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Construction and Characterization of Chimaeric Human Immunodeficiency Virus Type 1 Subtype C Gag Virus-like Particles

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ABSTRACT

In this study I explored the possibility of making HIV-1 subtype C Pr55^{gag}-based chimaeric virus-like particles (VLPs) as a boost to the HIV-1C multigene DNA vaccine pTHr.grttnC, which encodes a modified Gag-RT-Tat-Nef fusion protein (GRTTN). Furthermore, an attempt was made to produce VLP analogues to the HIV-1A polyepitope DNA vaccine pTHr.HIVA. A range of in-frame fusions with the C-termini of myristylation-competent p6-truncated Gag and native Pr55^{gag} were made to test how the length of polypeptide and its sequence might affect VLP formation and structure. The fused peptides included an artificial HIV-1A polyepitope string (PE, 155 aa); the truncated protein sequences 3'RT (113 aa) and 3'TN (169 aa), and the entire RT (450 aa) and TN (322 aa) and RTTN portions (778 aa) of GRTTN. Baculovirus-expressed chimaeric proteins were examined by western blot and electron microscopy. Pr55^{gag} without a myristylation signal was localized to the insect cell nucleus where aggregations of multilayered Gag structures were observed. Removal of p6 in addition to the myristylation signal resulted in only partial nuclear localization with individual Gag structures also present in the cytoplasm. All Gag chimaeras, except for the Gag-PE fusions, localized to the cell membrane and formed regular budded structures that could be purified from the culture medium. Particle diameter increased with protein molecular weight, from ~100 nm for native Pr55^{gag} to ~250 nm for Gag-RTTN fusions. The presence or absence of the Gag p6 region did not obviously affect VLP formation or appearance. Gag-PE fusions produced large irregular budded structures, up to 450 nm across. The sequence of the polypeptide fusion appeared to have a greater influence on VLP formation than polypeptide length, possibly due to protein folding constraints. It would appear that chimaeric Gag fusions must be empirically tested for VLP formation, and that depending on the sequence composition, large C-terminal Gag fusions can produce chimaeric VLPs for vaccine purposes. This has important implications for candidate HIV-1 vaccine design. The ability to make particles with all or part of the protein sequence specified by a DNA vaccine will allow prime-boost vaccination regimens which should significantly enhance cellular responses.

ABBREVIATIONS

| | |
|-------|--|
| aa | amino acids |
| AcNPV | <i>Autographa californica</i> nuclear polyhedrosis virus |
| AIDS | acquired immunodeficiency syndrome |
| apTEM | adsorbed particle transmission electron microscopy |
| bp | base pairs |
| BSA | bovine serum albumen |
| CA | capsid |
| CD4BD | CD4-binding domain |
| CTD | C-terminal domain |
| CTL | cytotoxic T-lymphocyte |
| DNA | deoxyribonucleic acid |
| dpi | days post infection |
| EM | electron microscopy |
| g | gram |
| HIV | human immunodeficiency virus |
| hr | hour |
| IEM | immunolectron microscopy |
| IIF | indirect immunofluorescence |
| kb | kilobase |
| kDa | kilo Dalton |
| MA | matrix |
| mAb | monoclonal antibody |
| MCS | multiple cloning site |
| mg | milligram |
| MHR | major homology region |
| min | minute |
| ml | millilitre |
| MOI | multiplicity of infection |
| MVA | modified vaccinia virus Ankara |
| MW | molecular weight |

| | |
|-------------------------|--|
| <i>myr</i> ⁺ | myristylation signal positive gene |
| <i>myr</i> ⁻ | myristylation signal negative gene |
| Myr ⁺ | myristylated |
| Myr ⁻ | non-myristoylated |
| NAb | neutralizing antibody |
| NC | nucleocapsid |
| ng | nanogram |
| NLS | nuclear localization signal |
| nt | nucleotide |
| NTD | N-terminal domain |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pPolh | polyhedron promoter |
| PR | protease |
| rAcNPV | recombinant <i>Autographa californica</i> nuclear polyhedrosis virus |
| RNA | ribonucleic acid |
| RT | reverse transcriptase |
| SCC | sucrose cushion centrifugation |
| SDS | sodium dodecyl sulphate |
| SGC | sucrose gradient centrifugation |
| SIV | simian immunodeficiency virus |
| SV5 | simian virus 5 |
| SXC | sucrose cushion and gradient centrifugation |
| TEM | transmission electron microscopy |
| tsTEM | thin section transmission electron microscopy |
| μg | microgram |
| μl | microlitre |
| V3 | third hypervariable loop of gp120 |
| VDP | virus-derived particle |
| VLP | virus-like particle |
| WT | wild-type |

CHAPTER 1

Literature Review

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1.1 Overview

Human immunodeficiency virus (HIV) was discovered in 1983 (Barre-Sinoussi *et al.*, 1983), and has been identified as the main causative agent of acquired immunodeficiency syndrome (AIDS) in humans. HIV type 1 (HIV-1), HIV-2 and simian immunodeficiency virus (SIV) are all lentiviruses within the family *Retroviridae*, but it is the major (M) group of HIV-1 (with 9 subtypes: A-K, no E or I) that has been responsible for the vast majority of the ~2.8 million AIDS deaths worldwide in 2005 (UNAIDS, 2006). The greatest global disease burden due to the HIV pandemic is currently borne in sub-Saharan Africa (~64% of the ~39 million global infections (UNAIDS, 2006)), where HIV-1 subtype C (HIV-1C) predominates (IAVI, 2006). In developed countries, treatment of AIDS patients with highly active antiretroviral therapy (HAART) has proven successful in suppression of viral load and disease, thereby reducing mortality. However, the cost, the often sub-optimal distribution conditions and the uncertainty of supply pertaining in many sub-Saharan countries militates against the widespread use of antiretrovirals. Thus, the best long term solution to the HIV pandemic, particularly in these regions, is the development of safe, effective and inexpensive HIV vaccines (Burgers & Williamson, 2005; Nkolola & Essex, 2006).

