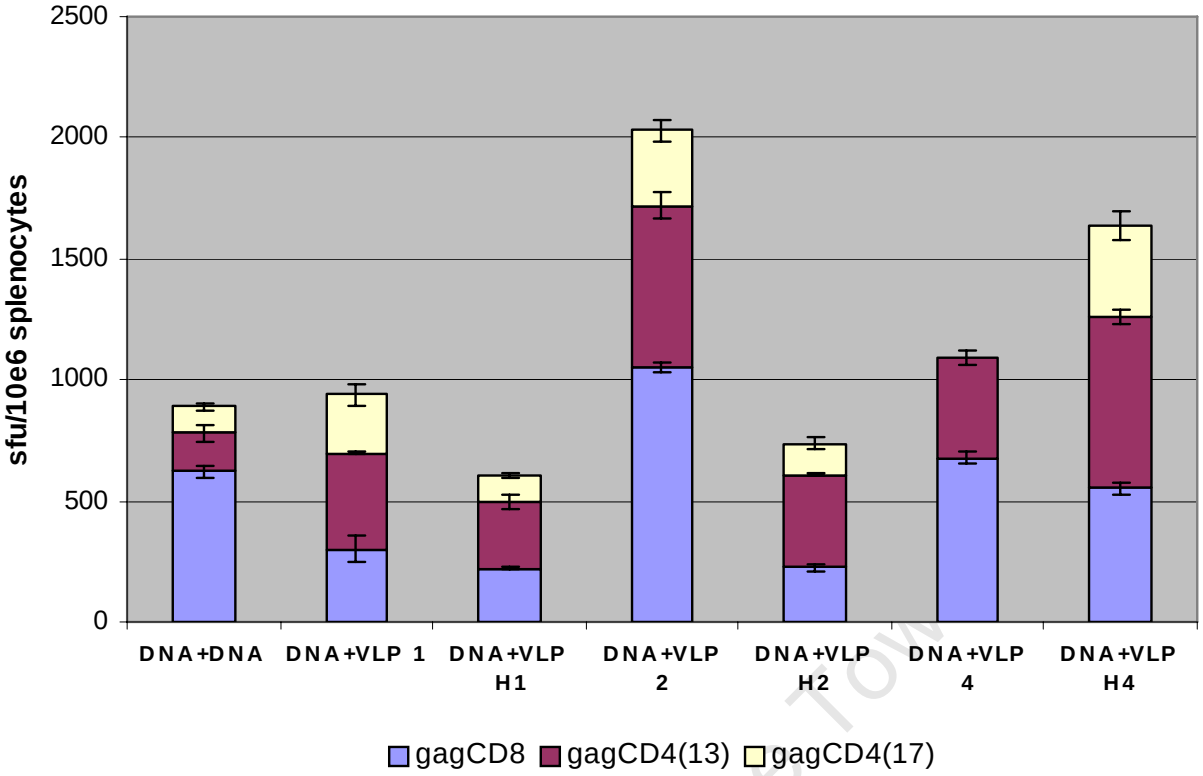
4.3.3 DNA primed/VLP boosted mouse ELISPOT results:

Positive IFN-γ and IL-2 ELISPOT data for DNA primed and VLP (heated and unheated) or DNA-boosted mice are displayed as cumulative responses for the both CD4 and the one CD8 peptide in Figure 4.3 (A) and (B) respectively. The IFN-γ ELISPOT results shown in Figure 4.3 (A) allowed direct comparisons of cellular responses stimulated by VLPs (heated, unheated) inocula, which were assessed as boosts when used in combination with a DNA prime. Results showed that DNA vaccine priming prior to boosting with VLP (heated/unheated) administration substantially enhanced the magnitude of cumulative responses (compare Figure 4.2 and Figure 4.3). The magnitude of responses was enhanced in terms of the response breadth, whereby all peptides (x1 CD8 and x2 CD4) stimulated responses as well as the overall cumulative response (≥ 600 SFU/10^6 splenocytes). When comparing responses from DNA prime/VLP boost (Figure 4.3) to VLP-only (Figure 4.2) inoculation regimens, the stark difference observed between immune responses stimulated by heated and unheated VLP-only inoculations appeared very much diminished when mice were primed with DNA.

Unlike the case with heated VLP-only inoculations, with DNA priming heated VLPs were capable of boosting cellular responses even at the lowest inoculum concentration (100ng) to a response equivalent to that stimulated by the DNA prime/DNA boost response. In this data set the strongest response was observed for the 200ng unheated VLP boost (cumulative response ~2000 SFU/10^6 splenocytes). The 200ng unheated VLP boost inoculum also displayed an enhanced response to CD8 peptide stimulation, which was not observed for any VLP only inoculations or heated VLP boosts. In this data set an unexpected response resulted from 400ng heated VLP boosted mice, which displayed a stronger IFN-γ response (~1400 SFU/10^6 splenocytes) for heated than the unheated VLP boost (~900 SFU/10^6 splenocytes). Cumulatively this response approached that observed for the optimal 200ng VLP boost (~ 2000 SFU/10^6 splenocytes).

IFN-γ ELISPOT assay

A



IL-2 ELISPOT assay

B

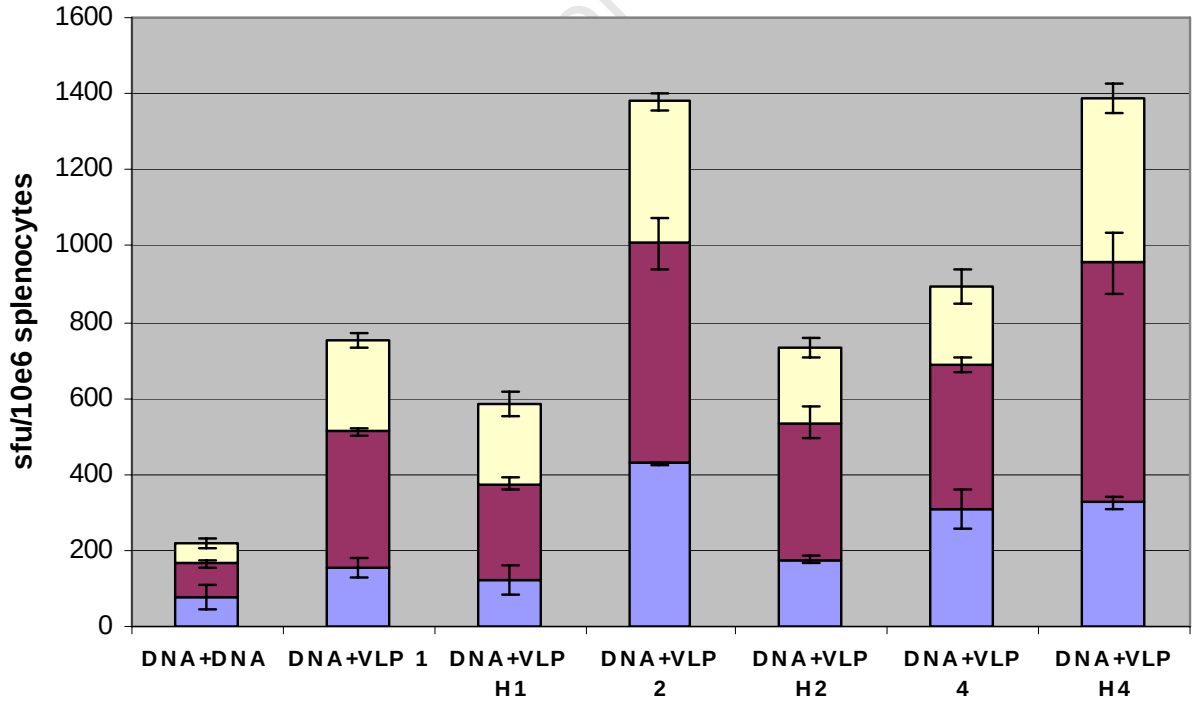


Figure 4.3 Comparative view of heated and unheated Gag VLP stimulated positive CD8 and CD4 T cell responses as measured by (A) IFN-γ and (B) IL-2 ELISPOT assays. Splenocytes used in assay were derived from mice primed with the pTHgagC DNA vaccine (DNA) and further boosted with 100; 200 or 400ng of heated or unheated Gag VLP (VLP 1, 2, 4 and VLP H1, H2, H4 respectively). Splenocytes were stimulated with the gagCD8, gagCD4(13) or gagCD4(17 peptides).

The IL-2 ELISPOT results of the DNA prime/VLP boost regimen shown in Figure 4.3 (B) allowed immunogenicity comparisons of heated and unheated VLPs when used as boosts in combination with a DNA prime. The cumulative cellular response to DNA prime and DNA boost had practically doubled when compared to the DNA only vaccination (~110 – 220 SFU/10^6 splenocytes). As with the corresponding IFN-γ ELISPOT data (Figure 4.3 (A)) from this graph it is evident that DNA priming prior to DNA or VLP boosting resulted in an increased overall cumulative response. Additionally an enhanced breadth of cellular responses was also observed here when compared to DNA- or VLP-only immunizations. In the prior IL-2 analysis (Figure 4.2 (B)), VLP-only inoculations only generated responses to the CD4(13) peptide. In contrast, when VLPs- heated or unheated were used to boost DNA-primed mice, the cumulative responses were several fold higher than VLP-only or DNA prime-DNA boost responses. Similarly, in all instances of VLP boosting, the breadth of the immune responses were larger than those observed for VLP- and DNA-only inoculations (responded to all 3 peptides). Unlike the case with VLP-only inoculations, CD8 peptide responses were enhanced for all (heated and unheated VLP) inoculations. As with the IFN-γ ELISPOT, the difference between heated and unheated VLP cellular responses appears diminished by use of the DNA vaccine prime. More specifically, 100ng and 200ng of unheated VLP responses decreased by ~ 20% and 45% respectively when heated. As with the corresponding IFN-γ ELISPOT prime-boost analysis, (Figure 4.3 (A)) the strongest VLP boost here too is elicited by 200ng of unheated VLPs. On the contrary the largest disparity between corresponding concentrations of heated and unheated VLP boost is also noted for the 200ng inoculum. As with the IFN-γ ELISPOT, unexpectedly the strong 200ng unheated VLP cumulative response was closely reproduced by the heated 400ng VLP boost.

4.4 Discussion:

Immune responses to VLPs presented in this chapter reiterate results presented in previous studies with respect to VLP immunogenicity. As with previous studies, VLP-only inoculations in the selected mouse model resulted in moderate cellular responses (Jaffray *et al*., 2004; Meyers *et al*., 2008). These responses appeared limited to CD4-specific Gag epitopes when assayed by IFN-γ and IL-2 ELISPOT and developed optimal (strongest) cumulative ELISPOT results when mice were inoculated with 400ng of unheated VLPs. Pseudotyped Gag VLPs, as discussed earlier (refer to **3.1.1**), should be able to stimulate MHC-1 directed CD8 cellular responses. Both IFN-γ and IL-2 ELISPOT results from VLP-only inoculated mice though failed to demonstrate these responses when splenocytes were stimulated with the GagCD8 peptide. When heated VLPs were used for the VLP-only inoculations, a substantial decrease in the overall cumulative cellular responses was observed (IFN-γ: ~80%; IL-2:~60%). These responses appeared to be unaffected by increased doses of heated VLPs for both IFN-γ and IL-2 ELISPOT, with responses limited to the GagCD4(13) epitope. Heated VLPs in single dose regimens would not be able to stimulate adequate immune responses as their stimulated responses fall short in terms of magnitude and breadth of the response.

Immune responses to the DNA vaccine-only inoculated mice are similar to those observed in other studies. The pTHGagC DNA vaccine has previously been shown to induce moderate levels of Gag-specific cellular immune responses in mice inoculated intramuscularly (van Harmelen *et al*., 2003; Jaffray *et al*., 2004; Meyers *et al*., 2008). In this study, when heated VLPs were used with a DNA vaccine (pTHGagC) in DNA-prime, VLP-boost regimens, results also reiterated those observed in previous studies (Meyers *et al*., 2008; Chege *et al*., 2008). As with previous studies, the overall magnitude and breadth of DNA-primed cellular responses stimulated by VLPs (heated and unheated) was significantly enhanced when assayed by IFN-γ and IL-2 ELISPOT, compared to the VLP-only case. Optimal cumulative IFN-γ and IL-2 ELISPOT results were ~ 5 fold higher and were stimulated by 200ng of unheated VLPs (half the amount necessary for responses in VLP-only inoculations). Prime-boost cellular responses using both heated and unheated

VLPs, unlike the VLP-only immunizations, stimulated responses to all CD4 and CD8 test peptides. Unheated VLP samples, though, were observed to stimulate/enhance the CD8 responses by larger increments when compared to equivalent concentrations of heated VLP boosts or the DNA boost. This may be a direct result of diminished Gp64 functionality on heated VLPs. As demonstrated in Chapter 3, VLP heat treatment had no effect on VLP cellular entry and CAT RNA expression in the HEK 293 cell line, possibly as a result of an alternate non-Gp64 mediated, non-degradative cell entry pathway. This possible alternate cell entry pathway may account for the lower levels of CD8 response enhancement by heated VLPs.

In general, the difference between cellular immune responses stimulated by heated and unheated VLPs was significantly diminished by the DNA boost. Heated VLP cellular responses in the prime-boost appeared to be enhanced by increasing VLP inoculum concentration in both IFN-γ an IL-2 ELISPOT. Specifically heated VLPs at 400ng inocula were observed to stimulate cellular responses, which virtually mirrored the optimal 200ng unheated VLP boost. Heated VLPs appeared suitable for vaccination when used in prime-boost regimens as they were able to stimulate cellular immune responses on par with lower concentrations of unheated VLP boosts.

Studies have been conducted to further characterize protective cellular immune responses to understand why, in the course of most HIV infections, cellular responses fail to protect. A review by Pantaleo *et al*. (2004) likened HIV infections to other chronic infection viruses like Epstein-Barr virus (EBV) and cytomegalovirus (CMV). CMV and EBV represent chronic infection viruses, which are successfully controlled by cellular immune responses. It was previously shown that EBV and CMV-specific cellular responses are characterized by the presence of 3 functionally distinct virus specific polyfunctional cell populations: cells that secrete IL-2 but not IFN-γ, that secrete both cytokines and cells that secrete IFN-γ only (Betts *et al*., 2001; Harari *et al*., 2003; Pitcher *et al*., 1999). Typically secretion of both cytokines is associated with protracted antigen exposure and low antigen load. While secretion of IFN-γ only frequently associated with the failure to clear antigen

and hence high antigen loads and IL-2 only is associated with antigen clearance.

HIV infections in which cellular immunity failed to control viraemia have characteristically had very low to non-existent titres of virus-specific polyfunctional CD4+ and CD8+ T cells that secrete more than one of these cytokines. Instead, lack of HIV infection control has been characterized by high frequencies of HIV-1 CD4 and CD8 T cells that secrete IFN-γ only.

On the contrary, individuals (humans and macaques) who remained healthy and managed to control viraemia (LTNPs), displayed HIV-1 specific CD4 and CD8 T cell responses that were polyfunctional (multi-cytokine producing: co-producing: IFN-γ; IL-2 and tumor necrosis factor (TNF)-α), similar to the protective EBV and CMV responses (Harari *et al*., 2003; Sadagopal *et al*., 2005; Kannanganat *et al*., 2007(A); (B); Boaz *et al*., 2003; Boaz *et al*., 2002; Iyasere *et al*., 2003; Younes *et al*., 2003; Robinson *et al*., 2007). The relevance of the multiple cytokine-producing T cells was further emphasized when individuals who managed to control viraemia developed HIV-specific CD4 T cells of which more than 50% produced more than one cytokine (IFN-γ; IL-2 and TNF-α) (Kannanganat *et al*., 2007). Individuals unable to control viraemia on the contrary developed HIV-specific T cells of which more than 75% produced only 1 cytokine; most of which were IFN-γ-positive.

It appears, therefore, that the success of the cellular immune response against an HIV infection is more a result of the type of immune response (single cytokine producing/multiple cytokine producing) rather than the quantity. Thus, multiple cytokine-producing immune T cell responses result in a superior level of viraemia control. However, in most cases there is a bias towards the development of single cytokine producing HIV-specific T cell populations.

Overall, this immunogenicity analysis demonstrated for the first time that heated VLPs, which have a limited capacity to deliver encapsidated RNAs for expression in mammalian cells are still efficient at stimulating cellular immune responses. Heated VLPs were capable of stimulating

strong IL-2 and IFN-γ responses to multiple HIV Gag epitopes (CD4 and CD8) when used in DNA prime/VLP boost regimens. These heated VLPs were required at higher concentrations when compared to unheated VLPs (400ng heated VLPs as apposed to 200ng unheated VLPs). The ELISPOT-generated IFN-γ and IL-2 results in this chapter could unfortunately not be used to determine co-production of cytokines in the splenocyte populations analysed: ELISPOT is a “single dimensional” assay, which is only able to determine if a cell population is producing a particular cytokine or not. The use of intracellular cytokine staining/flow cytometry is therefore recommended as a means of further analyzing heat treated VLP-stimulated cellular immune responses for multiple cytokine production.