It is increasingly recognized that an effective vaccine against HIV will need to elicit broad cell mediated and humoral immune responses in both the mucosal and systemic components of the immune system (Lehner, 2003). To date, no single HIV vaccine candidate has been shown to prevent establishment of HIV infection (Letvin, 2005). However, a number of mixed vaccines are being investigated, with the common immunogen prime–boost design often yielding strong cell-mediated immunological responses (McMichael & Hanke, 2003; Amara *et al.*, 2001; Amara *et al.*, 2005; Mwau *et al.*, 2004; Smith *et al.*, 2004; Goonetilleke *et al.*, 2006). The prime has usually been a DNA vaccine, while boost antigens have included subunit proteins, live vectors and particle-based vaccines, with a promising candidate being HIV-1 virus-like particles (VLPs) based on the Gag structural precursor polyprotein (Young & Ross, 2003; Montefiori *et al.*, 2001; Jaffray *et al.*, 2004; Paliard *et al.*, 2000; Doan *et al.*, 2005). Immune responses to Gag-based VLPs have been improved considerably by the incorporation of foreign antigenic proteins into the particle by two principal methods (Deml *et al.*, 2005), and one type of chimaeric Gag VLP was the focus of this work.

1.1.1 HIV-1 virion and proviral genome

The structural and regulatory proteins of HIV-1 have been extensively utilized as components in HIV-1 vaccines that have entered clinical trials (McMichael & Hanke, 2003), while the enzymatic proteins have been targeted by many therapeutic HIV-1 drugs (Pomerantz & Horn, 2003). The position of structural proteins within the mature HIV-1 virion and the genetic organization of the proviral genome are detailed in Figure. 1.1.

1.2 Rational design of HIV vaccines

Despite enormous efforts worldwide for over 20 years, there is still no protective HIV-1 vaccine, and progress in vaccine design has been hampered by a number of challenges. The error-prone reverse transcription of the viral RNA genome results in an extraordinarily high mutation rate, which allows for escape mutants that avoid recognition by both arms of the adaptive immune response (McMichael & Hanke, 2003). A compounding factor is the genetic and geographical diversity of HIV-1 subtypes, where prototype viruses have not been identified (Gaschen *et al.*, 2002). It is thought that vaccines may need to be directed at the predominant subtype within a given region to be effective (McMichael *et al.*, 2002). However, subtype specific vaccine design is further complicated by discrepancies between genotypes and immune properties (Nyambi *et al.*, 2000). Furthermore, the correlates of protection for HIV-1, including HIV-1C, are not well defined (Pantaleo & Koup, 2004). Non-human primate models are also of limited use in evaluating how existing vaccine designs can be improved, since HIV does not replicate in these animals (Burgers & Williamson, 2005).

The many attempts to generate vaccines eliciting neutralizing antibodies (NAbs) to HIV-1 isolates, primarily directed to regions of the envelope glycoprotein (Env, gp160), have been largely unsuccessful (Letvin, 2005; McMichael & Hanke, 2003). The third variable loop (V3) and CD4-binding domains (CD4BDs) of surface glycoprotein (gp120) have often been included in HIV-1 vaccines since these domains are recognized by antibodies that can neutralize HIV-1, albeit weakly or in an isolate-specific manner (Letvin & Walker, 2003). Although natural HIV-1 infection does lead to HIV-specific antibody production, few of the sera can neutralize heterologous strains (Bojak *et al.*, 2002). In the absence of NAbs that can broadly block viral infection, it is unlikely that HIV vaccines will be able to confer sterilizing immunity.

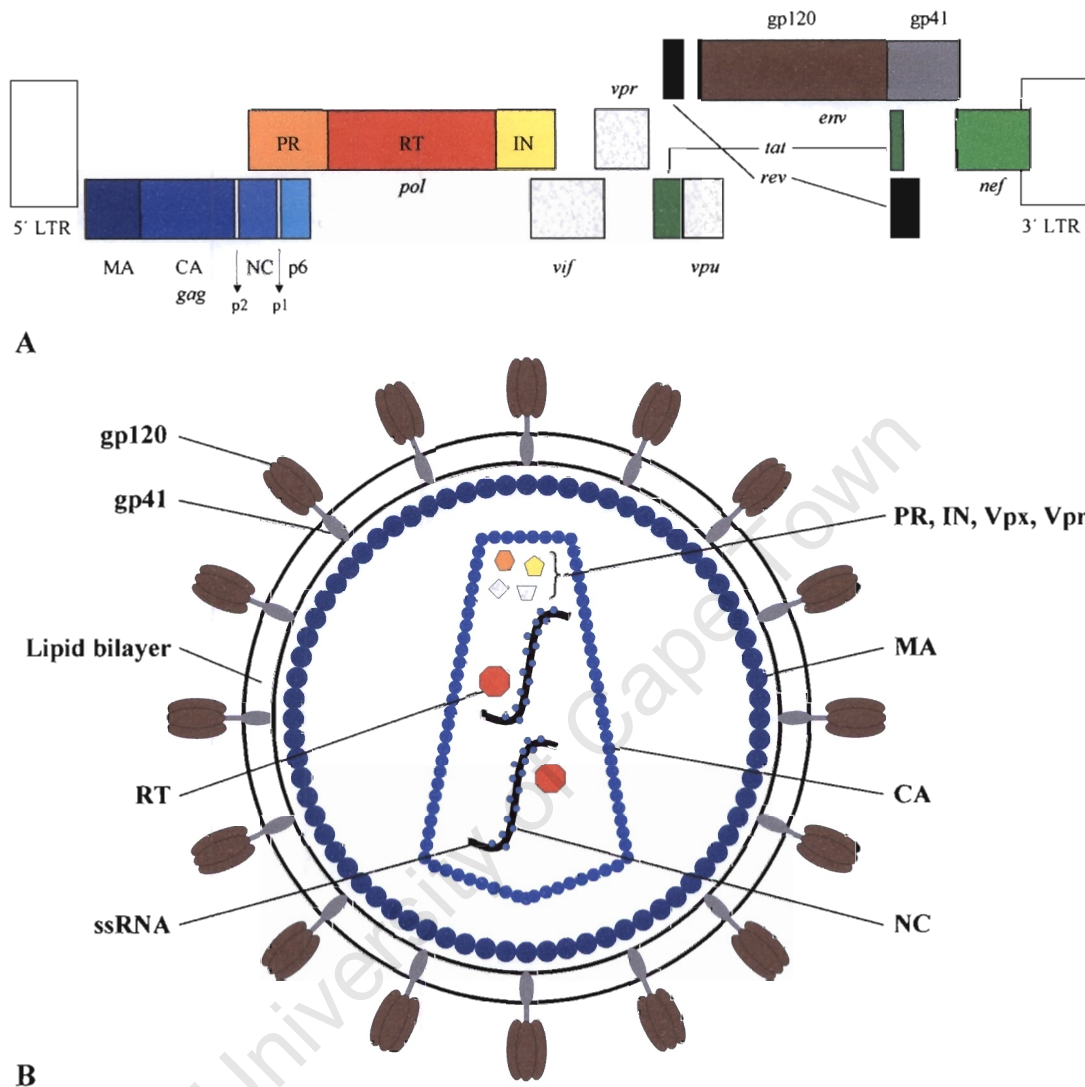


FIGURE 1.1. Schematic representation of the HIV-1 proviral genome and mature virion.

(A) The proviral genome (~9.2 kb) is flanked by two long terminal repeats (LTRs). There are 9 open reading frames encoding a total of 15 possible proteins. The *gag* gene is translated into the structural Gag precursor (Pr55^{gag}) which is cleaved into matrix (MA), capsid (CA), nucleocapsid (NC) and p6 (p2 and p1 are spacer peptides). The *pol* gene is translated by a (-1) ribosomal frameshift as a Gag-Pol precursor polypeptide that yields the enzymes reverse transcriptase (RT), integrase (IN) and protease (PR) upon cleavage. The *env* gene encodes another structural precursor (Env, gp160), which is cleaved into the surface (gp120) and transmembrane (gp41) glycoproteins. The regulatory (Nef, Tat, Rev) and accessory (Vpu, Vpr, Vif) proteins are encoded by spliced Env and Gag-Pol mRNAs. (B) In the mature HIV-1 virion, MA forms a layer beneath the host-derived lipid bilayer, CA forms the conical core and NC coats the two copies of viral single stranded (ss) RNA genome. Trimeric glycoprotein spikes composed of gp120 anchored to the lipid bilayer membrane by gp41 are exposed on the virion surface. Figures created based on data from Scarlata & Carter, 2003; Freed, 1998 & Luciw, 1996.

Early containment of acute HIV infection coincides with HIV-specific CD8⁺ cytotoxic T-lymphocyte (CTL) responses (Koup *et al.*, 1994; Letvin & Walker, 2003), which reduce viral load (Ogg *et al.*, 1998) and suppress viraemia (Gandhi & Walker, 2002). In conjunction with recent evidence highlighting the importance of CTL responses in controlling HIV infection, focus has shifted from producing vaccines that elicit NAbs to those that stimulate broad, cross-subtype cell mediated responses (Bojak *et al.*, 2002; McMichael & Hanke, 2003; Letvin, 2005; Nkolola & Essex, 2006). In addition, CD8⁺ CTL responses have often been favoured over CD4⁺ T-cell responses, since HIV replicates within CD4⁺ T cells. Nonetheless, there is evidence supporting the benefits of vaccine induced priming of CD4⁺ T-cell responses (Rosenberg *et al.*, 1997) which improve the effectiveness of the CD8⁺ CTL response (Kalams *et al.*, 1999). Ideally, vaccines should be designed to induce both systemic and mucosal responses, as HIV is spread venereally and through contaminated blood. Stimulation of innate immune responses further assists in combating infection and disease progression (Levy, 2001). Incorporating all these features into a single immunogen or into combination vaccine strategies remains a major challenge in HIV vaccine development.

1.2.1 Components of HIV vaccines

HIV-1 encodes 15 possible proteins (Fig. 1.1) and emerging vaccine designs for eliciting strong cell mediated responses have included components of Gag, Pol, Tat and Nef. The most conserved protein regions among HIV subtypes are within Gag and Pol, and cross-reactive anti-Pol and anti-Gag CTL responses are inversely correlated with disease progression (Ferrari *et al.*, 2000). Furthermore, defined epitopes recognized by CD8⁺ (Currier *et al.*, 2002; Xu *et al.*, 2002) and CD4⁺ (Boaz *et al.*, 2003) T cells in HIV-1 infected individuals have been identified in Gag. Both Tat and Nef induce strong cell mediated responses early in infection, and optimal CTL epitopes have been mapped in both of these regulatory proteins (Novitsky *et al.*, 2001). This temporary early immune activation may improve vaccine efficacy, and both these regulatory proteins are recognized as attractive vaccine components (Liang *et al.*, 2002b; Goldstein, 1996). Tat is cytotoxic and induces cellular apoptosis, while Nef causes immune system dysfunction in humans (Luciw, 1996). Therefore both proteins have usually been included in vaccines as inactivated forms.

1.3 HIV vaccine strategies

DNA vaccines, subunit vaccines (recombinant viral proteins or chemically synthesized peptides), live vectors (bacterial or non-HIV viruses carrying HIV proteins) and particle-based vaccines (inactivated virions, live attenuated vaccines, VLPs) have all been investigated as potential HIV vaccines (Giri *et al.*, 2004; Bojak *et al.*, 2002; McMichael & Hanke, 2003; Young & Ross, 2003; Nabel, 2002; Letvin, 2005; Girard *et al.*, 2006). As part of a heterologous prime/boost strategy, particle-based vaccines are complex antigens that can present a number of epitopes in a highly immunogenic formulation. Of these, VLPs are the most appealing in terms of safety and flexible vaccine design.

1.3.1 Particle-based vaccines

Inactivated vaccines

For many years, inactivated HIV virions did not result in vaccines which generate CTL responses in animal models (Letvin, 2005) and the immunogenicity of Env was generally destroyed during the inactivation process, precluding NAb formation (Girard *et al.*, 2006). In a recent report, a combined chemical and physical inactivation strategy yielded HIV-1 virions that elicited modest titres of NAbs in non-human primates (Poon *et al.*, 2005). However, the possibility of incomplete virion inactivation cannot be discounted, in which case vaccine formulations could still contain infectious virions. This overriding safety concern is likely to prevent this vaccine strategy from entering clinical trials.