Chapter 5

Final Discussion

5.1 Annual ARV Investment

Now, more than ever, there is a pressing need for the development of a protective or preventative measure against HIV infection. Infection rates, particularly in sub-Saharan Africa, have reached frightening levels. Internationally, billions of dollars are required each year for antiretroviral treatment of infected individuals. At the end of 2007, it was estimated that the global deficit between required and available funding for antiretroviral treatment was US $8.1 billion. Based on current infection rate trends it has also been estimated that funding will have to more than quadruple to US$ 35 billion in 2010 and to US$ 41 billion in 2015 (WHO website, 2008). The prices of most first-line antiretroviral drugs have decreased from 30% to 64% between 2004 and 2007 in the low and middle-income countries. Currently, in developing countries, the lowest price for the antiretroviral treatment of a single infected individual is around US $100 per person per year, but average prices are at least US $200 (WHO website, 2008). Despite the reduction in cost, these prices are still not economically viable for the poorest countries concentrated in sub-Saharan Africa and South-East Asia, which are affected the most by the AIDS pandemic. As these countries are not likely to be able to finance the treatment of infected individuals without foreign aid, the pricing of the drugs poses a barrier to the availability of antiretroviral treatment.

It has also become apparent that investment in HIV infection prevention methods (education, condoms and vaccine development) should ultimately be more sensible and cost effective than the partially effective antiretroviral treatment of infected individuals which is currently used. Although this study does not dispute the importance of continued antiretroviral therapy, it supports the use of the alternative protective, novel vaccine strategies against HIV infection, which may be more effective in the long term.

5.2 HIV vaccine Development

Novel HIV vaccine development has been aided significantly through the amalgamation of genetic engineering techniques with other research disciplines. The applied synergy of research disciplines and hence the fusion of ideas and technologies has lead to the development of important

technologies. Genetic engineering has played an important role in vaccine production and has aided in the development of plant, animal, insect, yeast and bacterial expression systems. These expression systems are able to express heterologous soluble peptides or protein bodies of interest at high concentrations for inoculation. Particular reference is made here to the production of inert Gag VLPs by means of the baculovirus-based expression vector systems in insect cell lines. When Gag VLPs were first produced in insect cells for vaccine purposes (Gheysen *et al*., 1989), researchers were probably oblivious to the high levels of baculovirus Gp64 envelope glycoproteins that would pseudotype the VLP when budding from cells. On the contrary, as the functionality of Gp64 in mediating virus entry into mammalian cells first documented in 1995, this may not have been regarded as a problem (Hoffmann *et al*., 1995).

5.3 HIV VLP candidate vaccines

These heterologous expression system-produced Gag VLPs have proven to be effective vaccine candidates. The primary advantages of VLPs as vaccines include that they present epitopes in a particulate form, which the immune system responds well to. Additionally, as they lack any replicative nucleic acids they avoid any complications typically introduced by the use of live attenuated and DNA vaccines. This study, though, has demonstrated the presence of large amounts of Gp64 envelope glycoprotein on the baculovirus expression system-produced Gag VLP surface. Thus, despite the lack of replicative nucleic acids in this system, it was highly probable that these pseudotyped Gag VLPs could enter mammalian cells by receptor mediated endocytosis as in related studies (Kumar *et al*., 2003; Oomens *et al*., 2004; Schauber *et al*., 2004). Recently, studies have suggested an important role for these Gp64 and envelope glycoproteins in general in mediating MHC-1 directed cellular immune responses by enabling VLP cellular entry (Table 1.1; Guo *et al*., 2003; Bellier *et al*. 2006; Marsac *et al*., 2002; Kuate *et al*., 2006; Granelli-Piperno *et al*., 2000; Schauber *et al*., 2004; Bellier *et al*., 2006; Ludwig *et al*., 2005). Wong *et al*. 2005 showed that VLPs that did not display any envelope glycoprotein were unable to stimulate immune responses which

were stronger than a DNA vaccine producing soluble peptides that were unable to assemble into particles.

5.4 Overview of Results

5.4.1 Gp64 Quantitation

Results from work carried out in this study confirmed the presence of Gp64 on the baculovirus produced VLPs. **While this was not the first time the presence of Gp64 has been documented on the Gag VLP surface, it was the first time to my knowledge that Gp64 concentration estimates were made.** Measured Gp64 concentration estimates in this study were fairly constant between several VLP preparations and were measured to be in the order **of ~1650 Gp64 molecules /Gag VLP**. This therefore indicated that an average VLP which contained ~5000 Gag/VLP would contain a ratio of **1 Gp64 for every 3 Gag molecules** in the VLP. These surprisingly high concentration estimates far exceeds recent estimates for the number of native HIV Gp120 envelope glycoproteins on the surface of HIV particles, which are between 2-14 Gp120 spikes per particle (Chertova *et al*., 2002; Zhu *et al*., 2003; 2006). This finding suggests that Gp64 could function as a more effective envelope glycoprotein at mediating cellular entry than Gp120, as it is present at much higher concentrations.

5.4.2 CAT RNA Encapsidation

Related studies have analysed the ability of Gp64 pseudotyped, live lentivirus expression vectors assembled and pseudotyped in mammalian cell lines (293T cell) to enter mammalian cells and express genomic RNAs (Kumar *et al*., 2003; Schauber *et al*., 2004). These mammalian cell expression systems expressed the Gp64 transgene to enable pseudotyping, while a reporter gene (GFP) was embedded in the viral genome. **This study on the contrary was unique as all work was carried out using a far less complex VLP system used to produce inert VLPs used for vaccine purposes.** HIV Gag VLPs used for vaccine purposes are predominantly produced using the insect cell culture-based baculovirus expression system. The expression system used in this study consisted only of the HIV *gag* gene co-expressed with a *CAT* gene

utilizing a baculovirus based, insect cell expression system. In this system, unlike previous studies, reporter genes consisted of randomly packaged reporter RNAs as no virus genomic RNA was present. **This study showed for the first time to my knowledge that the Gag VLPs produced in this type of expression system encapsidates highly variable levels of the CAT reporter RNA species (0.1-11 copies per VLP).**

5.4.3 Cell Uptake Assays

The encapsidated CAT reporter RNAs in this study were shown to be able to successfully enter mammalian cell lines (BHK-21; HeLa and HEK 293), where they were expressed at comparative levels between the different cell lines tested. Surprisingly the VLPs appeared to be more efficient RNA transfection agents than the commercially available non-lipid, cationic RNA transfection reagent. It was noted that the transfection of *in vitro*-transcribed CAT RNAs required much higher RNA concentrations to attain similar expression levels to the VLP encapsidated CAT RNAs (20-fold in some instances). **The results presented here are the first to my knowledge to have documented the Gp64 pseudotyped-VLP mediated transfer and expression of a reporter RNA species from the VLP expression system. Results suggest that there is a strong probability of the expression of randomly encapsidated, expression system-produced CAT RNAs in mammalian cell lines following VLP inoculation.** The results presented here question the definition of baculovirus/insect cell-produced Gag-HIV VLPs as inert particles. Typically they could be regarded as inert or non-infectious as they lack any replicative nucleic acids. Alternatively, despite not being able to replicate, VLPs are able to enter mammalian cell lines and deliver their expression system derived RNA contents for expression because of the presence of Gp64 on the VLP surface.

5.4.4 Control of RNA Encapsidation

Results from this study also therefore suggest that if a level of control can be attained with respect to the packaging of specific RNA species, these VLPs could serve as highly efficient transduction reagents for the transient production of certain gene products in mammalian cell lines. In this current

study it was found that the attachment of the HIV RNA packaging signal (ψ-site) to the 5' or 3' termini of CAT RNA species (results not shown) was unable to confer any bias for encapsidation of ψ-tagged CAT RNA. As indicated earlier (refer to **1.3.1**), the CAT mRNA concentrations in insect cell lines were probably so high that whether or not it had the ψ-site, it would be the predominant species encapsidated. As with non-ψ-tagged CAT RNA, ψ-tagged CAT levels were also variable between VLP preparations. Discussed earlier (refer to **1.5.2**), HIV-specific RNA packaging involves a complex system of functional protein elements and specific RNA sequence elements. A more complex system that has incorporated these elements into this VLP baculovirus/insect cell expression system could thus function in mediating the controlled encapsidation of specific RNAs. This type of system would though "blur the line" between a live attenuated viral vector as those used in previous studies and an inert VLP (Schauber *et al*., 2004). A pertinent question with respect to RNA packaging arose as to whether unpredictable levels of RNA packaging (both ψ-tagged and untagged) was specifically related to expression in the insect cell line. It would therefore be of interest to test a similar expression system in an alternate cell line.

As a result of the unpredictable levels of RNA packaged in this expression system we cannot be sure of how much RNA is being delivered when an individual is being inoculated. As discussed earlier (refer to **1.6.5**) baculoviral nucleic acids are presumed to function as adjuvants as a result of the CpG motifs. Thus, if different VLP inocula have variable levels of packaged RNAs we cannot be sure if the different VLP preparations, despite being at the same concentration, will stimulate an equivalent immune response.

5.4.5 Virus/VLP inactivation

The baculovirus expression system used in this study produced Gag VLPs with highly variable RNA contents. This would not be a favorable trait with regard to vaccine regulatory bodies, which require consistency between VLP batches produced for vaccine purposes be shown. As it appears to be impossible to exert any particular level of control on the encapsidated RNA concentrations the alternative would be to remove the potential for RNA expression subsequent to VLP inoculation. Established virus inactivation

techniques were therefore used in the current study to prevent expression of the packaged RNAs. The heat inactivation technique traditionally functions by the disruption of viral surface envelope glycoproteins. An alternate method used short wavelength UV irradiation to disrupt the viral nucleic acids. UV treatment of VLPs was shown to only partially reduce CAT expression levels (50% of non UV irradiated) in the tested cell line. The UV irradiation of *in vitro*-transcribed CAT RNA resulted in a ~80% reduction in CAT expression levels, which suggested a possible shielding effect by the VLP. The heat treatment of VLPs practically blocked all CAT expression in the BHK-21 and HeLa cell lines through the possible disruption of Gp64 envelope glycoproteins. CAT expression in contrast in the HEK 293 cell line appeared unaffected by the VLP heat treatment.

This finding was extremely interesting as it suggested a possible alternate mammalian cell entry pathway exploited by VLPs to gain entry into the HEK 293 cell line subsequent to Gp64 disruption. As mentioned (refer to 3.3.2) the virus heat inactivation protocol derived from Poon *et al*. (2005) pretreated the virus particles with formaldehyde as a fixing/cross-linking reagent prior to heating to prevent shedding of envelope glycoprotein. As the retention of envelope glycoprotein was of no priority in this study, VLPs were not treated with formaldehyde. The finding that HEK 293 VLP uptake and hence CAT RNA expression was unaffected by heat treatment suggested the presence of an unidentified VLP surface element that is unaffected by heat treatment. In retrospect, the addition of formaldehyde during virus inactivation could have facilitated the cross linking of such an element and could have inactivated cell uptake in this cell line as well.

An additional control for these uptake studies would require parallel heat treatment and uptake assays using Gag VLPs produced in different expression systems (yeast). Results here would give a good indication of whether it is in fact Gp64 inactivation through VLP heat treatment that prevents CAT RNA expression. Similarly it would also be of significant interest if VLPs derived from an alternate expression system are still capable of entering HEK 293 cell lines following heat treatment. Results from such an

analysis would indicate whether the alternate cell entry pathway is a baculovirus/insect cell element conferred pathway.

Overall, VLP heat treatment did not prevent RNA expression in all cell lines but reduced the range of cell lines that the VLPs could enter and express the encapsidated RNAs. It is therefore suggested that a combination of the virus inactivation methods used in this study be used to inactivate RNA expression from these VLPs. Further studies are also recommended using a broader range of mammalian cell lines in combination with various cell entry inhibitors to determine precise cell entry pathways utilized by the BEVS-produced VLPs.

Other than the Gp64-mediated cell entry ability, it is currently unknown whether the high concentration of Gp64 on the surface of the VLP has any bias on the stimulated immune response. The deletion of the *gp64* gene from the baculovirus expression system, from a VLP immunogenicity aspect, seems justified as it may have a considerable influence on immunodominant epitopes on the VLP. On the other hand, Gp64 on the VLP surface was shown to also mediate cellular entry in this and other studies, which could potentially stimulate the necessary MHC-1 cellular responses required to control HIV infection. Studies have shown that the simple deletion of Gp64 is not a viable means of redirecting immune responses towards the Gag epitopes in the VLP. *Gp64* gene deletion causes a severe reduction in baculovirus virion production and virions that are produced are non-infectious (Monsma *et al*., 1996; Oomens *et al*., 1999). Similarly, anti-Gp64 antibodies have been shown to neutralize baculovirus infectivity and would therefore negatively affect the VLP expression system (Zhou *et al*., 2006). A study by Zhou *et al*, (2008) has identified the functional domains of Gp64 which, were included in the development of a minimal construct that still permitted baculovirus budding and infection. The minimal construct (G-stem) is capable of substituting functionality of the full length Gp64 consisted of 38 amino acids of the Gp64 ectodomain in combination with 52 amino acids of the Gp64 transmembrane stem domain. The G-stem minimal construct produced in this study can be used to display foreign epitopes (eGFP; influenza N-terminal HA ectodomain) on the baculovirus virion surface. A recombinant G-stem construct could therefore potentially be used for the presentation of HIV epitopes on the VLP

surface if substituted for the native Gp64 in the baculovirus expression system. In the quantitation analysis, in this study, Gp64 showed high, consistent levels of incorporation into VLPs.

5.4.6 Immunology

While a vaccine that provides a completely sterilizing protection against HIV infection is the ideal (humoral), protective vaccine candidates that stimulate T-cell based responses have shown promise (Table 1.1; Jaffray *et al*., 2004; Marsac *et al*., 2002; Bachmann *et al*., 1996; Buonaguro *et al*., 2002; 2006; Ruedl *et al*., 2002; 2005, Kuate *et al*., 2006; Deml *et al*., 1997; Paliard *et al*., 2000; Deml *et al* 2005). These HIV-specific cellular immune responses may not clear an HIV infection but are able to help lower the initial burst viremia following infection, slow the progression towards disease and reduce transmissibility.

Bearing the significance of cellular immune responses stimulated by pseudotyped VLP in mind, this study sought to assess any changes to stimulated VLP immune responses subsequent to heat treatment. Heat treatment was expected to disrupt the cellular immune response due to the absence of functional envelope glycoprotein. On the contrary, the finding of an alternate cell entry pathway in the HEK 293 cell line suggested that the possibility still existed to stimulate adequate cellular immune responses. Mice were therefore inoculated with VLP-only (heated or non-heated) or DNA vaccine prime/VLP boost (heated or non-heated) regimens.

Mice inoculated with only heated VLPs displayed a clearly reduced cumulative cellular immune responses when compared to mice inoculated with unheated VLPs (IFN-γ and IL-2 ELISPOT assays). On average, heated VLPs displayed a 60% to 80% reduction in cumulative responses compared to the non-heated VLPs. These weak responses were limited to a single CD4-specific epitope and appeared unaffected by VLP dose (remained low irrelevant of the heated VLP dose). Heated VLPs are therefore unable to stimulate adequate cellular immune responses directed at HIV epitopes when immunizing mice with VLP-only inoculation regimens.

In contrast to the heated VLP-only inoculation regimen, the DNA prime/heated VLP boost yielded more promising results. The overall magnitude and breadth of cellular responses stimulated by heated VLPs in this instance was significantly enhanced (IFN-γ and IL-2 ELISPOT responses to all epitopes). Inoculation of mice with unheated VLPs though, enhanced responses to the CD8 epitope by larger increments than those inoculated with the heated VLPs. This may be a direct result of the heat reduced functionality of Gp64 but heat treated VLPs were nevertheless able to enhance the cellular response to the CD8 epitope stimulated by the DNA vaccine. Interestingly, the difference between cellular immune responses stimulated by heated and unheated VLPs in the prime/boost inoculation regimen were not as large as the VLP-only inoculations. The 100 and 200ng heated VLP boost displayed a 20% and 45% reduction in the cumulative response compared to the unheated VLPs. The 400ng heated VLP boost surprisingly stimulated almost equivalent responses to those stimulated by the optimal unheated VLP boost (200ng).

Unexpectedly, when mice were boosted with higher heated VLP doses following a DNA prime, cellular responses were almost equivalent to the optimal responses stimulated by non-heated VLPs. **This novel finding therefore suggests that VLPs can be heat treated to reduce its ability to transduce mammalian cell lines and express encapsidated RNAs, while retaining its immunogenicity. The only *proviso* would be the use of slightly higher inoculation doses of the VLPs in a prime/boost inoculation regimen as this reproduces the optimal type of immune response resulting from unheated VLP boosts.**

5.5 Conclusion

To conclude, results presented in this study have shown that Gp64 is incorporated in high concentrations (~1650 Gp64/VLP) into baculovirus produced HIV Gag VLPs. It is evident that VLPs pseudotyped by Gp64 is capable of productively entering mammalian cell lines and expressing the randomly encapsidated expression system derived RNAs. The range of target cells that the VLPs can enter and express their RNAs in can be limited by VLP

heat treatment. VLP immunogenicity subsequent to heat treatment can still be maintained provided that they are used in highly inoculation doses in a prime/boost inoculation regimen.

The development of a prophylactic VLP-based HIV vaccine therefore remains a worthwhile endeavour. Typically safety issues concerning vaccines arise as we attempt to fully control/manipulate virtually every aspect of the administered vaccine. As the delivery of foreign RNA species during vaccination remains a contemptuous issue, results from this study have shown that it is possible to modulate the transmission of foreign RNAs without affecting VLP immunogenicity. As the VLPs produced in this and many other studies are produced in insect cell-based expression systems, they are unlikely to carry any human pathogens, which also make them viable candidates for further investment as long term vaccine prospects.

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

Appendix A

Standard Methods

1. Bacterial growth media, conditions, antibiotics and strains

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

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

Ampicillin was used as a selective marker at 100μg/ml concentrations in both LB media and Luria agar to select for the ampicillin-resistant clones.
Liquid cultures were all grown at 37^{o}C with agitation.