Live attenuated vaccines

Many studies have been conducted on live attenuated primate retrovirus vaccines, but the most encouraging results have been obtained from SIV-HIV (SHIV) viruses, which are generalized as recombinant SIVs displaying HIV-1 Env. Live attenuated SHIV (*nef/vpr* deleted) led to protection of rhesus macaques challenged with highly pathogenic SHIV-89.6P (Ui *et al.*, 1999). Analogously, sequential immunization of rhesus macaques with two SHIV vaccines (*nef/vpu* deleted and then *vpu* deleted) conferred protection to mucosal challenge with a combination of SHIV and SIV challenge viruses (Kumar *et al.*, 2002). Despite such promising results, live attenuated vaccines for HIV have not been widely pursued (Girard *et al.*, 2006) since the degree of attenuation seems inversely correlated with vaccine efficacy and protection from disease (Young & Ross, 2003). This has been conclusively demonstrated for SIV (Johnson *et al.*, 1999),

and therefore the most promising vaccine candidates are also the least safe, with greatest risk of reversion to virulence. Additional caveats for live attenuated HIV vaccines include: (i) recombination with virulent forms, (ii) proviral integration into the host genome and (iii) disruption of immune system regulation (Young & Ross, 2003; Johnson, 1999).

1.3.2 Virus-like particles (VLPs)

VLPs can be generally defined as “self assembling, non-replicating, non-pathogenic genomeless particles of similar size and conformation to intact virions” (Young & Ross, 2003). VLPs have a number of safety, production and immunogenicity advantages over other HIV vaccine candidates (Noad & Roy, 2003; Doan *et al.*, 2005; Deml *et al.*, 2005). Primarily, they are non-infectious, lacking undesirable regulatory proteins or replicative genetic material (Deml *et al.*, 2005), and potentially can be produced to high yield and purity in heterologous expression systems. The particulate nature of VLPs (composed of structural proteins in a native conformation) predisposes toward high immunogenicity (Doan *et al.*, 2005; Persson *et al.*, 1998; Vzorov *et al.*, 1991). Furthermore, VLPs appear to have intrinsic adjuvant properties (Michel *et al.*, 1990), allowing them to stimulate both humoral and cellular immune responses in rodents and non-human primates, without extraneous addition of adjuvants (Wagner *et al.*, 1996a; Paliard *et al.*, 2000; Buonaguro *et al.*, 2002; Layton *et al.*, 1993). In the context of HIV, VLPs are based on the Gag precursor polyprotein, which can also be used as a carrier of foreign antigenic proteins to make chimaeric VLPs that may improve VLP immunogenicity.

1.4 Pr55^{gag} VLPs

The unprocessed HIV-1 Gag precursor polyprotein, Pr55^{gag}, is transported to the cell membrane, where it spontaneously assembles into particles which bud externally to form 100-120 nm VLPs (Fig. 1.2). Moreover, Pr55^{gag} VLPs can form in the absence of all other HIV proteins (Gheysen *et al.*, 1989), and have the same size, ultrastructural morphology and sedimentation properties as immature HIV virions (Wagner *et al.*, 1992). Analogous features apply to SIV Pr57^{gag} VLPs (Delchambre *et al.*, 1989; Yamshchikov *et al.*, 1995).

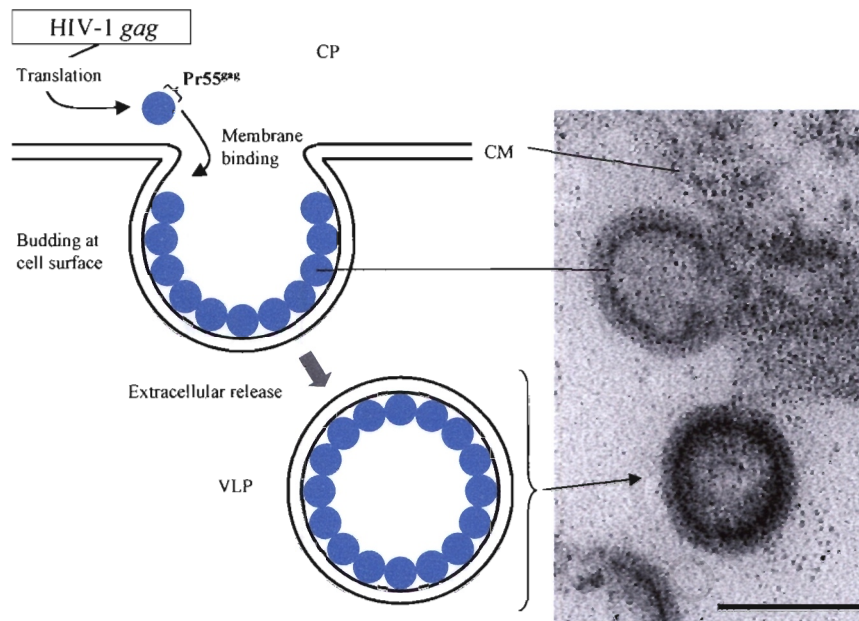


FIGURE 1.2. **Pr55^{gag} VLP formation.** HIV-1 *gag* is transcribed and translated into cytosolic Pr55^{gag} (Gag precursor protein) that is targeted to the inner leaflet of the cell membrane. Stable membrane binding facilitates the subsequent budding of enveloped VLPs (100-120 nm), which are released from the cell surface. The electron micrograph (from this study) shows the budding of Pr55^{gag} VLPs from Sf21 insect cells (*Spodoptera frugiperda*). Bar = 100 nm.

1.4.1 Expression systems

Expression of Pr55^{gag} in yeast spheroplasts (Sakuragi *et al.*, 2002; Tsunetsugu-Yokota *et al.*, 2003) and in mammalian cells using recombinant adenovirus (Vernon *et al.*, 1991), recombinant vaccinia virus (Wagner *et al.*, 1992; Karacostas *et al.*, 1989; Haffar *et al.*, 1990; von *et al.*, 1993; Hu *et al.*, 1990; Vzorov *et al.*, 1991; Shioda & Shibuta, 1990) and *rev* dependent expression vectors (Mergener *et al.*, 1992; Smith *et al.*, 1990) has led to VLP formation and budding.