2. DNA extraction procedures

2.1 Small-scale plasmid DNA extraction

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

2.2 Large-scale plasmid DNA isolation

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

3. Agarose gel electrophoresis of DNA and RNA

Gel electrophoresis of both DNA and RNA was carried out using 1% agarose gels containing 0.5μg/ml of ethidium bromide in a 1x Tris-Borate EDTA buffer at 95 volts. DNA fragment size was estimated using the Hyperladder I DNA size marker (Bioline), which gives a size estimation range of between 200bp and 10000bp. For RNA electrophoresis, all reagents were prepared under RNase-free conditions (used DEPC treated water for reagents).

4. Gel and endonuclease reaction purification of DNA

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

5. Transformation

E.cloni® 10G DUOs (Lucigen®) chemically competent *E. coli* cells were used for transformation, which was carried out using the method of Chung and Miller (1989). Transformations were conducted by adding 2μl ligation reaction DNA to 20μl of competent cells. The DNA and competent cells were mixed in a 1.5ml Eppendorf tube and chilled on ice for 30 minutes. The cells were then heat-shocked at 37^{o}C for 25 seconds, followed by incubation on ice for 2 minutes. 700μl of LB was then added to the transformation mix, which was then incubated at 37^{o}C for 1 hour 30 minutes. 100μl volumes of each transformation mix was then plated on Luria agar plates containing 100μg/ml ampicillin as a selective marker.

6. DNA sequencing

Purified miniprep (small scale plasmid DNA extraction) DNA was used for the sequencing reactions by means of a technique derived form the dideoxy chain terminating method of Sanger *et al*. (1977). This service was provided by the Macrogen Inc DNA sequencing service, Gasan-dong Geumchun-gu Seoul, Korea 153-021. Analysis of the generated sequences was done using the DNAMAN program for Windows version 5.2.9 (Lynnon BioSoft, © 1994-2001).

6.1 Sequencing primers used:

P10 forward: 5' AGA TCT ATG TCG TAC TAC CAT CAT CAC CAC CAC 3'
P10 reverse: 5' AGA TCT CAT AGC GCG GGT TCC TTC CGG TAT TG 3'

PH forward: 5' AGA TCT TAC TAC CAT CAC CAT CAC CAT CAC G 3'
PH reverse: 5' AGA TCT TTA TGA TCC TCT AGT ACT TCT CGA C 3'

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

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

8. Western Blot

Protein from SDS-PAGE gels was transferred onto Hybond™-ECL™ nitrocellulose membranes (Amersham Biosciences) using a semi-dry blotting electroblotter (BioRad^{TM}). Blotting was carried out for 60 minutes at 15 volts, after which membranes were blocked for 30 minutes at room temperature in a 1% Tween-20, 5% milk powder in phosphate buffered saline solution. Membranes were probed overnight in an appropriate dilution of primary antibody at 4^{o}C. Blots were subsequently washed with blocking buffer 4X for 15 minutes/wash before being probed with an appropriate dilution of alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich) for 2 hours at room temperature. The blots were next washed with blocking buffer lacking milk powder as above. Detection was via a colorimetric reaction using the

SIGMAFAST™, 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) containing tablets as per supplier's directions (Roche).

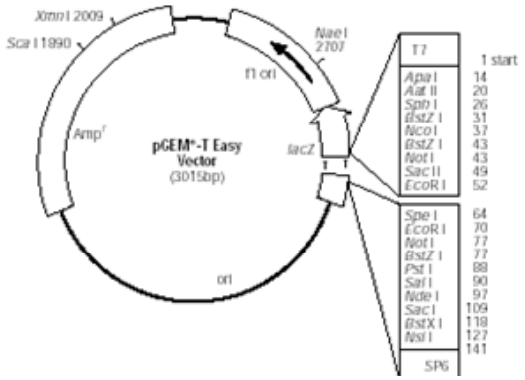
9. CAT ELISA

A CAT ELISA kit (Roche) was used for this assay, according to the manufacturer's protocol. ELISA plates were analysed using the PowerWave™ XS ELISA plate reader (BioTek®) and results were analysed using the KCL™ (version 3.4) program (BioTek®).

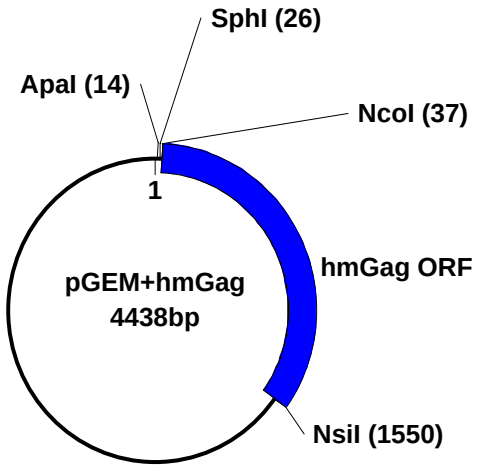
Appendix B

Vectors used

1. pGEM-T Easy Vector maps



A



B

Figure B1(A and B) Map of pGEM®-T-easy cloning vector (Promega) that contained the hmGag ORF.

2. Sequence of hmGag ORF in pGem®-T easy cloning vector

pGEM®-T-easy multiple cloning site — NcoI site — Start codon — hmGag ORF — Stop codon

```
1     TGTAATACGA CTCACTATAG GGCGAATTGG GCCCGACGTC GCATGCTCCC GGCCGACCAT +
61    GG GTGCTCGC GCATCTATCC TCAGAGGCGA AAAGTTGGAT AAGTGGGAAA AAATCAGACT
121   CAGGCCAGGA GGTAAAAAAC ACTACATGCT GAAGCATATC GTGTGGGCAT CTAGGGAGTT
181   GGAGAGATTT GCACTGAACC CCGGACTGCT GGAAACCTCA GAGGGCTGTA AGCAAATCAT
241   GAAACAGCTC CAACCAGCCT TGCAGACCGG AACAGAAGAG CTGAAGTCCC TTTACAATAC
301   CGTGGCAACC CTCTATTGCG TCCACGAGAA GATCGAGGTG AGAGACACAA AGGAGGCCCT
361   GGACAAAATC GAGGAGGAGC AGAATAAGTG CCAGCAGAAG ACCCAGCAGG CAAAGGCTGC
421   TGACGGAAAG GTCTCTCAGA ACTATCCTAT CGTTCAGAAC CTTCAGGGGC AGATGGTGCA
481   CCAAGCAATC AGCCCTAGAA CCCTGAACGC ATGGGTGAAG GTGATCGAGG AGAAAGCCTT
541   TTCTCCCGAG GTTATCCCCA TGTTTACCGC CCTGAGCGAA GGCGCCACTC CTCAAGACCT
601   GAACACTATG CTGAACACAG TGGGAGGACA CCAGGCCGCT ATGCAGATGT TGAAGGATAC
661   CATCAACGAG GAGGCAGCCG AATGGGACCG CCTCCACCCC GTGCACGCCG GACCTATCGC
721   CCCCGGACAA ATGAGAGAAC CTCGCGGAAG TGATATTGCC GGTACTACCA GCACCCTTCA
781   AGAGCAGATT GCTTGGATGA CCAGCAACCC ACCCATCCCA GTGGGCGATA TTTACAAAAG
841   GTGGATTATT CTGGGGCTGA ACAAAATTGT GAGAATGTAC TCCCCCGTCT CCATCCTCGA
901   CATCCGCCAA GGACCCAAGG AGCCTTTTAG GGATTACGTG GACAGATTCT TCAAAACCCT
961   TAGAGCTGAG CAAGCCACTC AGGAGGTTAA GAACTGGATG ACAGATACTC TGCTCGTGCA
1021  AAACGCTAAC CCCGATTGCA AAACCATCTT GAGAGCTCTC GGTCCAGGTG CCACCCTTGA
1081  GGAAATGATG ACAGCATGTC AAGGCGTGGG AGGACCTGGG CACAAGGCCA GAGTTCTCGC
1141  TGAGGCCATG AGCCAGACAA ACTCAGGCAA TATCATGATG CAGAGGAGTA ACTTTAAGGG
1201  TCCCAGGAGA ATCGTCAAGT GCTTCAATTG TGGCAAGGAG GGTCACATTG CCAGGAACTG
1261  CCGCGCCCCC AGGAAGAAAG GCTGCTGGAA GTGTGGCAAA GAGGGCCACC AGATGAAGGA
1321  TTGCACCGAG CGCCAAGCAA ACTTCCTGGG AAAGATTTGG CCCAGTCATA AGGGCCGCCC
1381  TGGCAACTTC CTTCAAAACA GACCCGAGCC TACCGCCCCC CCCGCTGAGT CTTTCAGATT
1441  TGAGGAGACC ACCCCCGCTC CAAAGCAGGA GCCAATTGAG AGAGAGCCTC TCACCAGTCT
1501  CAAAAGCCTC TTTGGTAGCG ACCCCCTCAG CCAATAAGAA TTCAATCACT AGTGATAAGC
```

```
1561    GCCGG ATGCAT AGCTTGAGT ATTCTATAGT GTCACCTAAA T
              NsiI site      pGEM®-T-easy multiple cloning site
```

3. pcDNA3.1/Zeo/CAT cloning vector map

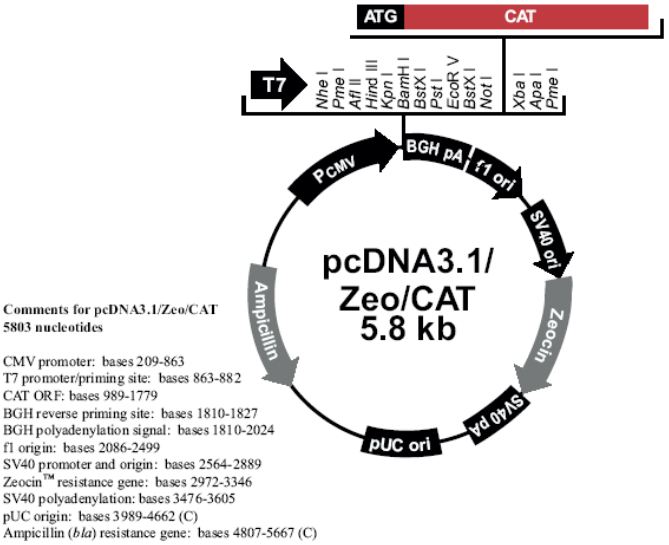
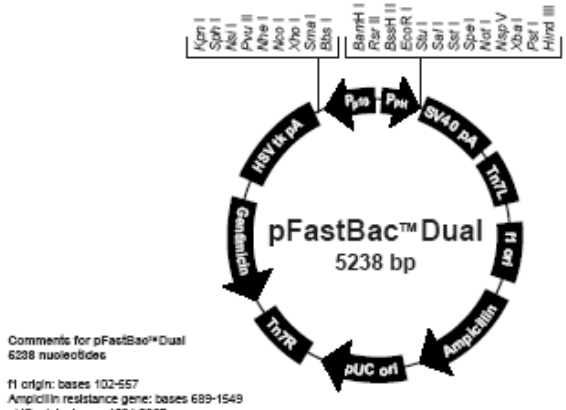


Figure B3 Map of the pcDNA3.1/Zeo/CAT cloning vector containing the CAT ORF used for cloning (Vector map derived from vector product manual (Invitrogen)).

4. Sequence of CAT ORF in the pcDNA3.1/Zeo/CAT cloning vector

```
        NotI site                                                  Start codonCAT ORF
1       GCGGCCGC TCGAGCTTCGAC GAGATTTTCA GGAGCTAAGG AAGCTAAAATG GAGAAAAAA
61      ATCACTGGAT ATACCACCGT TGATATATCC CAATGGCATC GTAAAGAACA TTTTGAGGCA
121     TTTCAGTCAG TTGCTCAATG TACCTATAAC CAGACCGTTC AGCTGGATAT TACGGCCTTT
181     TTAAAGACCG TAAAGAAAAA TAAGCACAAG TTTTATCCGG CCTTTATTCA CATTCTTGCC
241     CGCCTGATGA ATGCTCATCC GGAATTCCGT ATGGCAATGA AAGACGGTGA GCTGGTGATA
301     TGGGATAGTG TTCACCCTTG TTACACCGTT TTCCATGAGC AAACTGAAAC GTTTTCATCG
361     CTCTGGAGTG AATACCACGA CGATTTCCGG CAGTTTCTAC ACATATATTC GCAAGATGTG
421     GCGTGTTACG GTGAAAACCT GGCCTATTTC CCTAAAGGGT TTATTGAGAA TATGTTTTTC
481     GTCTCAGCCA ATCCCTGGGT GAGTTTCACC AGTTTTGATT TAAACGTGGC CAATATGGAC
541     AACTTCTTCG CCCCCGTTTT CACCATGGGC AAATATTATA CGCAAGGCGA CAAGGTGCTG
601     ATGCCGCTGG CGATTCAGGT TCATCATGCC GTCTGTGATG GCTTCCATGT CGGCAGAATG
661     CTTAATGAAT TACAACAGTA CTGCGATGAG TGGCAGGGCG GGGCGTAATT TTTTTAAGGC
                                                           Stop Codon
721     AGTTATTGGT GCCCTTAAAC GCCTGGTGCT ACGCCTGAAT AAGTGATAAT AAGCGGATGA
781     ATGGCAGAAA TTCGTCGAAG CTCTAGA
                                   XbaI site
```

5. pFastBac Dual Vector



Comments for pFastBac™Dual
6238 nucleotides

f1 origin: bases 102-557
Ampicillin resistance gene: bases 689-1549
pUC origin: bases 1694-2367
Tn7R: bases 2611-2835
Gentamicin resistance gene: bases 2902-3435 (complementary strand)
HSV tk polyadenylation signal: bases 3992-4274 (complementary strand)
Multiple cloning site: bases 4274-4337 (complementary strand)
p10 promoter (P_{p10}): bases 4338-4459 (complementary strand)
Polyhedrin promoter (P_{PH}): bases 4478-4606
Multiple cloning site: bases 4606-4704
SV40 polyadenylation signal: bases 4722-4962
Tn7L: bases 4991-5155

A

Multiple cloning site downstream of the PH promoter

Start of Transcription
Polyhedrin promoter
4481 ATGGAGATAA TTAAAATGAT AACCATCTCG CAAATAAATA AGTATTTTAC
wild-type ATG mutated to ATT
4531 TGTTTTCGTA ACAGTTTTGT AATAAAAAAA CCTATAAATA TTCCGGATTA
Bam H I Rsr II BssH II EcoR I
4581 TTCATACCGT CCCACCATCG GGCGCGGATC CCGGTCCGAA GCGCGCGGAA
Stu I Sal I Sst I Spe I Not I Nsp V Xba I
4631 TTCAAAGGCC TACGTCGACG AGCTCACTAG TCGCGGCCGC TTTCGAATCT
Pst I Xho I Hind III
4681 AGAGCCTGCA GTCTCGACAA GCTTGTCGAG AAGTACTAGA GGATCATAAT
SV40 polyadenylation signal
4731 CAGCCATACC ACATTTGTAG AGGTTTTACT TGCTTTAAAA AACCTCCCAC

Multiple cloning site downstream of the p10 promoter

Start of Transcription
p10 promoter
4460 TATACGGACC TTTAATTCAA CCCAACACAA TATATTATAG TTAAATAAGA
4410 ATTATTATCA AATCATTTGT ATATTAATTA AAATACTATA CTGTAAATTA
Bbs II Sma I Xho I
4360 CATTTTATTT ACAATCACTC GACGAAGACT TGATCACCCG GGATCTCGAG
Nco I Nhe I Pvu II Nsi I Sph I Kpn I
4310 CCATGGTGCT AGCAGCTGAT GCATAGCATG CGGTACCGGG AGATGGGGGA
HSV tk polyadenylation signal
4260 GGCTAACTGA AACACGGAAG GAGACAATAC CGGAAGGAAC CCGCGCTATG

B

Figure B5 pFastBac™ Dual insect cell expression vector. (A) Vector map, (B) Position of restriction sites in the PH and p10 promoter multiple cloning sites (Vector map and multiple cloning site sequence derived from vector product manual (Invitrogen))