Production of Pr55^{gag} VLPs in insect cells using recombinant baculovirus has been widely reported (Tobin *et al.*, 1996b; Wagner *et al.*, 1992; Gheysen *et al.*, 1989; Royer *et al.*, 1991; Jaffray *et al.*, 2004). A range of transfer vectors and isolation procedures facilitate the easy generation and selection of recombinant baculovirus to express a gene of interest. Due to the availability of commercial reagents, advances in insect cell culture and amenability to scale-up; the baculovirus-insect cell expression system has been used for the routine production of numerous recombinant proteins (Kost *et al.*, 2005). This expression system is referred to in this study as the ‘baculovirus system’, and has often been used to produce batches of Pr55^{gag} VLPs for

immunogenicity testing. Herein, the protease (PR) within Pol can be over-expressed by strong baculovirus promoters and lead to premature Pr55^{gag} cleavage that prevents VLP assembly (Morikawa *et al.*, 1991; Hughes *et al.*, 1993). Thus, for efficient VLP production in the baculovirus system, Pr55^{gag} is usually expressed from only *gag* gene sequences, rather than the full *gag-pol* gene.

1.4.2 Immunogenicity of Pr55^{gag} VLPs

Humoral responses to Pr55^{gag} VLPs have been recorded in rabbits (Wagner *et al.*, 1992; Wagner *et al.*, 1996b; Deml *et al.*, 1997b) and rhesus macaques (Montefiori *et al.*, 2001). The antibody titres in these studies were often high, but the sera were non-neutralizing. Vaccination of rhesus macaques with Pr55^{gag} VLPs led to long lived CD8⁺ CTL responses against multiple epitopes in CA, NC and p6 (Paliard *et al.*, 2000). Furthermore, numerous T-cell epitopes recognized by CD8⁺ (Currier *et al.*, 2002; Xu *et al.*, 2002), and CD4⁺ (Boaz *et al.*, 2002; Rosenberg *et al.*, 1997) cells in HIV-1 infected individuals, have been identified in Pr55^{gag}. In our research group, HIV-1C Pr55^{gag} VLPs have been used successfully to boost the antibody and cellular immune responses in mice (Jaffray *et al.*, 2004) and baboons primed with the DNA vaccine pTHgagC (R. Thomas, G. Chege, E. Shephard, E. Rybicki, A. Williamson, unpublished).

Recent studies have indicated that VLPs can activate the innate immune system through 'danger signals' and lead to dendritic cell maturation and increased cytokine production (Tsunetsugu-Yokota *et al.*, 2003). This is thought to be a result of the particulate nature of VLPs and residual components of the cell expression system used in production. For example, baculoviral gp64, which is incorporated into the membrane of Gag VLPs produced in insect cells (Deml *et al.*, 2005), induces interferon production in mammalian cells (Gronowski *et al.*, 1999).

1.4.3 Safety of Pr55^{gag} VLPs

A number of studies have highlighted the relative safety of Gag-based VLPs compared to other particulate HIV candidate vaccines (Persson *et al.*, 1998; Deml *et al.*, 2005; Doan *et al.*, 2005; Young & Ross, 2003; Noad & Roy, 2003). The main concern which remains is that in the absence of genomic viral RNA, Gag VLPs can incorporate cellular RNAs from the expression system (Khorchid *et al.*, 2002), contrary to an earlier report (Vzorov *et al.*, 1991). Removal of the ϕ -site upstream of *gag* and disruption of the nucleic acid binding domains in NC has reduced the already low RNA content of VLPs by ~95% (Persson *et al.*, 1998).

The presence of minimal quantities of non-infectious RNA is certainly preferable in comparison to some of the formidable safety concerns associated with other particulate HIV vaccines (1.3.1)

1.5 Pr55^{gag} polyprotein precursor

In natural HIV-1 infection, Pr55^{gag} (translated from the *gag* gene, Fig. 1.1) is proteolytically cleaved during or immediately after budding into matrix (MA or p17), capsid (CA or p24), p2, nucleocapsid (NC or p7), p1 and p6 by the HIV-1 encoded PR (Henderson *et al.*, 1992) (Freed, 1998). This processing is required for infectivity but not particle assembly or release (Rose *et al.*, 1995). In the mature virion (Fig. 1.1), MA remains associated with the inner membrane surface while NC coats the genomic viral RNA within the CA core (Freed, 1998; Scarlata & Carter, 2003). However, each domain has functions within the Gag precursor prior to maturation, and some of these also pertain to VLP assembly and release (Fig. 1.3).

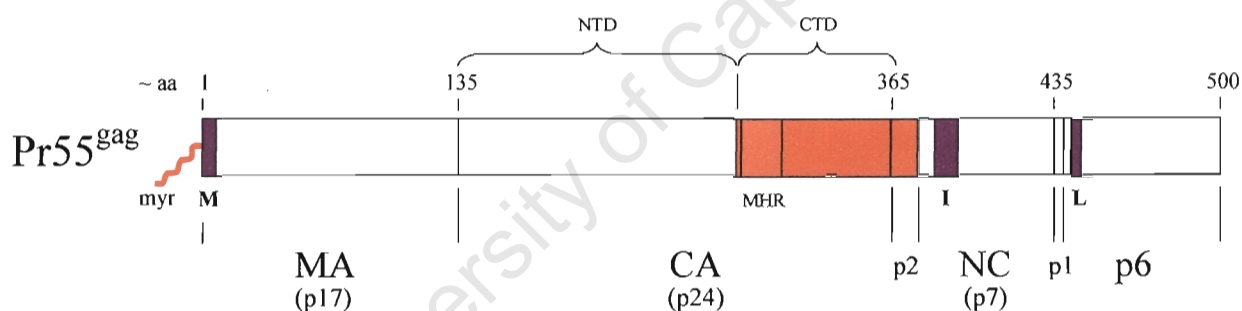


FIGURE 1.3. Minimal Gag domains involved in VLP formation.