6. Sequence of the hmGag ORF in the pFastBac™ dual insect cell expression vector under the p10 promoter multiple cloning site

p10 promoter MCS

```
1       TATACGGACC TTTAATTCAA CCCAACACAA TATATTATAG TTAAATAAGA ATTATTATCA
61      AATCATTTGT ATATTAATTA AAATACTATA CTGTAAATTA CATTTTATTT ACAATCACTC

                                        NcoI site +Start codon      hmGag ORF
121     GACGAAGACT TGATCACCCG GGATCTCGAG CCATGGGTGC TCGCGCATCT ATCCTCAGAG
181     GCGAAAAGTT GGATAAGTGG GAAAAAATCA GACTCAGGCC AGGAGGTAAA AAACACTACA
241     TGCTGAAGCA TATCGTGTGG GCATCTAGGG AGTTGGAGAG ATTTGCACTG AACCCCGGAC
301     TGCTGGAAAC CTCAGAGGGC TGTAAGCAAA TCATGAAACA GCTCCAACCA GCCTTGCAGA
        CCGGAACAGA AGAGCTGAAG TCCCTTTACA ATACCGTGGC AACCCTCTAT TGCGTCCACG
421     AGAAGATCGA GGTGAGAGAC ACAAAGGAGG CCCTGGACAA AATCGAGGAG GAGCAGAATA
481     AGTGCCAGCA GAAGACCCAG CAGGCAAAGG CTGCTGACGG AAAGGTCTCT CAGAACTATC
541     CTATCGTTCA GAACCTTCAG GGGCAGATGG TGCACCAAGC AATCAGCCCT AGAACCCTGA
601     ACGCATGGGT GAAGGTGATC GAGGAGAAAG CCTTTTCTCC CGAGGTTATC CCCATGTTTA
661     CCGCCCTGAG CGAAGGCGCC ACTCCTCAAG ACCTGAACAC TATGCTGAAC ACAGTGGGAG
721     GACACCAGGC CGCTATGCAG ATGTTGAAGG ATACCATCAA CGAGGAGGCA GCCGAATGGG
781     ACCGCCTCCA CCCCGTGCAC GCCGGACCTA TCGCCCCCGG ACAAATGAGA GAACCTCGCG
841     GAAGTGATAT TGCCGGTACT ACCAGCACCC TTCAAGAGCA GATTGCTTGG ATGACCAGCA
901     ACCCACCCAT CCCAGTGGGC GATATTTACA AAAGGTGGAT TATTCTGGGG CTGAACAAAA
961     TTGTGAGAAT GTACTCCCCC GTCTCCATCC TCGACATCCG CCAAGGACCC AAGGAGCCTT
1021    TTAGGGATTA CGTGGACAGA TTCTTCAAAA CCCTTAGAGC TGAGCAAGCC ACTCAGGAGG
1081    TTAAGAACTG GATGACAGAT ACTCTGCTCG TGCAAAACGC TAACCCCGAT TGCAAAACCA
1141    TCTTGAGAGC TCTCGGTCCA GGTGCCACCC TTGAGGAAAT GATGACAGCA TGTCAAGGCG
1201    TGGGAGGACC TGGGCACAAG GCCAGAGTTC TCGCTGAGGC CATGAGCCAG ACAAACTCAG
1261    GCAATATCAT GATGCAGAGG AGTAACTTTA AGGGTCCCAG GAGAATCGTC AAGTGCTTCA
1321    ATTGTGGCAA GGAGGGTCAC ATTGCCAGGA ACTGCCGCGC CCCCAGGAAG AAAGGCTGCT
1381    GGAAGTGTGG CAAAGAGGGC CACCAGATGA AGGATTGCAC CGAGCGCCAA GCAAACTTCC
1441    TGGGAAAGAT TTGGCCCAGT CATAAGGGCC GCCCTGGCAA CTTCCTTCAA AACAGACCCG
1501    AGCCTACCGC CCCCCCCGCT GAGTCTTTCA GATTTGAGGA GACCACCCCC GCTCCAAAGC
1561    AGGAGCCAAT TGAGAGAGAG CCTCTCACCA GTCTCAAAAG CCTCTTTGGT AGCGACCCCC
1621    TCAGCCAAT A AGAATTCAAT CACTAGTGAT AAGCGCCGGA TGCATCGGTA CCGGGAGATG
          Stop codon                                 NsiI site

1681    GGGGAGGCTA ACTGAAACAC GGAAGGAGAC AATACCGGAA GGAACCCGCG CTATG
        p10 promoter MCS
```

7. Sequence of the CAT ORF in the pFastBac™ dual insect cell expression vector under the Polyhedrin (PH) promoter multiple cloning site

PH promoter MCS

```
1       ATGGAGATAA TTAAAATGAT AACCATCTCG CAAATAAATA AGTATTTTAC TGTTTTCGTA
61      ACAGTTTTGT AATAAAAAAA CCTATAAATA TTCCGGATTA TTCATACCGT CCCACCATCG
121     GGCGCGGATC CCGGTCCGAA GCGCGCGGAA TTCAAAGGCC TACGTCGACG AGCTCACTAG
```

NotI site — **Start codon** — **CAT ORF**

```
181     TC GCGGCCGC TCGAGCTTCG ACGAGATTTT CAGGAGCTAA GGAAGCTAAA ATGGAGAAAA
241     AAATCACTGG ATATACCACC GTTGATATAT CCCAATGGCA TCGTAAAGAA CATTTTGAGG
301     CATTTCAGTC AGTTGCTCAA TGTACCTATA ACCAGACCGT TCAGCTGGAT ATTACGGCCT
361     TTTTAAAGAC CGTAAAGAAA AATAAGCACA AGTTTTATCC GGCCTTTATT CACATTCTTG
421     CCCGCCTGAT GAATGCTCAT CCGGAATTCC GTATGGCAAT GAAAGACGGT GAGCTGGTGA
481     TATGGGATAG TGTTCACCCT TGTTACACCG TTTTCCATGA GCAAACTGAA ACGTTTTCAT
541     CGCTCTGGAG TGAATACCAC GACGATTTCC GGCAGTTTCT ACACATATAT TCGCAAGATG
601     TGGCGTGTTA CGGTGAAAAC CTGGCCTATT TCCCTAAAGG GTTTATTGAG AATATGTTTT
661     TCGTCTCAGC CAATCCCTGG GTGAGTTTCA CCAGTTTTGA TTTAAACGTG GCCAATATGG
721     ACAACTTCTT CGCCCCCGTT TTCACCATGG GCAAATATTA TACGCAAGGC GACAAGGTGC
781     TGATGCCGCT GGCGATTCAG GTTCATCATG CCGTCTGTGA TGGCTTCCAT GTCGGCAGAA
841     TGCTTAATGA ATTACAACAG TACTGCGATG AGTGGCAGGG CGGGGCGTAA TTTTTTTAAG
```

Stop Codon

```
901     GCAGTTATTG GTGCCCTTAA ACGCCTGGTG CTACGCCTGA ATAAGTGATA ATAAGCGGAT
961     GAATGGCAGA AATTCGTCGA AGCTCTAGA GCCTGCAGTCT CGACAAGCTT GTCGAGAAGT
```

XbaI site

```
1021    ACTAGAGGAT CATAATCAGC CATACCACAT TTGTAGAGGT TTTACTTGCT TTAAAAAACC
1081    TCCCAC
```

PH promoter MCS

8. CAT open reading frame

Start of Psi sequence

BamHI

```
61        GGATCCC GGGTCTCTCT GGTTAGACCA GATCTGAGCC TGGGAGCTCT CTGGCTAACT
121     AGGGAACCCA CTGCTTAAGC CTCAATAAAG CTTGCCTTGA GTGCTTCAAG TAGTGTGTGC
181     CCGTCTGTTG TGTGACTCTG GTAACTAGAG ATCCCTCAGA CCCTTTTAGT CAGTGTGGAA
241     AATCTCTAGC AGTGGCGCCC GAACAGGGAC CTGAAAGCGA AAGGGAAACC AGAGGAGCTC
301     TCTCGACGCA GGACTCGGCT TGCTGAAGCG CGCACGGCAA GAGGCGAGGG GCGGCGACTG
361     GTGAGTACGC CAAAAATTTT GACTAGCGGA GGCTAGAAGG AGAGAGATGG GTGCGAGAGC
421     GTCAGTATTA GGAATTCCAA TCACTAGTGA ATTCGCGGCC GCCTGCAGGT CGACCATATG
```

NdeI

End of Psi sequence

References

Anonymous., 2003. VaxGen vaccine trial fails the test but may offer insights. AIDS Alert. 18, 41, 43-41, 45.

Abbink, T. E., Berkhout, B., 2003. A novel long distance base-pairing interaction in human immunodeficiency virus type 1 RNA occludes the Gag start codon. J.Biol.Chem. 278, 11601-11611.

Abdurahman, S., Hoglund, S., Goobar-Larsson, L., Vahlne, A., 2004. Selected amino acid substitutions in the C-terminal region of human immunodeficiency virus type 1 capsid protein affect virus assembly and release. Journal of General Virology 85, 2903-2913.

Abe, T., Takahashi, H., Hamazaki, H., Miyano-Kurosaki, N., Matsuura, Y., Takaku, H., 2003. Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. J.Immunol. 171, 1133-1139.

AIDS info (2008). Vaccine trials. http://aidsinfo.nih.gov/vaccines/.

Airenne, K. J., Hiltunen, M. O., Turunen, M. P., Turunen, A. M., Laitinen, O. H., Kulomaa, M. S., Yla-Herttuala, S., 2000. Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. Gene Ther. 7, 1499-1504.

Aires, K. A., Cianciarullo, A. M., Carneiro, S. M., Villa, L. L., Boccardo, E., Perez-Martinez, G., Perez-Arellano, I., Oliveira, M. L., Ho, P. L., 2006. Production of human papillomavirus type 16 L1 virus-like particles by recombinant Lactobacillus casei cells. Appl.Environ.Microbiol. 72, 745-752.

Akahata, W., Yang, Z. Y., Nabel, G. J., 2005. Comparative immunogenicity of human immunodeficiency virus particles and corresponding polypeptides in a DNA vaccine. Journal of Virology 79, 626-631.

Aldovini, A., Young, R. A., 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. Journal of Virology 64, 1920-1926.

Alfadhli, A., Dhenub, T. C., Still, A., Barklis, E., 2005. Analysis of human immunodeficiency virus type 1 Gag dimerization-induced assembly. Journal of Virology 79, 14498-14506.

Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., Earl, P. L., Smith, J. M., Ma, H. L., Grimm, B. D., Hulsey, M. L., McClure, H. M., McNicholl, J. M., Moss, B., Robinson, H. L., 2002. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Vaccine 20, 1949-1955.

Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., Cavacini, L. A., Posner, M. R., Katinger, H., Stiegler, G., Bernacky, B. J., Rizvi, T. A., Schmidt, R., Hill, L. R., Keeling, M. E., Lu, Y., Wright, J. E., Chou, T. C., Ruprecht, R. M., 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat.Med. 6, 200-206.

Bachmann, M. F., Lutz, M. B., Layton, G. T., Harris, S. J., Fehr, T., Rescigno, M., Ricciardi-Castagnoli, P., 1996. Dendritic cells process exogenous viral proteins and virus-like particles for class I presentation to CD8+ cytotoxic T lymphocytes. Eur.J.Immunol. 26, 2595-2600.

Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., Lewis, M. G., Emini, E. A., Shiver, J. W., Letvin, N. L., 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science 290, 486-492.

Barsoum, J., Brown, R., McKee, M., Boyce, F. M., 1997. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum.Gene Ther. 8, 2011-2018.

Baudin, F., Marquet, R., Isel, C., Darlix, J. L., Ehresmann, B., Ehresmann, C., 1993. Functional sites in the 5' region of human immunodeficiency virus type 1 RNA form defined structural domains. J.Mol.Biol. 229, 382-397.

Bellier, B., Dalba, C., Clerc, B., Desjardins, D., Drury, R., Cosset, F. L., Collins, M., Klatzmann, D., 2006. DNA vaccines encoding retrovirus-based virus-like particles induce efficient immune responses without adjuvant. Vaccine 24, 2643-2655.

Belyakov, I. M., Hel, Z., Kelsall, B., Kuznetsov, V. A., Ahlers, J. D., Nacsa, J., Watkins, D. I., Allen, T. M., Sette, A., Altman, J., Woodward, R., Markham, P. D., Clements, J. D., Franchini, G., Strober, W., Berzofsky, J. A., 2001. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. Nat.Med. 7, 1320-1326.

Berkowitz, R., Fisher, J., Goff, S. P., 1996. RNA packaging. Curr.Top.Microbiol.Immunol. 214, 177-218.

Berkowitz, R. D., Luban, J., Goff, S. P., 1993. Specific binding of human immunodeficiency virus type 1 gag polyprotein and nucleocapsid protein to viral RNAs detected by RNA mobility shift assays. Journal of Virology 67, 7190-7200.

Berkowitz, R. D., Goff, S. P., 1994. Analysis of binding elements in the human immunodeficiency virus type 1 genomic RNA and nucleocapsid protein. Virology 202, 233-246.

Berkowitz, R. D., Ohagen, A., Hoglund, S., Goff, S. P., 1995. Retroviral nucleocapsid domains mediate the specific recognition of genomic viral RNAs by chimeric Gag polyproteins during RNA packaging in vivo. Journal of Virology 69, 6445-6456.

Betts, M. R., Ambrozak, D. R., Douek, D. C., Bonhoeffer, S., Brenchley, J. M., Casazza, J. P., Koup, R. A., Picker, L. J., 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. Journal of Virology 75, 11983-11991.

Blissard, G. W., Wenz, J. R., 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. Journal of Virology 66, 6829-6835.

Blissard, G. W., 1996. Baculovirus--insect cell interactions. Cytotechnology 20, 73-93.

Boaz, M. J., Waters, A., Murad, S., Easterbrook, P. J., Vyakarnam, A., 2002. Presence of HIV-1 Gag-specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection. J.Immunol. 169, 6376-6385.

Boaz, M. J., Waters, A., Murad, S., Easterbrook, P. J., D'Sousa, E., van, W. C., Vyakarnam, A., 2003. CD4 responses to conserved HIV-1 T helper epitopes show both negative and positive associations with virus load in chronically infected subjects. Clin.Exp.Immunol. 134, 454-463.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M., Oldstone, M. B., 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. Journal of Virology 68, 6103-6110.

Borsetti, A., Ohagen, A., Gottlinger, H. G., 1998. The C-terminal half of the human immunodeficiency virus type 1 Gag precursor is sufficient for efficient particle assembly. Journal of Virology 72, 9313-9317.

Bowzard, J. B., Bennett, R. P., Krishna, N. K., Ernst, S. M., Rein, A., Wills, J. W., 1998. Importance of basic residues in the nucleocapsid sequence for retrovirus Gag assembly and complementation rescue. Journal of Virology 72, 9034-9044.

Boyce, F. M., Bucher, N. L., 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc.Natl.Acad.Sci.U.S.A 93, 2348-2352.

Briggs, J. A., Simon, M. N., Gross, I., Krausslich, H. G., Fuller, S. D., Vogt, V. M., Johnson, M. C., 2004. The stoichiometry of Gag protein in HIV-1. Nat.Struct.Mol.Biol. 11, 672-675.

Bruck, C., Thiriart, C., Fabry, L., Francotte, M., Pala, P., Van, O. O., Culp, J., Rosenberg, M., De, W. M., Heidt, P., Heeney, J., 1994. HIV-1 envelope-elicited neutralizing antibody titres correlate with protection and virus load in chimpanzees. Vaccine 12, 1141-1148.

Bukrinskaya, A. G., Ghorpade, A., Heinzinger, N. K., Smithgall, T. E., Lewis, R. E., Stevenson, M., 1996. Phosphorylation-dependent human immunodeficiency virus type 1 infection and nuclear targeting of viral DNA. Proc.Natl.Acad.Sci.U.S.A 93, 367-371.

Buonaguro, L., Racioppi, L., Tornesello, M. L., Arra, C., Visciano, M. L., Biryahwaho, B., Sempala, S. D., Giraldo, G., Buonaguro, F. M., 2002. Induction of neutralizing antibodies and cytotoxic T lymphocytes in Balb/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A. Antiviral Res. 54, 189-201.

Buonaguro, L., Visciano, M. L., Tornesello, M. L., Tagliamonte, M., Biryahwaho, B., Buonaguro, F. M., 2005. Induction of systemic and mucosal cross-clade neutralizing antibodies in BALB/c mice immunized with human immunodeficiency virus type 1 clade A virus-like particles administered by different routes of inoculation. Journal of Virology 79, 7059-7067.

Buonaguro, L., Tornesello, M. L., Tagliamonte, M., Gallo, R. C., Wang, L. X., Kamin-Lewis, R., Abdelwahab, S., Lewis, G. K., Buonaguro, F. M., 2006. Baculovirus-derived human immunodeficiency virus type 1 virus-like particles activate dendritic cells and induce ex vivo T-cell responses. Journal of Virology 80, 9134-9143.

Burniston, M. T., Cimarelli, A., Colgan, J., Curtis, S. P., Luban, J., 1999. Human immunodeficiency virus type 1 Gag polyprotein multimerization requires the nucleocapsid domain and RNA and is promoted by the capsid-dimer interface and the basic region of matrix protein. Journal of Virology 73, 8527-8540.

Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., Yee, J. K., 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc.Natl.Acad.Sci.U.S.A 90, 8033-8037.

Cadet, J., 1991, The photochemistry of nucleic acids, 2-272, In H Morrison (ed.), Bioorganic photochemistry, vol1. Wiley intescience, Hoboken, N.J.

Campbell, S., Vogt, V. M., 1995. Self-assembly in vitro of purified CA-NC proteins from Rous sarcoma virus and human immunodeficiency virus type 1. Journal of Virology 69, 6487-6497.

Campbell, S., Vogt, V. M., 1997. In vitro assembly of virus-like particles with Rous sarcoma virus Gag deletion mutants: identification of the p10 domain as a morphological determinant in the formation of spherical particles. Journal of Virology 71, 4425-4435.

Campbell, S., Rein, A., 1999. In vitro assembly properties of human immunodeficiency virus type 1 Gag protein lacking the p6 domain. Journal of Virology 73, 2270-2279.

Carriere, C., Gay, B., Chazal, N., Morin, N., Boulanger, P., 1995. Sequence requirements for encapsidation of deletion mutants and chimeras of human immunodeficiency virus type 1 Gag precursor into retrovirus-like particles. Journal of Virology 69, 2366-2377.

Chan, D. C., Fass, D., Berger, J. M., Kim, P. S., 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89, 263-273.

Chan, D. C., Kim, P. S., 1998. HIV entry and its inhibition. Cell 93, 681-684.

Chege, G. K., Shephard, E. G., Meyers, A., van Harmelen, J., Williamson, C., Lynch, A., Gray, C. M., Rybicki, E. P., Williamson A. –L., 2008. HIV-1 subtype C Pr55gag virus-like particle vaccine efficiently boosts baboons primed with a matched DNA vaccine. Journal of General Virology 89, 2497-2501.

Chen, X., IJkel, W. F., Tarchini, R., Sun, X., Sandbrink, H., Wang, H., Peters, S., Zuidema, D., Lankhorst, R. K., Vlak, J. M., Hu, Z., 2001. The sequence of the Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus genome. Journal of General Virology 82, 241-257.

Chen, Y. L., Ts'ai, P. W., Yang, C. C., Wang, C. T., 1997. Generation of infectious virus particles by transient co-expression of human immunodeficiency virus type 1 gag mutants. Journal of General Virology 78 (Pt 10), 2497-2501.

Chernomordik, L., Leikina, E., Cho, M. S., Zimmerberg, J., 1995. Control of baculovirus gp64-induced syncytium formation by membrane lipid composition. Journal of Virology 69, 3049-3058.

Chertova, E., Bess, J. J., Jr., Crise, B. J., Sowder II, R. C., Schaden, T. M., Hilburn, J. M., Hoxie, J. A., Benveniste, R. E., Lifson, J. D., Henderson, L. E., Arthur, L. O., 2002. Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), Is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. Journal of Virology 76, 5315-5325.

Chiu, H. C., Yao, S. Y., Wang, C. T., 2002. Coding sequences upstream of the human immunodeficiency virus type 1 reverse transcriptase domain in Gag-Pol are not essential for incorporation of the Pr160(gag-pol) into virus particles. Journal of Virology 76, 3221-3231.

Chung, C. T., Niemela, S. L., Miller, R. H., 1989. One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. Proc.Natl.Acad.Sci.U.S.A 86, 2172-2175.

Cimarelli, A., Sandin, S., Hoglund, S., Luban, J., 2000. Basic residues in human immunodeficiency virus type 1 nucleocapsid promote virion assembly via interaction with RNA. Journal of Virology 74, 3046-3057.

Clavel, F., Orenstein, J. M., 1990. A mutant of human immunodeficiency virus with reduced RNA packaging and abnormal particle morphology. Journal of Virology 64, 5230-5234.

Clever, J. L., Wong, M. L., Parslow, T. G., 1996. Requirements for kissing-loop-mediated dimerization of human immunodeficiency virus RNA. Journal of Virology 70, 5902-5908.

Clever, J. L., Eckstein, D. A., Parslow, T. G., 1999. Genetic dissociation of the encapsidation and reverse transcription functions in the 5' R region of human immunodeficiency virus type 1. Journal of Virology 73, 101-109.

Clever, J. L., Taplitz, R. A., Lochrie, M. A., Polisky, B., Parslow, T. G., 2000. A heterologous, high-affinity RNA ligand for human immunodeficiency virus Gag protein has RNA packaging activity. Journal of Virology 74, 541-546.

Clever, J. L., Miranda, J. D., Jr., Parslow, T. G., 2002. RNA structure and packaging signals in the 5' leader region of the human immunodeficiency virus type 1 genome. J Virol 76, 12381-12387.

Cosson, P., 1996. Direct interaction between the envelope and matrix proteins of HIV-1. EMBO J. 15, 5783-5788.

Cranage, M. P., Baskerville, A., Ashworth, L. A., Dennis, M., Cook, N., Sharpe, S., Farrar, G., Rose, J., Kitchin, P. A., Greenaway, P. J., 1992. Intrarectal challenge of macaques vaccinated with formalin-inactivated simian immunodeficiency virus. Lancet 339, 273-274.

Daecke, J., Fackler, O. T., Dittmar, M. T., Krausslich, H. G., 2005. Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. Journal of Virology 79, 1581-1594.

Dalgleish, A. G., Habeshaw, J., Manca, F., 1991. HIV and tropism: implications for pathogenesis. Mol.Aspects Med. 12, 267-282.

Dannull, J., Surovoy, A., Jung, G., Moelling, K., 1994. Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. EMBO J. 13, 1525-1533.

Darlix, J. L., Lapadat-Tapolsky, M., de, R. H., Roques, B. P., 1995. First glimpses at structure-function relationships of the nucleocapsid protein of retroviruses. J.Mol.Biol. 254, 523-537.

Das, A. T., Klaver, B., Klasens, B. I., van Wamel, J. L., Berkhout, B., 1997. A conserved hairpin motif in the R-U5 region of the human immunodeficiency virus type 1 RNA genome is essential for replication. Journal of Virology 71, 2346-2356.

Dawson, L., Yu, X. F., 1998. The role of nucleocapsid of HIV-1 in virus assembly. Virology 251, 141-157.

Dayton, A. I., Sodroski, J. G., Rosen, C. A., Goh, W. C., Haseltine, W. A., 1986. The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. Cell 44, 941-947.

De Guzman, R. N., Wu, Z. R., Stalling, C. C., Pappalardo, L., Borer, P. N., Summers, M. F., 1998. Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. Science 279, 384-388.

De Roda Husman, A. M., Bijkerk, P., Lodder, W., Van Den, B. H., Pribil, W., Cabaj, A., Gehringer, P., Sommer, R., Duizer, E., 2004. Calicivirus inactivation by nonionizing (253.7-nanometer-wavelength [UV]) and ionizing (gamma) radiation. Appl.Environ.Microbiol. 70, 5089-5093.

de Rocquigny. H., Gabus, C., Vincent, A., Fournie-Zaluski, M. C., Roques, B., Darlix, J. L., 1992. Viral RNA annealing activities of human immunodeficiency virus type 1 nucleocapsid protein require only peptide domains outside the zinc fingers. Proc.Natl.Acad.Sci.U.S.A 89, 6472-6476.

Delaney, W. E., Isom, H. C., 1998. Hepatitis B virus replication in human HepG2 cells mediated by hepatitis B virus recombinant baculovirus. Hepatology 28, 1134-1146.

Deml, L., Schirmbeck, R., Reimann, J., Wolf, H., Wagner, R., 1997. Recombinant human immunodeficiency Pr55gag virus-like particles presenting chimeric envelope glycoproteins induce cytotoxic T-cells and neutralizing antibodies. Virology 235, 26-39.

Deml, L., Wild, J., Wagner, R., 2004. Virus-like particles: a novel tool for the induction and monitoring of both T-helper and cytotoxic T-lymphocyte activity. Methods Mol.Med. 94, 133-157.

Deml, L., Speth, C., Dierich, M. P., Wolf, H., Wagner, R., 2005. Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses. Mol.Immunol. 42, 259-277.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di, M. P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., Landau, N. R., 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature 381, 661-666.

DePolo, N. J., Reed, J. D., Sheridan, P. L., Townsend, K., Sauter, S. L., Jolly, D. J., Dubensky, T. W., Jr., 2000. VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. Mol.Ther. 2, 218-222.

Dimitrov, A. S., Xiao, X., Dimitrov, D. S., Blumenthal, R., 2001. Early intermediates in HIV-1 envelope glycoprotein-mediated fusion triggered by CD4 and co-receptor complexes. J.Biol.Chem. 276, 30335-30341.

Dimitrov, A. S., Louis, J. M., Bewley, C. A., Clore, G. M., Blumenthal, R., 2005. Conformational changes in HIV-1 gp41 in the course of HIV-1 envelope glycoprotein-mediated fusion and inactivation. Biochemistry 44, 12471-12479.

Dorfman, T., Luban, J., Goff, S. P., Haseltine, W. A., Gottlinger, H. G., 1993. Mapping of functionally important residues of a cysteine-histidine box in the human immunodeficiency virus type 1 nucleocapsid protein. Journal of Virology 67, 6159-6169.

Dorfman, T., Bukovsky, A., Ohagen, A., Hoglund, S., Gottlinger, H. G., 1994. Functional domains of the capsid protein of human immunodeficiency virus type 1. Journal of Virology 68, 8180-8187.

Dorfman, T., Mammano, F., Haseltine, W. A., Gottlinger, H. G., 1994. Role of the matrix protein in the virion association of the human immunodeficiency virus type 1 envelope glycoprotein. Journal of Virology 68, 1689-1696.

Duisit, G., Saleun, S., Douthe, S., Barsoum, J., Chadeuf, G., Moullier, P., 1999. Baculovirus vector requires electrostatic interactions including heparan sulfate for efficient gene transfer in mammalian cells. J.Gene Med. 1, 93-102.

Dumais, N., Patrick, A., Moss, R. B., Davis, H. L., Rosenthal, K. L., 2002. Mucosal immunization with inactivated human immunodeficiency virus plus CpG oligodeoxynucleotides induces genital immune responses and protection against intravaginal challenge. J.Infect.Dis. 186, 1098-1105.

Dupraz, P., Spahr, P. F., 1992. Specificity of Rous sarcoma virus nucleocapsid protein in genomic RNA packaging. Journal of Virology 66, 4662-4670.

Durbeej, B., Eriksson, L. A., 2003. On the formation of cyclobutane pyrimidine dimers in UV-irradiated DNA: why are thymines more reactive? Photochem.Photobiol. 78, 159-167.

Bermann-Leitner, E. S., Leitner, W. W., 2004. Danger, death and DNA vaccines. Microbes and Infection. 6, 319-327.

Ernst, W., Schinko, T., Spenger, A., Oker-Blom, C., Grabherr, R., 2006. Improving baculovirus transduction of mammalian cells by surface display of a RGD-motif. J.Biotechnol. 126, 237-240.

Evans, N. A., Savige, W. E., McLaren, A. D., 1969. Photochemistry of tobacco mosaic virus RNA. Effect of HCN. Photochem.Photobiol. 9, 515-526.

Facciabene, A., Aurisicchio, L., La Monica, N., 2004. Baculovirus vectors elicit antigen-specific immune responses in mice. Journal of Virology 78, 8663-8672.

Facke, M., Janetzko, A., Shoeman, R. L., Krausslich, H. G., 1993. A large deletion in the matrix domain of the human immunodeficiency virus gag gene redirects virus particle assembly from the plasma membrane to the endoplasmic reticulum. Journal of Virology 67, 4972-4980.

Fackler, O. T., Peterlin, B. M., 2000. Endocytic entry of HIV-1. Curr.Biol. 10, 1005-1008.

Farzan, M., Choe, H., Desjardins, E., Sun, Y., Kuhn, J., Cao, J., Archambault, D., Kolchinsky, P., Koch, M., Wyatt, R., Sodroski, J., 1998. Stabilization of human immunodeficiency virus type

1 envelope glycoprotein trimers by disulfide bonds introduced into the gp41 glycoprotein ectodomain. Journal of Virology 72, 7620-7625.

Fauci, A. S., 2007. 25 years of HIV/AIDS science: reaching the poor with research advances. Cell 131, 429-432.

Fauquet, C. M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., 2008. Baculoviridae, 177. Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic press, Amsterdam, Boston, Heidelberg, London, New York, Oxford, Paris, Singapore, San Diego, San Francisco, Sydney, Tokyo.

Fields, B. N., Knipe, D. M., Howly, P. M., Chanock, R. M., Melnick, J. L., Monath, T. P., Roizman, B. and Straus, S. E., 1996. Fields-Virology 60, 1881-1952. Lippincott-Raven publishers; Philadelphia, New York.

Franke, E. K., Yuan, H. E., Luban, J., 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature 372, 359-362.

Fredericksen, B. L., Wei, B. L., Yao, J., Luo, T., Garcia, J. V., 2002. Inhibition of endosomal/lysosomal degradation increases the infectivity of human immunodeficiency virus. Journal of Virology 76, 11440-11446.

Freed, E. O., Orenstein, J. M., Buckler-White, A. J., Martin, M. A., 1994. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. Journal of Virology 68, 5311-5320.

Freed, E. O., Martin, M. A., 1995. Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. Journal of Virology 69, 1984-1989.

Freed, E. O., Martin, M. A., 1996. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. Journal of Virology 70, 341-351.

Freed, E. O., 2002. Viral late domains. Journal of Virology 76, 4679-4687.

Furuta, R. A., Shimano, R., Ogasawara, T., Inubushi, R., Amano, K., Akari, H., Hatanaka, M., Kawamura, M., Adachi, A., 1997. HIV-1 capsid mutants inhibit the replication of wild-type virus at both early and late infection phases. FEBS Lett. 415, 231-234.

Gallay, P., Swingler, S., Aiken, C., Trono, D., 1995. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. Cell 80, 379-388.

Gamble, T. R., Vajdos, F. F., Yoo, S., Worthylake, D. K., Houseweart, M., Sundquist, W. I., Hill, C. P., 1996. Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell 87, 1285-1294.

Gamble, T. R., Yoo, S., Vajdos, F. F., von Schwedler, U. K., Worthylake, D. K., Wang, H., McCutcheon, J. P., Sundquist, W. I., Hill, C. P., 1997. Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein. Science 278, 849-853.

Ganser, B. K., Li, S., Klishko, V. Y., Finch, J. T., Sundquist, W. I., 1999. Assembly and analysis of conical models for the HIV-1 core. Science 283, 80-83.

Gao, H., Wang, Y., Li, N., Peng, W. P., Sun, Y., Tong, G. Z., Qiu, H. J., 2007. Efficient gene delivery into mammalian cells mediated by a recombinant baculovirus containing a whispovirus ie1 promoter, a novel shuttle promoter between insect cells and mammalian cells. J.Biotechnol. 131, 138-143.

Garcea, R. L., Gissmann, L., 2004. Virus-like particles as vaccines and vessels for the delivery of small molecules. Curr.Opin.Biotechnol. 15, 513-517.

Garland, S. M., Hernandez-Avila, M., Wheeler, C. M., Perez, G., Harper, D. M., Leodolter, S., Tang, G. W., Ferris, D. G., Steben, M., Bryan, J., Taddeo, F. J., Railkar, R., Esser, M. T., Sings, H. L., Nelson, M., Boslego, J., Sattler, C., Barr, E., Koutsky, L. A., 2007. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. N.Engl.J.Med. 356, 1928-1943.

Garnier, L., Ratner, L., Rovinski, B., Cao, S. X., Wills, J. W., 1998. Particle size determinants in the human immunodeficiency virus type 1 Gag protein. Journal of Virology 72, 4667-4677.

Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszka, D. G., Sundquist, W. I., 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell 107, 55-65.

Gelderblom, H. R., Ozel, M., Pauli, G., 1989. Morphogenesis and morphology of HIV. Structure-function relations. Arch.Virol. 106, 1-13.

Gelderblom, H. R., 1991. Assembly and morphology of HIV: potential effect of structure on viral function. AIDS 5, 617-637.

Gheysen, D., Jacobs, E., de Foresta, F., Thiriart, C., Francotte, M., Thines, D., De Wilde, M., 1989. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. Cell 59, 103-112.

Gorelick, R. J., Nigida, S. M., Jr., Bess, J. W., Jr., Arthur, L. O., Henderson, L. E., Rein, A., 1990. Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA. Journal of Virology 64, 3207-3211.

Gorelick, R. J., Chabot, D. J., Rein, A., Henderson, L. E., Arthur, L. O., 1993. The two zinc fingers in the human immunodeficiency virus type 1 nucleocapsid protein are not functionally equivalent. Journal of Virology 67, 4027-4036.

Gorelick, R. J., Chabot, D. J., Ott, D. E., Gagliardi, T. D., Rein, A., Henderson, L. E., Arthur, L. O., 1996. Genetic analysis of the zinc finger in the Moloney murine leukemia virus nucleocapsid domain: replacement of zinc-coordinating residues with other zinc-coordinating residues yields noninfectious particles containing genomic RNA. Journal of Virology 70, 2593-2597.

Gottlinger, H. G., Sodroski, J. G., Haseltine, W. A., 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc.Natl.Acad.Sci.U.S.A 86, 5781-5785.

Gottlinger, H. G., Dorfman, T., Sodroski, J. G., Haseltine, W. A., 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc.Natl.Acad.Sci.U.S.A 88, 3195-3199.

Granelli-Piperno, A., Zhong, L., Haslett, P., Jacobson, J., Steinman, R. M., 2000. Dendritic cells, infected with vesicular stomatitis virus-pseudotyped HIV-1, present viral antigens to CD4+ and CD8+ T cells from HIV-1-infected individuals. J.Immunol. 165, 6620-6626.

Grewe, C., Beck, A., Gelderblom, H. R., 1990. HIV: early virus-cell interactions. J.Acquir.Immune.Defic.Syndr. 3, 965-974.

Gross, I., Hohenberg, H., Krausslich, H. G., 1997. In vitro assembly properties of purified bacterially expressed capsid proteins of human immunodeficiency virus. Eur.J.Biochem. 249, 592-600.

Grovit-Ferbas, K., Hsu, J. F., Ferbas, J., Gudeman, V., Chen, I. S., 2000. Enhanced binding of antibodies to neutralization epitopes following thermal and chemical inactivation of human immunodeficiency virus type 1. Journal of Virology 74, 5802-5809.

Guo, L., Lu, X., Kang, S. M., Chen, C., Compans, R. W., Yao, Q., 2003. Enhancement of mucosal immune responses by chimeric influenza HA/SHIV virus-like particles. Virology 313, 502-513.

Guo, X., Hu, J., Whitney, J. B., Russell, R. S., Liang, C., 2004. Important role for the CA-NC spacer region in the assembly of bovine immunodeficiency virus Gag protein. Journal of Virology 78, 551-560.

Guo, X., Roldan, A., Hu, J., Wainberg, M. A., Liang, C., 2005. Mutation of the SP1 sequence impairs both multimerization and membrane-binding activities of human immunodeficiency virus type 1 Gag. Journal of Virology 79, 1803-1812.