The Pr55^{gag} polyprotein precursor is cleaved into MA, CA, p2, NC, p1 and p6 during HIV maturation. The positions of the M-, I-, L-domains along with the NTD, CTD and MHR within CA are shown. Approximate amino acid (aa) positions are indicated, myr = myristyl moiety. Regions in orange have been shown to be essential for VLP formation, and those in purple may be replaced by functional heterologous sequences (1.5.2 and 1.5.3). White regions are thought to be dispensable for VLP assembly, based on data from a number of studies using different expression systems.

1.5.1 Myristylation

Myristylation of cytosolic Pr55^{gag} occurs co-translationally (Wilcox *et al.*, 1987) through the removal of the N-terminal methionine and covalent attachment of myristic acid (CH₃(CH₂)₁₂COOH) to gly2 (Henderson *et al.*, 1992), which is conserved among HIV-1 subtypes (Bryant & Ratner, 1990). Assembly and replication of HIV-1 are critically dependent on Pr55^{gag} myristylation, without which progeny virions are not produced during infection (Gottlinger *et al.*,

1989; Bryant & Ratner, 1990). Mutation of the myristylation signal also prevents the budding of Gag VLPs from mammalian (Wagner *et al.*, 1992) and insect cells (Wagner *et al.*, 1992; Gheysen *et al.*, 1989; Royer *et al.*, 1991) as well as yeast spheroplasts (Sakuragi *et al.*, 2002).

Myristylation plays a central role in the targeting of Gag to the cell membrane (Freed, 1998; Scarlata & Carter, 2003), but it is not critical in this process since limited, weak membrane association occurs without myristylation (Bryant & Ratner, 1990). The primary function of myristylation is rather to facilitate the stable binding of Pr55^{Gag} to the cell membrane during virion and VLP assembly (Bryant & Ratner, 1990). This stable binding also requires a second signal consisting of basic residues in MA (Zhou *et al.*, 1994; Spearman *et al.*, 1997). Although myristylation is not directly involved in Gag multimerization (Morikawa *et al.*, 1996), it has a key function in converting Gag multimers into Gag VLPs (Morikawa *et al.*, 2000).

Accumulated evidence suggests that a myristyl switch regulates the reversible binding of HIV-1 Gag to membranes (Resh, 2004). In brief, Gag multimerization co-operatively exposes the myristyl moiety and enhances membrane binding prior to particle budding. Conversely, MA cleavage decreases membrane affinity, so that MA dissociates into the cell during infection.

1.5.2 Assembly domains of Gag

The roles of Gag proteins (corresponding to domains within Pr55^{Gag}) in the HIV-1 life cycle are numerous and complex (Modrow *et al.*, 1994; Freed, 1998; Scarlata & Carter, 2003; Wills & Craven, 1991). Contradictory domain functions have been reported in different cell lines and expression systems, but the main differences are evident when comparing particle types, especially virions and VLPs. Only those functions involved in particle assembly and subsequent release are described here.

1.5.2.1 Matrix (MA)

An N-terminal basic domain in MA together with the myristyl moiety (the M-domain) regulate the targeting and binding of Pr55^{Gag} to the cell membrane (Spearman *et al.*, 1994; Wang & Barklis, 1993) and appear to interact with membrane phospholipids (Zhou *et al.*, 1994). However, this function can be carried out by replacing MA with a heterologous membrane targeting signal (Reil *et al.*, 1998), and large C-terminal portions of MA have been removed without preventing virion assembly (Wang *et al.*, 2000; Wang *et al.*, 1993). Even complete MA deletion did not

prevent virion assembly, provided a myristylation signal was retained (Accola *et al.*, 2000; Lee & Linial, 1994). In several other studies of virion and VLP assembly, using different expression systems, deletions or mutations of MA regions have disrupted or abolished extracellular particle formation (Wagner *et al.*, 1994a; Morikawa *et al.*, 1995; Spearman *et al.*, 1994; Freed *et al.*, 1994), and both Gag assembly and targeting signals have been identified throughout MA (Freed *et al.*, 1994).

1.5.2.2 Capsid (CA)

CA condenses into the mature virion core (Freed, 1998) and contains the highly conserved major homology region (MHR) which has been implicated in membrane affinity (Ebbets-Reed *et al.*, 1996). The N-terminal domain (NTD) of CA is required for mature core formation (Dorfman *et al.*, 1994) and possible regulation of overall particle morphology (Reicin *et al.*, 1996), but is not necessary for virion or VLP assembly (Wagner *et al.*, 1994a; Borsetti *et al.*, 1998; Wang & Barklis, 1993; Reicin *et al.*, 1995). Conversely, the C-terminal domain (CTD) of CA (including MHR) is essential for HIV particle assembly (Kattenbeck *et al.*, 1997; Borsetti *et al.*, 1998; Dorfman *et al.*, 1994; Mammano *et al.*, 1994; Reicin *et al.*, 1995; Zhang *et al.*, 1996) and VLP formation (von *et al.*, 1993; Jowett *et al.*, 1992; Kattenbeck *et al.*, 1996). Furthermore, the C-terminal 11 amino acids (aa) of CA are involved in both Gag multimerization and membrane binding (Liang *et al.*, 2003).

1.5.2.3 Nucleocapsid (NC)

A distinguishing feature of NC is the presence of two conserved cysteine-histidine boxes (or zinc finger motifs) for RNA packaging (Dorfman *et al.*, 1993), which show a preference for ϕ -site-containing viral RNA (Amarasinghe *et al.*, 2001). NC appears to aid in the tight packing of adjacent Gag molecules during particle assembly, since NC deletions resulted in low-density particles (Bennett *et al.*, 1993). The N-terminal basic portion of NC, which contains an interaction (I)-domain, is responsible for self-interaction of Gag molecules (Zabransky *et al.*, 2002) but apparently not membrane binding (Ono *et al.*, 2005) as previously proposed (Sandefur *et al.*, 1998). NC, and the I-domain in particular, are implicated in HIV-1 assembly (Dawson & Yu, 1998; Dorfman *et al.*, 1993) and possibly in VLP formation as well (Jowett *et al.*, 1992; Hoshikawa *et al.*, 1991; Gheysen *et al.*, 1989), although other studies showed that NC deletions only reduced VLP production levels (Zhang & Barklis, 1997; Wang *et al.*, 1998).