Haglund, K., Forman, J., Krausslich, H. G., Rose, J. K., 2000. Expression of human immunodeficiency virus type 1 Gag protein precursor and envelope proteins from a vesicular stomatitis virus recombinant: high-level production of virus-like particles containing HIV envelope. Virology 268, 112-121.

Haider, T., Sommer, R., Knasmuller, S., Eckl, P., Pribil, W., Cabaj, A., Kundi, M., 2002. Genotoxic response of Austrian groundwater samples treated under standardized UV (254 nm)--disinfection conditions in a combination of three different bioassays. Water Res. 36, 25-32.

Halsey, R. J., Tanzer, F. L., Meyers, A., Pillay, S., Lynch, A., Shephard, E., Williamson, A. L., Rybicki, E. P., 2008. Chimaeric HIV-1 subtype C Gag molecules with large in-frame C-terminal polypeptide fusions form virus-like particles. Virus Res. 133, 259-268.

Harari, A., Petitpierre, S., Vallelian, F., Pantaleo, G., 2004. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. Blood 103, 966-972.

Harm, W., 1980. Biological effects of UV radiation, 217. In IUPAB Biophysics series. Cambridge university press, United Kingdom.

Harrison, G. P., Lever, A. M., 1992. The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure. Journal of Virology 66, 4144-4153.

Harrison, G. P., Miele, G., Hunter, E., Lever, A. M., 1998. Functional analysis of the core human immunodeficiency virus type 1 packaging signal in a permissive cell line. Journal of Virology 72, 5886-5896.

Hefferon, K. L., Oomens, A. G., Monsma, S. A., Finnerty, C. M., Blissard, G. W., 1999. Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. Virology 258, 455-468.

Helga-Maria, C., Hammarskjold, M. L., Rekosh, D., 1999. An intact TAR element and cytoplasmic localization are necessary for efficient packaging of human immunodeficiency virus type 1 genomic RNA. Journal of Virology 73, 4127-4135.

Henderson, L. E., Bowers, M. A., Sowder, R. C., Serabyn, S. A., Johnson, D. G., Bess, J. W., Jr., Arthur, L. O., Bryant, D. K., Fenselau, C., 1992. Gag proteins of the highly replicative MN strain of human immunodeficiency virus type 1: posttranslational modifications, proteolytic processings, and complete amino acid sequences. Journal of Virology 66, 1856-1865.

Hermida-Matsumoto, L., Resh, M. D., 1999. Human immunodeficiency virus type 1 protease triggers a myristoyl switch that modulates membrane binding of Pr55(gag) and p17MA. Journal of Virology 73, 1902-1908.

Hervas-Stubbs, S., Rueda, P., Lopez, L., Leclerc, C., 2007. Insect baculoviruses strongly potentiate adaptive immune responses by inducing type I IFN. J.Immunol. 178, 2361-2369.

Hill, C. P., Worthylake, D., Bancroft, D. P., Christensen, A. M., Sundquist, W. I., 1996. Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. Proc.Natl.Acad.Sci.U.S.A 93, 3099-3104.

HIV Vaccines Trials Network (2008). Current Vaccine Trials. http://www.hvtn.org/ science/ trials.html.

Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P., Strauss, M., 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc.Natl.Acad.Sci.U.S.A 92, 10099-10103.

Hohmann, A. W., Faulkner, P., 1983. Monoclonal antibodies to baculovirus structural proteins: determination of specificities by Western blot analysis. Virology 125, 432-444.

Huang, M., Martin, M. A., 1997. Incorporation of Pr160(gag-pol) into virus particles requires the presence of both the major homology region and adjacent C-terminal capsid sequences within the Gag-Pol polyprotein. Journal of Virology 71, 4472-4478.

Huang, Z., Elkin, G., Maloney, B. J., Beuhner, N., Arntzen, C. J., Thanavala, Y., Mason, H. S., 2005. Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses. Vaccine 23, 1851-1858.

Huthoff, H., Berkhout, B., 2001. Two alternating structures of the HIV-1 leader RNA. RNA. 7, 143-157.

HVTN, HIV vaccine strategies, HIV vaccine types www.hvtn.org/science/ strategies.html.

Imaoka, K., Miller, C. J., Kubota, M., McChesney, M. B., Lohman, B., Yamamoto, M., Fujihashi, K., Someya, K., Honda, M., McGhee, J. R., Kiyono, H., 1998. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. J.Immunol. 161, 5952-5958.

Iyasere, C., Tilton, J. C., Johnson, A. J., Younes, S., Yassine-Diab, B., Sekaly, R. P., Kwok, W. W., Migueles, S. A., Laborico, A. C., Shupert, W. L., Hallahan, C. W., Davey, R. T., Jr., Dybul, M., Vogel, S., Metcalf, J., Connors, M., 2003. Diminished proliferation of human immunodeficiency virus-specific CD4+ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. Journal of Virology 77, 10900-10909.

Jacque, J. M., Mann, A., Enslen, H., Sharova, N., Brichacek, B., Davis, R. J., Stevenson, M., 1998. Modulation of HIV-1 infectivity by MAPK, a virion-associated kinase. EMBO J. 17, 2607-2618.

Jaffray, A., Shephard, E., van, H. J., Williamson, C., Williamson, A. L., Rybicki, E. P., 2004. Human immunodeficiency virus type 1 subtype C Gag virus-like particle boost substantially improves the immune response to a subtype C gag DNA vaccine in mice. Journal of General Virology 85, 409-413.

Jeang, K. T., 1998. Tat, Tat-associated kinase, and transcription. J.Biomed.Sci. 5, 24-27.

Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J., Weinberger, L., Kostrikis, L. G., Zhang, L., Perelson, A. S., Ho, D. D., 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J.Exp.Med. 189, 991-998.

Johnson, J. E., Schnell, M. J., Buonocore, L., Rose, J. K., 1997. Specific targeting to CD4+ cells of recombinant vesicular stomatitis viruses encoding human immunodeficiency virus envelope proteins. Journal of Virology 71, 5060-5068.

Johnson, J. E., Rodgers, W., Rose, J. K., 1998. A plasma membrane localization signal in the HIV-1 envelope cytoplasmic domain prevents localization at sites of vesicular stomatitis virus budding and incorporation into VSV virions. Virology 251, 244-252.

Johnson, P. R., Montefiori, D. C., Goldstein, S., Hamm, T. E., Zhou, J., Kitov, S., Haigwood, N. L., Misher, L., London, W. T., Gerin, J. L., ., 1992. Inactivated whole SIV vaccine in macaques: evaluation of protective efficacy against challenge with cell-free virus or infected cells. AIDS Res.Hum.Retroviruses 8, 1501-1505.

Jones, K. A., 1997. Taking a new TAK on tat transactivation. Genes Dev. 11, 2593-2599.

Kang, S. M., Compans, R. W., 2003. Enhancement of mucosal immunization with virus-like particles of simian immunodeficiency virus. J Virol 77, 3615-3623.

Kannanganat, S., Ibegbu, C., Chennareddi, L., Robinson, H. L., Amara, R. R., 2007. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. Journal of Virology 81, 8468-8476.

Kannanganat, S., Kapogiannis, B. G., Ibegbu, C., Chennareddi, L., Goepfert, P., Robinson, H. L., Lennox, J., Amara, R. R., 2007. Human immunodeficiency virus type 1 controllers but not noncontrollers maintain CD4 T cells coexpressing three cytokines. Journal of Virology 81, 12071-12076.

Kaplan, A. H., Manchester, M., Swanstrom, R., 1994. The activity of the protease of human immunodeficiency virus type 1 is initiated at the membrane of infected cells before the release of viral proteins and is required for release to occur with maximum efficiency. Journal of Virology 68, 6782-6786.

Karacostas, V., Nagashima, K., Gonda, M. A., Moss, B., 1989. Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. Proc.Natl.Acad.Sci.U.S.A 86, 8964-8967.

Kaul, R., Dong, T., Plummer, F. A., Kimani, J., Rostron, T., Kiama, P., Njagi, E., Irungu, E., Farah, B., Oyugi, J., Chakraborty, R., MacDonald, K. S., Bwayo, J. J., McMichael, A., Rowland-Jones, S. L., 2001. CD8(+) lymphocytes respond to different HIV epitopes in seronegative and infected subjects. J.Clin.Invest 107, 1303-1310.

Kaul, R., Rowland-Jones, S. L., Kimani, J., Dong, T., Yang, H. B., Kiama, P., Rostron, T., Njagi, E., Bwayo, J. J., MacDonald, K. S., McMichael, A. J., Plummer, F. A., 2001. Late seroconversion in HIV-resistant Nairobi prostitutes despite pre-existing HIV-specific CD8+ responses. J.Clin.Invest 107, 341-349.

Kaye, J. F., Richardson, J. H., Lever, A. M., 1995. cis-acting sequences involved in human immunodeficiency virus type 1 RNA packaging. Journal of Virology 69, 6588-6592.

Keddie, B. A., Volkman, L. E., 1985. Infectivity difference between the two phenotypes ofAutographa californica nuclear polyhedrosis virus: importance of the 64K envelope glycoprotein.Journal of General Virology. 66,1195-1200.

Kim, H. J., Lee, K., O'Rear, J. J., 1994. A short sequence upstream of the 5' major splice site is important for encapsidation of HIV-1 genomic RNA. Virology 198, 336-340.

Kingsley, D. H., Behbahani, A., Rashtian, A., Blissard, G. W., Zimmerberg, J., 1999. A discrete stage of baculovirus GP64-mediated membrane fusion. Mol.Biol.Cell 10, 4191-4200.

Kitagawa, Y., Tani, H., Limn, C. K., Matsunaga, T. M., Moriishi, K., Matsuura, Y., 2005. Ligand-directed gene targeting to mammalian cells by pseudotype baculoviruses. Journal of Virology 79, 3639-3652.

Kitajima, M., Hamazaki, H., Miyano-Kurosaki, N., Takaku, H., 2006. Characterization of baculovirus Autographa californica multiple nuclear polyhedrosis virus infection in mammalian cells. Biochem.Biophys.Res.Commun. 343, 378-384.

Ko, G., Cromeans, T. L., Sobsey, M. D., 2005. UV inactivation of adenovirus type 41 measured by cell culture mRNA RT-PCR. Water Res. 39, 3643-3649.

Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A., Scolnick, E. M., Sigal, I. S., 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc.Natl.Acad.Sci.U.S.A 85, 4686-4690.

Kost, T. A., Condreay, J. P., 2002. Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol. 20, 173-180.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C., Ho, D. D., 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. Journal of Virology 68, 4650-4655.

Krausslich, H. G., Ochsenbauer, C., Traenckner, A. M., Mergener, K., Facke, M., Gelderblom, H. R., Bosch, V., 1993. Analysis of protein expression and virus-like particle formation in mammalian cell lines stably expressing HIV-1 gag and env gene products with or without active HIV proteinase. Virology 192, 605-617.

Krausslich, H. G., Facke, M., Heuser, A. M., Konvalinka, J., Zentgraf, H., 1995. The spacer peptide between human immunodeficiency virus capsid and nucleocapsid proteins is essential for ordered assembly and viral infectivity. Journal of Virology 69, 3407-3419.

Kuate, S., Stahl-Hennig, C., Haaft, P., Heeney, J., Uberla, K., 2003. Single-cycle immunodeficiency viruses provide strategies for uncoupling in vivo expression levels from viral replicative capacity and for mimicking live-attenuated SIV vaccines. Virology 313, 653-662.

Kuate, S., Stahl-Hennig, C., Stoiber, H., Nchinda, G., Floto, A., Franz, M., Sauermann, U., Bredl, S., Deml, L., Ignatius, R., Norley, S., Racz, P., Tenner-Racz, K., Steinman, R. M., Wagner, R., Uberla, K., 2006. Immunogenicity and efficacy of immunodeficiency virus-like particles pseudotyped with the G protein of vesicular stomatitis virus. Virology 351, 133-144.

Kuluncsics, Z., Perdiz, D., Brulay, E., Muel, B., Sage, E., 1999. Wavelength dependence of ultraviolet-induced DNA damage distribution: involvement of direct or indirect mechanisms and possible artefacts. J.Photochem.Photobiol.B 49, 71-80.

Kumar, M., Bradow, B. P., Zimmerberg, J., 2003. Large-scale production of pseudotyped lentiviral vectors using baculovirus GP64. Hum.Gene Ther. 14, 67-77.

Lanzavecchia, A., 1996. Mechanisms of antigen uptake for presentation. Curr.Opin.Immunol. 8, 348-354.

Lapadat-Tapolsky, M., de, R. H., Van, G. D., Roques, B., Plasterk, R., Darlix, J. L., 1993. Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle. Nucleic Acids Res. 21, 831-839.

Laughrea, M., Jette, L., 1994. A 19-nucleotide sequence upstream of the 5' major splice donor is part of the dimerization domain of human immunodeficiency virus 1 genomic RNA. Biochemistry 33, 13464-13474.

Lee, Y. M., Tian, C. J., Yu, X. F., 1998. A bipartite membrane-binding signal in the human immunodeficiency virus type 1 matrix protein is required for the proteolytic processing of Gag precursors in a cell type-dependent manner. Journal of Virology 72, 9061-9068.

Leikina, E., Onaran, H. O., Zimmerberg, J., 1992. Acidic pH induces fusion of cells infected with baculovirus to form syncytia. FEBS Lett. 304, 221-224.

Letvin, N. L., 2005. Progress toward an HIV vaccine. Annu.Rev.Med. 56, 213-223.

Levy, J. A., 2001. The importance of the innate immune system in controlling HIV infection and disease. Trends Immunol. 22, 312-316.

Li, S., Hill, C. P., Sundquist, W. I., Finch, J. T., 2000. Image reconstructions of helical assemblies of the HIV-1 CA protein. Nature 407, 409-413.

Liang, C., Song, J., Chen, X., 2005. The GP64 protein of Autographa californica multiple nucleopolyhedrovirus rescues Helicoverpa armigera nucleopolyhedrovirus transduction in mammalian cells. Journal of General Virology 86, 1629-1635.

Limberis, M., Anson, D. S., Fuller, M., Parsons, D. W., 2002. Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer. Hum.Gene Ther. 13, 1961-1970.

Lindwasser, O. W., Resh, M. D., 2001. Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains. Journal of Virology 75, 7913-7924.

Linial, M. L., Miller, A. D., 1990. Retroviral RNA packaging: sequence requirements and implications. Curr.Top.Microbiol.Immunol. 157, 125-152.

Liu, M. L., Winther, B. L., Kay, M. A., 1996. Pseudotransduction of hepatocytes by using concentrated pseudotyped vesicular stomatitis virus G glycoprotein (VSV-G)-Moloney murine leukemia virus-derived retrovirus vectors: comparison of VSV-G and amphotropic vectors for hepatic gene transfer. Journal of Virology 70, 2497-2502.

Long, G., Pan, X., Kormelink, R., Vlak, J. M., 2006. Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. Journal of Virology 80, 8830-8833.

Lu, M., Blacklow, S. C., Kim, P. S., 1995. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nat.Struct.Biol. 2, 1075-1082.

Luban, J., Goff, S. P., 1991. Binding of human immunodeficiency virus type 1 (HIV-1) RNA to recombinant HIV-1 gag polyprotein. Journal of Virology 65, 3203-3212.

Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., Goff, S. P., 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell 73, 1067-1078.

Luban, J., Goff, S. P., 1994. Mutational analysis of cis-acting packaging signals in human immunodeficiency virus type 1 RNA. Journal of Virology 68, 3784-3793.

Ludwig, C., Wagner, R., 2007. Virus-like particles-universal molecular toolboxes. Curr.Opin.Biotechnol. 18, 537-545.

Lytle, C. D., Sagripanti, J. L., 2005. Predicted inactivation of viruses of relevance to biodefense by solar radiation. Journal of Virology 79, 14244-14252.

Mammano, F., Ohagen, A., Hoglund, S., Gottlinger, H. G., 1994. Role of the major homology region of human immunodeficiency virus type 1 in virion morphogenesis. Journal of Virology 68, 4927-4936.

Mammano, F., Kondo, E., Sodroski, J., Bukovsky, A., Gottlinger, H. G., 1995. Rescue of human immunodeficiency virus type 1 matrix protein mutants by envelope glycoproteins with short cytoplasmic domains. Journal of Virology 69, 3824-3830.

Mangor, J. T., Monsma, S. A., Johnson, M. C., Blissard, G. W., 2001. A GP64-null baculovirus pseudotyped with vesicular stomatitis virus G protein. Journal of Virology 75, 2544-2556.

Marechal, V., Clavel, F., Heard, J. M., Schwartz, O., 1998. Cytosolic Gag p24 as an index of productive entry of human immunodeficiency virus type 1. Journal of Virology 72, 2208-2212.

Marechal, V., Prevost, M. C., Petit, C., Perret, E., Heard, J. M., Schwartz, O., 2001. Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. Journal of Virology 75, 11166-11177.

Markovic, I., Pulyaeva, H., Sokoloff, A., Chernomordik, L. V., 1998. Membrane fusion mediated by baculovirus gp64 involves assembly of stable gp64 trimers into multiprotein aggregates. J.Cell Biol. 143, 1155-1166.

Marquet, R., Paillart, J. C., Skripkin, E., Ehresmann, C., Ehresmann, B., 1994. Dimerization of human immunodeficiency virus type 1 RNA involves sequences located upstream of the splice donor site. Nucleic Acids Res. 22, 145-151.