In addition, the I-domain function can be replaced by a leucine zipper (Accola *et al.*, 2000; Zhang *et al.*, 1998). The process of RNA packaging seems to concentrate Gag monomers and promote multimerization (Scarlata & Carter, 2003), but this is not required for VLP formation (Zhang & Barklis, 1997).

1.5.2.4 p6

The p6 region is the most variable domain in Gag, but contains a conserved proline-rich motif PTAP(P) within the late (L)-domain. This motif plays a crucial role in the release of mature HIV virions from the cell (Scarlata & Carter, 2003). Mutations throughout the L-domain severely impair virion budding, and assembled particles remain tethered to the exterior of human cells (Gottlinger *et al.*, 1991; Freed, 2002). Subsequent studies confirmed this role of p6 for detaching virions from the cell (Huang *et al.*, 1995; Yu *et al.*, 1995). The L-domain interacts with a host protein, Tsg101, which is involved in the intracellular trafficking of membrane-associated proteins and exocytic pathways (Freed, 2002; VerPlank *et al.*, 2001). Ubiquitin binding to Tsg101 is required for viral release (Goff *et al.*, 2003). In other retroviruses, late domain functions have been identified (Freed, 2002) which can be functionally exchanged between unrelated Gag proteins (Parent *et al.*, 1995). A secondary late (L₂)-domain (LXXLF) has been identified in p6 that binds a cellular protein AIP1 (Strack *et al.*, 2003), which like Tsg101, facilitates viral budding through the use of vacuolar protein sorting machinery.

Despite this requirement for the L-domain in HIV maturation, p6 is not required for efficient Gag-VLP formation in the baculovirus system (Griffiths *et al.*, 1993; Royer *et al.*, 1991; Jowett *et al.*, 1992) or the vaccinia virus system (Spearman *et al.*, 1994). An L-domain function was required for VLP release in HeLa cells (Accola *et al.*, 2000) but not in 293T cells (Wang *et al.*, 1998) - although VLP production was low. Huang *et al.* noted that the requirement of p6 for virion release was suppressed when PR was inactivated (Huang *et al.*, 1995), and that this may partly account for the results obtained when Gag was expressed alone for VLP production. In contrast, Accola *et al.* subsequently reported that the inclusion of p6 enhanced VLP production in the absence of PR processing (Accola *et al.*, 2000). Therefore, the requirement of L-domains for particle release is apparently dependent on the expression system, cell type and the type of particle (VLP or virion) produced.

1.5.2.5 Spacer regions, p2 and p1

The CA-NC spacer, p2 (or SP1, 14 aa), is crucial in HIV assembly (Liang *et al.*, 2002a) and may be required for structurally ordered assembly of particles. Deletion of p2 has been shown to have no effect on Gag membrane binding (Guo & Liang, 2005), but led to heterogeneous sized virions (Krausslich *et al.*, 1995) or tubular structures instead of VLPs (Morikawa *et al.*, 2000) or virions (Gross *et al.*, 2000). Mutations in p2 restricted both Gag multimerization and binding to cell membranes (Guo *et al.*, 2005), and have caused aberrant VLP structures (Accola *et al.*, 1998). There are no reports to date on the functions of the NC-p6 spacer, p1 (or SP2).

1.5.3 Minimal Gag constructs for VLP formation

The data available on the assembly domains of Gag indicates that large portions of *gag* can potentially be deleted without preventing Gag VLP formation. This has application in the design of chimaeric Gag VLPs, where dispensable regions for VLP assembly may be replaced by heterologous sequences.

VLPs comprising C-terminally truncated Gag

Two groups have produced p6-truncated HIV-1 Gag VLPs using the baculovirus system (Royer *et al.*, 1991; Griffiths *et al.*, 1993). Luo *et al.* expressed high levels of VLPs using recombinant baculovirus from an HIV-2 *gag* gene without the overlapping *pol* sequence (Luo *et al.*, 1990). HIV-1 Gag C-terminal truncations including p2 (Fig. 1.1) abolished particle formation in insect (Gheysen *et al.*, 1989; Morikawa *et al.*, 2000) and human cells (Accola *et al.*, 2000) or yielded considerably reduced levels of particles (Wang *et al.*, 1998) in human cells.

Minimal Gag-containing VLPs

Sequence between the myristyl anchor and MHR was shown to be dispensable for VLP formation in human cells (Borsetti *et al.*, 1998), and limited VLPs were produced in the absence of NC-p1-p6 (Wang *et al.*, 1998). Based on this, and the knowledge of Gag domain functions in assembly (1.5.2), the minimal Gag regions required for VLP assembly can be estimated (Fig. 1.3). Accola *et al.* demonstrated that a 16 kDa Gag protein retaining only the myristylation signal and the C-terminus of the CA-p2 domain could form extracellular VLPs in human cells if the I- and L-domain functions were provided by heterologous sequences (Accola *et al.*, 2000).

1.6 Chimaeric Gag VLPs

VLPs based on Pr55^{Gag} or truncated Gag derivatives are highly immunogenic particulate carriers for foreign (non-Gag) polypeptides. Two principal strategies have been defined for displaying antigenic epitopes and peptides (Deml *et al.*, 2005). Type I VLPs have foreign polypeptides included within the VLP as integrations or fusions with Gag, whereas type II VLPs have foreign proteins (primarily Env) incorporated into the outer surface of the Gag VLP (Fig. 1.4). The majority of type I VLPs have been produced by expressing heterologous sequences as in-frame insertions within *gag* or as in-frame fusions to the 3'-end of a *gag* gene. More recently, foreign sequences have been expressed in the Gag-Pol open reading frame as a result of ribosomal frameshifting (Tobin *et al.*, 1997). In this study, these different chimaeric Gag VLPs are referred to as: type II VLPs, type I frameshift VLPs and type I in-frame VLPs. Since the latter were the focus of this study, the preceding two are only described briefly for comparison.