Marsac, D., Loirat, D., Petit, C., Schwartz, O., Michel, M. L., 2002. Enhanced Presentation of Major Histocompatibility Complex Class I-Restricted Human Immunodeficiency Virus Type 1 (HIV-1) Gag-Specific Epitopes after DNA Immunization with Vectors Coding for Vesicular Stomatitis Virus Glycoprotein- Pseudotyped HIV-1 Gag Particles. The Journal of Virology 76, 7544-7553.

Mascola, J. R., Lewis, M. G., Stiegler, G., Harris, D., VanCott, T. C., Hayes, D., Louder, M. K., Brown, C. R., Sapan, C. V., Frankel, S. S., Lu, Y., Robb, M. L., Katinger, H., Birx, D. L., 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. Journal of Virology 73, 4009-4018.

Matilainen, H., Makela, A. R., Riikonen, R., Saloniemi, T., Korhonen, E., Hyypia, T., Heino, J., Grabherr, R., Oker-Blom, C., 2006. RGD motifs on the surface of baculovirus enhance transduction of human lung carcinoma cells. J.Biotechnol. 125, 114-126.

McBride, M. S., Panganiban, A. T., 1996. The human immunodeficiency virus type 1 encapsidation site is a multipartite RNA element composed of functional hairpin structures. Journal of Virology 70, 2963-2973.

McBride, M. S., Schwartz, M. D., Panganiban, A. T., 1997. Efficient encapsidation of human immunodeficiency virus type 1 vectors and further characterization of cis elements required for encapsidation. Journal of Virology 71, 4544-4554.

McDougal, J. S., Martin, L. S., Cort, S. P., Mozen, M., Heldebrant, C. M., Evatt, B. L., 1985. Thermal inactivation of the acquired immunodeficiency syndrome virus, human T lymphotropic virus-III/lymphadenopathy-associated virus, with special reference to antihemophilic factor. J.Clin.Invest 76, 875-877.

Melamed, D., Mark-Danieli, M., Kenan-Eichler, M., Kraus, O., Castiel, A., Laham, N., Pupko, T., Glaser, F., Ben-Tal, N., Bacharach, E., 2004. The conserved carboxy terminus of the capsid domain of human immunodeficiency virus type 1 gag protein is important for virion assembly and release. Journal of Virology 78, 9675-9688.

Mergener, K., Facke, M., Welker, R., Brinkmann, V., Gelderblom, H. R., Krausslich, H. G., 1992. Analysis of HIV particle formation using transient expression of subviral constructs in mammalian cells. Virology 186, 25-39.

Mervis, R. J., Ahmad, N., Lillehoj, E. P., Raum, M. G., Salazar, F. H., Chan, H. W., Venkatesan, S., 1988. The gag gene products of human immunodeficiency virus type 1: alignment within the gag open reading frame, identification of posttranslational modifications, and evidence for alternative gag precursors. Journal of Virology 62, 3993-4002.

Meyers, A., Chakauya, E., Shephard, E., Tanzer, F. L., Maclean, J., Lynch, A., Williamson, A. L., Rybicki, E. P., 2008. Expression of HIV-1 antigens in plants as potential subunit vaccines. BMC.Biotechnol. 8, 53.

Monsma, S. A., Blissard, G. W., 1995. Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein. Journal of Virology 69, 2583-2595.

Monsma, S. A., Oomens, A. G., Blissard, G. W., 1996. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. Journal of Virology 70, 4607-4616.

Montefiori, D. C., Safrit, J. T., Lydy, S. L., Barry, A. P., Bilska, M., Vo, H. T., Klein, M., Tartaglia, J., Robinson, H. L., Rovinski, B., 2001. Induction of neutralizing antibodies and gag-specific cellular immune responses to an R5 primary isolate of human immunodeficiency virus type 1 in rhesus macaques. Journal of Virology 75, 5879-5890.

Muriaux, D., Fosse, P., Paoletti, J., 1996. A kissing complex together with a stable dimer is involved in the HIV-1Lai RNA dimerization process in vitro. Biochemistry 35, 5075-5082.

Muriaux, D., Mirro, J., Harvin, D., Rein, A., 2001. RNA is a structural element in retrovirus particles. Proc.Natl.Acad.Sci.U.S.A 98, 5246-5251.

Muriaux, D., Mirro, J., Nagashima, K., Harvin, D., Rein, A., 2002. Murine leukemia virus nucleocapsid mutant particles lacking viral RNA encapsidate ribosomes. Journal of Virology 76, 11405-11413.

Murphey-Corb, M., 1997. Live-attenuated HIV vaccines: how safe is safe enough? Nat.Med. 3, 17-18.

Myers, G., Berzofsky, J. A., Korber, B., Smith, R. F. & Pavlakis, G. N., 1991. Human Retroviruses and AIDS: a Compilation and Analysis of Nucleic and Amino Acid Sequences. Los Alamos, NM: Los Alamos National Laboratory.

National Institute of Allergy and Infectious Diseases (2008). How HIV causes AIDS. www.aegis.com/topics/ basics/hivandaids.html.

National Institute of Allergy and Infectious Diseases (2008). http://www3.niaid.nih.gov/ news/QA/step_ qa.htm).

Nguyen, D. H., Hildreth, J. E., 2000. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. Journal of Virology 74, 3264-3272.

Nicklas, W., 1992. Aluminum salts. Res.Immunol. 143, 489-494.

Ogg, G. S., Jin, X., Bonhoeffer, S., Dunbar, P. R., Nowak, M. A., Monard, S., Segal, J. P., Cao, Y., Rowland-Jones, S. L., Cerundolo, V., Hurley, A., Markowitz, M., Ho, D. D., Nixon, D. F., McMichael, A. J., 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 279, 2103-2106.

Ono, A., Freed, E. O., 2001. Plasma membrane rafts play a critical role in HIV-1 assembly and release. Proc.Natl.Acad.Sci.U.S.A 98, 13925-13930.

Oomens, A. G., Monsma, S. A., Blissard, G. W., 1995. The baculovirus GP64 envelope fusion protein: synthesis, oligomerization, and processing. Virology 209, 592-603.

Oomens, A. G., Blissard, G. W., 1999. Requirement for GP64 to drive efficient budding of Autographa californica multicapsid nucleopolyhedrovirus. Virology 254, 297-314.

Oomens, A. G., Wertz, G. W., 2004. The baculovirus GP64 protein mediates highly stable infectivity of a human respiratory syncytial virus lacking its homologous transmembrane glycoproteins. Journal of Virology 78, 124-135.

Ooms, M., Huthoff, H., Russell, R., Liang, C., Berkhout, B., 2004. A riboswitch regulates RNA dimerization and packaging in human immunodeficiency virus type 1 virions. Journal of Virology 78, 10814-10819.

O'Reilly, D. R., Miller, L. K., Luckow, V. A., 1994. Baculovirus expression vectors - A Laboratory manual 1-2, 1-50. Oxford University Press; Oxford; New York.

Ory, D. S., Neugeboren, B. A., Mulligan, R. C., 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc.Natl.Acad.Sci.U.S.A 93, 11400-11406.

Ott, D. E., Coren, L. V., Gagliardi, T. D., 2005. Redundant roles for nucleocapsid and matrix RNA-binding sequences in human immunodeficiency virus type 1 assembly. Journal of Virology 79, 13839-13847.

Paavonen, J., Jenkins, D., Bosch, F. X., Naud, P., Salmeron, J., Wheeler, C. M., Chow, S. N., Apter, D. L., Kitchener, H. C., Castellsague, X., de Carvalho, N. S., Skinner, S. R., Harper, D. M., Hedrick, J. A., Jaisamrarn, U., Limson, G. A., Dionne, M., Quint, W., Spiessens, B., Peeters, P., Struyf, F., Wieting, S. L., Lehtinen, M. O., Dubin, G., 2007. Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. Lancet 369, 2161-2170.

Paillart, J. C., Marquet, R., Skripkin, E., Ehresmann, B., Ehresmann, C., 1994. Mutational analysis of the bipartite dimer linkage structure of human immunodeficiency virus type 1 genomic RNA. J.Biol.Chem. 269, 27486-27493.

Paillart, J. C., Skripkin, E., Ehresmann, B., Ehresmann, C., Marquet, R., 1996. A loop-loop "kissing" complex is the essential part of the dimer linkage of genomic HIV-1 RNA. Proc.Natl.Acad.Sci.U.S.A 93, 5572-5577.

Paillart, J. C., Gottlinger, H. G., 1999. Opposing effects of human immunodeficiency virus type 1 matrix mutations support a myristyl switch model of gag membrane targeting. Journal of Virology 73, 2604-2612.

Paliard, X., Liu, Y., Wagner, R., Wolf, H., Baenziger, J., Walker, C. M., 2000. Priming of strong, broad, and long-lived HIV type 1 p55gag-specific CD8+ cytotoxic T cells after administration of a virus-like particle vaccine in rhesus macaques. AIDS Res.Hum.Retroviruses 16, 273-282.

Pantaleo, G., Koup, R. A., 2004. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. Nat.Med. 10, 806-810.

Parker, S. D., Hunter, E., 2000. A cell-line-specific defect in the intracellular transport and release of assembled retroviral capsids. Journal of Virology 74, 784-795.

Parolin, C., Dorfman, T., Palu, G., Gottlinger, H., Sodroski, J., 1994. Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes. Journal of Virology 68, 3888-3895.

Pauza, C. D., Price, T. M., 1988. Human immunodeficiency virus infection of T cells and monocytes proceeds via receptor-mediated endocytosis. J.Cell Biol. 107, 959-968.

Pearson, M. N., Groten, C., Rohrmann, G. F., 2000. Identification of the lymantria dispar nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the Baculoviridae. Journal of Virology 74, 6126-6131.

Pepinsky, R. B., Vogt, V. M., 1984. Fine-structure analyses of lipid-protein and protein-protein interactions of gag protein p19 of the avian sarcoma and leukemia viruses by cyanogen bromide mapping. Journal of Virology 52, 145-153.

Pieroni, L., Maione, D., La, M. N., 2001. In vivo gene transfer in mouse skeletal muscle mediated by baculovirus vectors. Hum.Gene Ther. 12, 871-881.

Piszkiewicz, D., Thomas, W., Lieu, M., Cabradilla, C. D., Andrews, J., Kim, J., Bourret, E., McDougal, J. S., Cort, S. P., 1986. Heat inactivation of human immunodeficiency virus in lyophilized anti-inhibitor coagulant complex (Autoplex). Thromb.Res. 44, 701-707.

Pitcher, C. J., Quittner, C., Peterson, D. M., Connors, M., Koup, R. A., Maino, V. C., Picker, L. J., 1999. HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. Nat.Med. 5, 518-525.

Plonsky, I., Cho, M. S., Oomens, A. G., Blissard, G., Zimmerberg, J., 1999. An analysis of the role of the target membrane on the Gp64-induced fusion pore. Virology 253, 65-76.

Poon, B., Hsu, J. F., Gudeman, V., Chen, I. S., Grovit-Ferbas, K., 2005. Formaldehyde-treated, heat-inactivated virions with increased human immunodeficiency virus type 1 env can be used to induce high-titer neutralizing antibody responses. Journal of Virology 79, 10210-10217.

Poon, B., Safrit, J. T., McClure, H., Kitchen, C., Hsu, J. F., Gudeman, V., Petropoulos, C., Wrin, T., Chen, I. S., Grovit-Ferbas, K., 2005. Induction of humoral immune responses following vaccination with envelope-containing, formaldehyde-treated, thermally inactivated human immunodeficiency virus type 1. Journal of Virology 79, 4927-4935.

Poon, D. T., Wu, J., Aldovini, A., 1996. Charged amino acid residues of human immunodeficiency virus type 1 nucleocapsid p7 protein involved in RNA packaging and infectivity. Journal of Virology 70, 6607-6616.

Poon, D. T., Li, G., Aldovini, A., 1998. Nucleocapsid and matrix protein contributions to selective human immunodeficiency virus type 1 genomic RNA packaging. Journal of Virology 72, 1983-1993.

Poon, D. T., Chertova, E. N., Ott, D. E., 2002. Human immunodeficiency virus type 1 preferentially encapsidates genomic RNAs that encode Pr55(Gag): functional linkage between translation and RNA packaging. Virology 293, 368-378.

Porter, D. C., Melsen, L. R., Compans, R. W., Morrow, C. D., 1996. Release of virus-like particles from cells infected with poliovirus replicons which express human immunodeficiency virus type 1 Gag. Journal of Virology 70, 2643-2649.

Prats, A. C., Housset, V., de, B. G., Cornille, F., Prats, H., Roques, B., Darlix, J. L., 1991. Viral RNA annealing activities of the nucleocapsid protein of Moloney murine leukemia virus are zinc independent. Nucleic Acids Res. 19, 3533-3541.

Quinnan, G. V., Jr., Wells, M. A., Wittek, A. E., Phelan, M. A., Mayner, R. E., Feinstone, S., Purcell, R. H., Epstein, J. S., 1986. Inactivation of human T-cell lymphotropic virus, type III by heat, chemicals, and irradiation. Transfusion 26, 481-483.

Race, E., Frezza, P., Stephens, D. M., Davis, D., Polyanskaya, N., Cranage, M., Oxford, J. S., 1995. An experimental chemically inactivated HIV-1 vaccine induces antibodies that neutralize homologous and heterologous viruses. Vaccine 13, 54-60.

Race, E., Stein, C. A., Wigg, M. D., Baksh, A., Addawe, M., Frezza, P., Oxford, J. S., 1995. A multistep procedure for the chemical inactivation of human immunodeficiency virus for use as an experimental vaccine. Vaccine 13, 1567-1575.

Redfield, R. R., Wright, D. C., James, W. D., Jones, T. S., Brown, C., Burke, D. S., 1987. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N.Engl.J.Med. 316, 673-676.

Richardson, J. H., Child, L. A., Lever, A. M., 1993. Packaging of human immunodeficiency virus type 1 RNA requires cis-acting sequences outside the 5' leader region. Journal of Virology 67, 3997-4005.

Robinson, H. L., 2002. New hope for an AIDS vaccine. Nat.Rev.Immunol. 2, 239-250.

Robinson, H. L., Sharma, S., Zhao, J., Kannanganat, S., Lai, L., Chennareddi, L., Yu, T., Montefiori, D. C., Amara, R. R., Wyatt, L. S., Moss, B., 2007. Immunogenicity in macaques of the clinical product for a clade B DNA/MVA HIV vaccine: elicitation of IFN-gamma, IL-2, and TNF-alpha coproducing CD4 and CD8 T cells. AIDS Res.Hum.Retroviruses 23, 1555-1562.

Rosenberg, E. S., Altfeld, M., Poon, S. H., Phillips, M. N., Wilkes, B. M., Eldridge, R. L., Robbins, G. K., D'Aquila, R. T., Goulder, P. J., Walker, B. D., 2000. Immune control of HIV-1 after early treatment of acute infection. Nature 407, 523-526.

Rowland-Jones, S., Tan, R., McMichael, A., 1997. Role of cellular immunity in protection against HIV infection. Adv.Immunol. 65, 277-346.

Rowland-Jones, S. L., Dong, T., Fowke, K. R., Kimani, J., Krausa, P., Newell, H., Blanchard, T., Ariyoshi, K., Oyugi, J., Ngugi, E., Bwayo, J., MacDonald, K. S., McMichael, A. J., Plummer, F. A., 1998. Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. J.Clin.Invest 102, 1758-1765.

Royer, M., Hong, S. S., Gay, B., Cerutti, M., Boulanger, P., 1992. Expression and extracellular release of human immunodeficiency virus type 1 Gag precursors by recombinant baculovirus-infected cells. Journal of Virology 66, 3230-3235.

Ruedl, C., Storni, T., Lechner, F., Bachi, T., Bachmann, M. F., 2002. Cross-presentation of virus-like particles by skin-derived CD8(-) dendritic cells: a dispensable role for TAP. Eur.J.Immunol. 32, 818-825.

Ruedl, C., Schwarz, K., Jegerlehner, A., Storni, T., Manolova, V., Bachmann, M. F., 2005. Virus-like particles as carriers for T-cell epitopes: limited inhibition of T-cell priming by carrier-specific antibodies. Journal of Virology 79, 717-724.

Russell, R. S., Hu, J., Laughrea, M., Wainberg, M. A., Liang, C., 2002. Deficient dimerization of human immunodeficiency virus type 1 RNA caused by mutations of the u5 RNA sequences. Virology 303, 152-163.

Russell, R. S., Hu, J., Beriault, V., Mouland, A. J., Laughrea, M., Kleiman, L., Wainberg, M. A., Liang, C., 2003. Sequences downstream of the 5' splice donor site are required for both packaging and dimerization of human immunodeficiency virus type 1 RNA. Journal of Virology 77, 84-96.

Russell, R. S., Liang, C., Wainberg, M. A., 2004. Is HIV-1 RNA dimerization a prerequisite for packaging? Yes, no, probably? Retrovirology. 1, 23.

Sadagopal, S., Amara, R. R., Montefiori, D. C., Wyatt, L. S., Staprans, S. I., Kozyr, N. L., McClure, H. M., Moss, B., Robinson, H. L., 2005. Signature for long-term vaccine-mediated control of a Simian and human immunodeficiency virus 89.6P challenge: stable low-breadth and low-frequency T-cell response capable of coproducing gamma interferon and interleukin-2. Journal of Virology 79, 3243-3253.

Sailaja, G., Skountzou, I., Quan, F. S., Compans, R. W., Kang, S. M., 2007. Human immunodeficiency virus-like particles activate multiple types of immune cells. Virology 362, 331-341.

Sakuragi, J., Sakai, H., Kawamura, M., Tokunaga, K., Ueda, S., Adachi, A., 1995. Generation and characterization of a host cell-dependent gag gene mutant of human immunodeficiency virus type 1. Virology 212, 251-254.

Sakuragi, S., Goto, T., Sano, K., Morikawa, Y., 2002. HIV type 1 Gag virus-like particle budding from spheroplasts of Saccharomyces cerevisiae. Proc.Natl.Acad.Sci.U.S.A 99, 7956-7961.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (2). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.

Swanstrom, R., Wills, J. W., 1997. Synthesis, assembly and processing of viral proteins, 263-334. Coffin, J. M., Hughs, S. H., Varmus, H.E. (eds), Retroviruses. Cold Spring Harbour Lab Press, Cold Spring Harbour, NY.

Schaeffer, E., Geleziunas, R., Greene, W. C., 2001. Human immunodeficiency virus type 1 Nef functions at the level of virus entry by enhancing cytoplasmic delivery of virions. Journal of Virology 75, 2993-3000.

Schaeffer, E., Soros, V. B., Greene, W. C., 2004. Compensatory link between fusion and endocytosis of human immunodeficiency virus type 1 in human CD4 T lymphocytes. Journal of Virology 78, 1375-1383.

Schauber, C. A., Tuerk, M. J., Pacheco, C. D., Escarpe, P. A., Veres, G., 2004. Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro. Gene Ther. 11, 266-275.

Schmalzbauer, E., Strack, B., Dannull, J., Guehmann, S., Moelling, K., 1996. Mutations of basic amino acids of NCp7 of human immunodeficiency virus type 1 affect RNA binding in vitro. Journal of Virology 70, 771-777.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L., Reimann, K. A., 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 283, 857-860.

Senior, K., 2002. Adeno-associated virus vectors under scrutiny. Lancet 359, 1216.

Shapiro, S. Z., 2002. The HIV/AIDS vaccine researchers' orientation to the process of preparing a US FDA application for an investigational new drug (IND): what it is all about and how you start by preparing for your pre-IND meeting. Vaccine 20, 1261-1280.

Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z. Q., Simon, A. J., Trigona, W. L., Dubey, S. A., Huang, L., Harris, V. A., Long, R. S., Liang, X., Handt, L., Schleif, W. A., Zhu, L., Freed, D. C., Persaud, N. V., Guan, L., Punt, K. S., Tang, A., Chen, M., Wilson, K. A., Collins, K. B., Heidecker, G. J., Fernandez, V. R., Perry, H. C., Joyce, J. G., Grimm, K. M., Cook, J. C., Keller, P. M., Kresock, D. S., Mach, H., Troutman, R. D., Isopi, L. A., Williams, D. M., Xu, Z., Bohannon, K. E., Volkin, D. B., Montefiori, D. C., Miura, A., Krivulka, G. R., Lifton, M. A., Kuroda, M. J., Schmitz, J. E., Letvin, N. L., Caulfield, M. J., Bett, A. J., Youil, R., Kaslow, D. C., Emini, E. A., 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 415, 331-335.

Shoji, I., Aizaki, H., Tani, H., Ishii, K., Chiba, T., Saito, I., Miyamura, T., Matsuura, Y., 1997. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. Journal of General Virology 78 (Pt 10), 2657-2664.

Simonet, J., Gantzer, C., 2006. Inactivation of poliovirus 1 and F-specific RNA phages and degradation of their genomes by UV irradiation at 254 nanometers. Appl.Environ.Microbiol. 72, 7671-7677.

Sinn, P. L., Burnight, E. R., Hickey, M. A., Blissard, G. W., McCray, P. B., Jr., 2005. Persistent gene expression in mouse nasal epithelia following feline immunodeficiency virus-based vector gene transfer. Journal of Virology 79, 12818-12827.

Skountzou, I., Quan, F. S., Gangadhara, S., Ye, L., Vzorov, A., Selvaraj, P., Jacob, J., Compans, R. W., Kang, S. M., 2007. Incorporation of glycosylphosphatidylinositol-anchored granulocyte- macrophage colony-stimulating factor or CD40 ligand enhances immunogenicity of chimeric simian immunodeficiency virus-like particles. Journal of Virology 81, 1083-1094.

Skripkin, E., Paillart, J. C., Marquet, R., Ehresmann, B., Ehresmann, C., 1994. Identification of the primary site of the human immunodeficiency virus type 1 RNA dimerization in vitro. Proc.Natl.Acad.Sci.U.S.A 91, 4945-4949.

Smith, A. J., Srinivasakumar, N., Hammarskjold, M. L., Rekosh, D., 1993. Requirements for incorporation of Pr160gag-pol from human immunodeficiency virus type 1 into virus-like particles. Journal of Virology 67, 2266-2275.

Song, J., Liang, C., Chen, X., 2006. Transduction of avian cells with recombinant baculovirus. J.Virol.Methods 135, 157-162.

South African Treatment Action Campaign website. (2008). Current ARV statistics. http://www.tac.org.za/community/keystatistics.

Spearman, P., Wang, J. J., Vander, H. N., Ratner, L., 1994. Identification of human immunodeficiency virus type 1 Gag protein domains essential to membrane binding and particle assembly. Journal of Virology 68, 3232-3242.

Spearman, P., Horton, R., Ratner, L., Kuli-Zade, I., 1997. Membrane binding of human immunodeficiency virus type 1 matrix protein in vivo supports a conformational myristyl switch mechanism. Journal of Virology 71, 6582-6592.

Srinivasakumar, N., Hammarskjold, M. L., Rekosh, D., 1995. Characterization of deletion mutations in the capsid region of human immunodeficiency virus type 1 that affect particle formation and Gag-Pol precursor incorporation. Journal of Virology 69, 6106-6114.

Stein, B. S., Gowda, S. D., Lifson, J. D., Penhallow, R. C., Bensch, K. G., Engleman, E. G., 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell 49, 659-668.

Stott, E. J., Chan, W. L., Mills, K. H., Page, M., Taffs, F., Cranage, M., Greenaway, P., Kitchin, P., 1990. Preliminary report: protection of cynomolgus macaques against simian immunodeficiency virus by fixed infected-cell vaccine. Lancet 336, 1538-1541.

Su, J., Palm, A., Wu, Y., Sandin, S., Hoglund, S., Vahlne, A., 2000. Deletion of the GPG motif in the HIV type 1 V3 loop does not abrogate infection in all cells. AIDS Res.Hum.Retroviruses 16, 37-48.

Tani, H., Nishijima, M., Ushijima, H., Miyamura, T., Matsuura, Y., 2001. Characterization of cell-surface determinants important for baculovirus infection. Virology 279, 343-353.

Tani, H., Limn, C. K., Yap, C. C., Onishi, M., Nozaki, M., Nishimune, Y., Okahashi, N., Kitagawa, Y., Watanabe, R., Mochizuki, R., Moriishi, K., Matsuura, Y., 2003. In vitro and in vivo gene delivery by recombinant baculoviruses. Journal of Virology 77, 9799-9808.

Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C. T., Sodroski, J., Gottlinger, H. G., 1994. Functional association of cyclophilin A with HIV-1 virions. Nature 372, 363-365.

The Step Study (2008). http://www.stepstudies.com/

Theilmann, D. A., Blissard, G. W., Bonning, B., Jehle, J. A., O'Reily, D. R., Rohrmann, G. F., Thiem, S., Vlak J. M., 2005. Baculoviridae, 177–185.*In* C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), VIIIth report of the International Committee on Taxonomy of Viruses. Elsevier, New York, N.Y.

Tsunetsugu-Yokota, Y., Morikawa, Y., Isogai, M., Kawana-Tachikawa, A., Odawara, T., Nakamura, T., Grassi, F., Autran, B., Iwamoto, A., 2003. Yeast-derived human immunodeficiency virus type 1 p55(gag) virus-like particles activate dendritic cells (DCs) and induce perforin expression in Gag-specific CD8(+) T cells by cross-presentation of DCs. Journal of Virology 77, 10250-10259.

UNAIDS – AIDS Epidemic update. (2008). http://www.unaids.org/en/ KnowledgeCentre/HIV Data/EpiUpdate/EpiUpdArchive/2007/default.asp

United States National Library of medicine. (2008). http://www.nlm.nih.gov/databases/alerts/hiv_step_study.html.

van Harmelen, J. H., Shephard, E., Thomas, R., Hanke, T., Williamson, A. L., Williamson, C., 2003. Construction and characterisation of a candidate HIV-1 subtype C DNA vaccine for South Africa. Vaccine 21, 4380-4389.

Vogt, V. M., 1996. Proteolytic processing and particle maturation. Curr.Top.Microbiol.Immunol. 214, 95-131.

Volkman, L. E., Goldsmith, P. A., Hess, R. T., Faulkner, P., 1984. Neutralization of budded Autographa californica NPV by a monoclonal antibody: Identification of the target antigen. Virology 133, 354-362.

von Schwedler, U. K., Stuchell, M., Muller, B., Ward, D. M., Chung, H. Y., Morita, E., Wang, H. E., Davis, T., He, G. P., Cimbora, D. M., Scott, A., Krausslich, H. G., Kaplan, J., Morham, S. G., Sundquist, W. I., 2003. The protein network of HIV budding. Cell 114, 701-713.

von, P. A., Wagner, R., Niedrig, M., Wanner, G., Wolf, H., Modrow, S., 1993. Identification of a region in the Pr55gag-polyprotein essential for HIV-1 particle formation. Virology 193, 981-985.

Wagner, R., Deml, L., Schirmbeck, R., Niedrig, M., Reimann, J., Wolf, H., 1996. Construction, expression, and immunogenicity of chimeric HIV-1 virus-like particles. Virology 220, 128-140.

Wagner, R., Teeuwsen, V. J., Deml, L., Notka, F., Haaksma, A. G., Jhagjhoorsingh, S. S., Niphuis, H., Wolf, H., Heeney, J. L., 1998. Cytotoxic T cells and neutralizing antibodies induced in rhesus monkeys by virus-like particle HIV vaccines in the absence of protection from SHIV infection. Virology 245, 65-74.

Wang, C. T., Zhang, Y., McDermott, J., Barklis, E., 1993. Conditional infectivity of a human immunodeficiency virus matrix domain deletion mutant. Journal of Virology 67, 7067-7076.

Wang, C. T., Barklis, E., 1993. Assembly, processing, and infectivity of human immunodeficiency virus type 1 gag mutants. Journal of Virology 67, 4264-4273.

Wang, C. T., Lai, H. Y., Li, J. J., 1998. Analysis of minimal human immunodeficiency virus type 1 gag coding sequences capable of virus-like particle assembly and release. J Virol 72, 7950-7959.

Wang, C. T., Chou, Y. C., Chiang, C. C., 2000. Assembly and processing of human immunodeficiency virus Gag mutants containing a partial replacement of the matrix domain by the viral protease domain. Journal of Virology 74, 3418-3422.

Wang, H., Norris, K. M., Mansky, L. M., 2003. Involvement of the matrix and nucleocapsid domains of the bovine leukemia virus Gag polyprotein precursor in viral RNA packaging. Journal of Virology 77, 9431-9438.

Wang, P., Hammer, D. A., Granados, R. R., 1997. Binding and fusion of Autographa californica nucleopolyhedrovirus to cultured insect cells. Journal of General Virology 78 (Pt 12), 3081-3089.

Wang, S., Kennedy, J. S., West, K., Montefiori, D. C., Coley, S., Lawrence, J., Shen, S., Green, S., Rothman, A. L., Ennis, F. A., Arthos, J., Pal, R., Markham, P., Lu, S., 2008. Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers. Vaccine 26, 1098-1110.

Wang, S. W., Aldovini, A., 2002. RNA incorporation is critical for retroviral particle integrity after cell membrane assembly of Gag complexes. Journal of Virology 76, 11853-11865.

WHO Towards universal access Scaling up priority HIV/AIDS interventions in the health sector Progress report, 2008. http://www.who.int/hiv/mediacentre/ 2008progressreport/en/index.html.

WHO sexually transmitted diseases, 2008. www.who.int/vaccine_research/diseases/soastd/en/index4.html.

Wilk, T., Gross, I., Gowen, B. E., Rutten, T., de, H. F., Welker, R., Krausslich, H. G., Boulanger, P., Fuller, S. D., 2001. Organization of immature human immunodeficiency virus type 1. Journal of Virology 75, 759-771.

Williamson, C., Morris, L., Maughan, M. F., Ping, L. H., Dryga, S. A., Thomas, R., Reap, E. A., Cilliers, T., van, H. J., Pascual, A., Ramjee, G., Gray, G., Johnston, R., Karim, S. A.,

Swanstrom, R., 2003. Characterization and selection of HIV-1 subtype C isolates for use in vaccine development. AIDS Res.Hum.Retroviruses 19, 133-144.

Wong, S. B., Siliciano, R. F., 2005. Contribution of virus-like particles to the immunogenicity of human immunodeficiency virus type 1 Gag-derived vaccines in mice. Journal of Virology 79, 1701-1712.

Wyatt, R., Sodroski, J., 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 280, 1884-1888.

Wyss, S., Dimitrov, A. S., Baribaud, F., Edwards, T. G., Blumenthal, R., Hoxie, J. A., 2005. Regulation of human immunodeficiency virus type 1 envelope glycoprotein fusion by a membrane-interactive domain in the gp41 cytoplasmic tail. Journal of Virology 79, 12231-12241.

Yamamoto, J. K., Okuda, T., Ackley, C. D., Louie, H., Pembroke, E., Zochlinski, H., Munn, R. J., Gardner, M. B., 1991. Experimental vaccine protection against feline immunodeficiency virus. AIDS Res.Hum.Retroviruses 7, 911-922.

Yamamoto, J. K., Hohdatsu, T., Olmsted, R. A., Pu, R., Louie, H., Zochlinski, H. A., Acevedo, V., Johnson, H. M., Soulds, G. A., Gardner, M. B., 1993. Experimental vaccine protection against homologous and heterologous strains of feline immunodeficiency virus. Journal of Virology 67, 601-605.

Yao, Q., Vuong, V., Li, M., Compans, R. W., 2002. Intranasal immunization with SIV virus-like particles (VLPs) elicits systemic and mucosal immunity. Vaccine 20, 2537-2545.

Yee, J. K., Friedmann, T., Burns, J. C., 1994. Generation of high-titer pseudotyped retroviral vectors with very broad host range. Methods Cell Biol. 43 Pt A, 99-112.

Yewdell, J. W., Norbury, C. C., Bennink, J. R., 1999. Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines. Adv.Immunol. 73, 1-77.

Yoo, S., Myszka, D. G., Yeh, C., McMurray, M., Hill, C. P., Sundquist, W. I., 1997. Molecular recognition in the HIV-1 capsid/cyclophilin A complex. J.Mol.Biol. 269, 780-795.

Younes, S. A., Yassine-Diab, B., Dumont, A. R., Boulassel, M. R., Grossman, Z., Routy, J. P., Sekaly, R. P., 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. J.Exp.Med. 198, 1909-1922.

Young, K. R., McBurney, S. P., Karkhanis, L. U., Ross, T. M., 2006. Virus-like particles: designing an effective AIDS vaccine. Methods 40, 98-117.

Yu, X., Yuan, X., Matsuda, Z., Lee, T. H., Essex, M., 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. Journal of Virology 66, 4966-4971.

Zhang, S., Hara, H., Kaji, H., Kaji, A., 1997. Inhibition of HIV type 1 RNA dimerization by antisense DNA corresponding to the 17-nucleotide sequence downstream from the splice donor site of HIV type 1 RNA. AIDS Res.Hum.Retroviruses 13, 865-873.

Zhang, W. H., Hockley, D. J., Nermut, M. V., Morikawa, Y., Jones, I. M., 1996. Gag-Gag interactions in the C-terminal domain of human immunodeficiency virus type 1 p24 capsid antigen are essential for Gag particle assembly. Journal of General Virology 77 (Pt 4), 743-751.

Zhang, Y., Barklis, E., 1995. Nucleocapsid protein effects on the specificity of retrovirus RNA encapsidation. Journal of Virology 69, 5716-5722.

Zhang, Y., Barklis, E., 1997. Effects of nucleocapsid mutations on human immunodeficiency virus assembly and RNA encapsidation. Journal of Virology 71, 6765-6776.

Zhou, J., Blissard, G. W., 2006. Mapping the conformational epitope of a neutralizing antibody (AcV1) directed against the AcMNPV GP64 protein. Virology 352, 427-437.

Zhou, J., Blissard, G. W., 2008. Display of heterologous proteins on gp64null baculovirus virions and enhanced budding mediated by a vesicular stomatitis virus G-stem construct. Journal of Virology 82, 1368-1377.

Zhou, W., Parent, L. J., Wills, J. W., Resh, M. D., 1994. Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. Journal of Virology 68, 2556-2569.

Zhu, P., Chertova, E., Bess, J., Jr., Lifson, J. D., Arthur, L. O., Liu, J., Taylor, K. A., Roux, K. H., 2003. Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. Proc.Natl.Acad.Sci.U.S.A 100, 15812-15817.

Zhu, P., Liu, J., Bess, J., Jr., Chertova, E., Lifson, J. D., Grise, H., Ofek, G. A., Taylor, K. A., Roux, K. H., 2006. Distribution and three-dimensional structure of AIDS virus envelope spikes. Nature 441, 847-852.

Zuber, G., McDermott, J., Karanjia, S., Zhao, W., Schmid, M. F., Barklis, E., 2000. Assembly of retrovirus capsid-nucleocapsid proteins in the presence of membranes or RNA. Journal of Virology 74, 7431-7441.