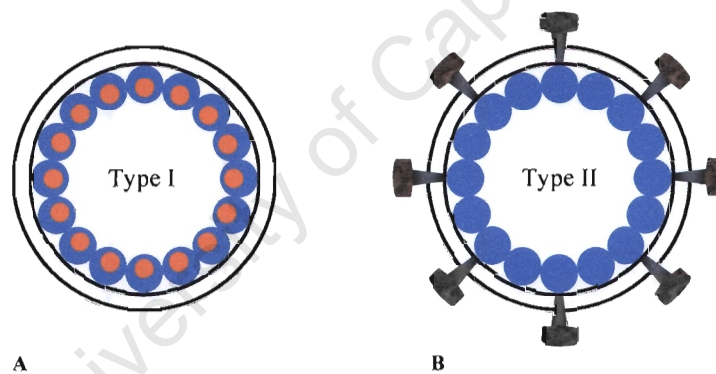


FIGURE 1.4. **Type I and Type II chimaeric Gag VLPs.**

(A) Type I VLPs are composed of Gag carrier proteins (blue) that contain foreign epitopes or polypeptides (orange). These can be inserted to replace deleted sequences within Gag that are not required for VLP formation, or fused to the C-terminus of full-length or truncated Gag. (B) Type II VLPs have Env proteins (brown) anchored to the outside of the particle by gp41 or a suitable transmembrane domain (grey). This nomenclature is based on work by Deml *et al.*, 2005.

1.6.1 Type II VLPs

Native HIV-1 Env, and derivatives thereof, can be incorporated into the host-derived membrane of VLPs by a suitable transmembrane domain, as occurs in HIV virions (Fig. 1.1). However, shedding of these Env proteins during VLP purification, due to unstable membrane anchoring, has been reported to reduce to the Env-specific immune responses to VLP preparations (Tobin *et al.*, 1996a).

Env components of type II VLPs

Type II VLPs incorporating full-length gp160, anchored by gp41, have been produced in vaccinia (Vzorov *et al.*, 1991; Haffar *et al.*, 1990) and baculovirus systems (Tobin *et al.*, 1996a; Wagner *et al.*, 1994b). Env incorporation into VLPs is known to be dramatically increased by partial or complete deletion of the C-terminal cytoplasmic tail of gp160 (Deml *et al.*, 2005) or by the use of heterologous membrane anchors (e.g. transmembrane domain of Epstein-Barr virus gp220/350 (Deml *et al.*, 1997b; Deml *et al.*, 1997a)). These strategies also circumvent possible immune dysfunction attributed to gp41 (Ruegg *et al.*, 1989).

Immune responses to type II VLPs

In addition to humoral and cellular responses to Gag, type II VLPs have stimulated both high titres of Env-specific antibodies and CTLs toward Env in mice (Buonaguro *et al.*, 2005; Buonaguro *et al.*, 2002; Deml *et al.*, 1997b), rabbits (Haffar *et al.*, 1991; Wagner *et al.*, 1996c) and rhesus macaques (Wagner *et al.*, 1998). Furthermore the immune sera exhibited neutralizing activity toward homologous (Deml *et al.*, 1997b; Wagner *et al.*, 1998) and heterologous (Buonaguro *et al.*, 2005; Buonaguro *et al.*, 2002) HIV-1 isolates, unlike type I VLPs. Despite these promising immune responses, vaccination of rhesus macaques did not confer protection to SHIV infection (Wagner *et al.*, 1998).

1.6.2 Type I frameshift VLPs

The Gag-Pol polyprotein (160 kDa) is translated through a (-1) ribosomal frameshift at the *gag-pol* frameshift signal within NC, and occurs at a frequency of ~5% (Jacks *et al.*, 1988; Wilson *et al.*, 1988). Therefore, *pol* genes are expressed as a fusion to a truncated Gag molecule, and CA is involved in incorporating Gag-Pol into HIV virion (Srinivasakumar *et al.*, 1995) and VLPs (Smith *et al.*, 1993). This process has been harnessed to create type I frameshift VLPs, where the DNA sequence encoding the foreign polypeptide is added downstream of *gag* in the same frame as *pol*. Using this method, only 5% or fewer of the Gag molecules in the resultant VLP would be expected to carry the extended protein sequence, which agrees with analyses of type I frameshift VLP protein content (Tobin *et al.*, 1997; Buonaguro *et al.*, 2001).

It has been suggested that it is this lower molar ratio of chimaeric protein to Gag that allows frame-shift VLPs to accept larger peptide fusions than type I in-frame VLPs (Tobin *et al.*, 1997). Examples of type I frameshift VLPs produced in the baculovirus system include a Gag-Pol-Nef

(~160 kDa) construct (Buonaguro *et al.*, 2001) and a Gag fusion to the C-terminal 273 aa of HIV-1 gp120 (Tobin *et al.*, 1997). Young *et al.* constructed DNA vaccine plasmids that expressed large Gag-Pol chimaeras which were capable of forming type I frameshift VLPs in primate cell lines (Young *et al.*, 2004).

The Gag-Env frame-shift VLPs elicited CTL responses to both Gag and Env epitopes in mice, whereas humoral responses, albeit strong, were limited to Gag (Tobin *et al.*, 1997). There is no reported immunological data for the Gag-Pol-Nef frameshift VLPs (Buonaguro *et al.*, 2001) or those expressed by the DNA vaccines (Young *et al.*, 2004).

1.6.3 Type I in-frame VLPs

A number of chimaeric Gag VLPs have been constructed by the direct incorporation of heterologous peptides into the Gag carrier, either to replace portions of Pr55^{gag} that are non-essential to VLP formation, or as fusions to a truncated Gag C-terminus. This is accomplished by gene insertions or fusions in-frame with *gag*. These strategies are thought to permit longer fusions than internal insertions (without replacement) or C-terminal fusions to Pr55^{gag}, both of which have been less successful in producing chimaeric VLPs (Luo *et al.*, 1992; Tobin *et al.*, 1996a) and have not been as well investigated (Deml *et al.*, 2005). In each case the fusion is in-frame with *gag*.

1.6.3.1 In-frame fusion positions and sequences

Fusion sequences

Approximately 80% of Pr55^{gag} is dispensable for VLP assembly (Accola *et al.*, 2000), which potentially allows for type I VLPs to be constructed with substantial lengths of heterologous antigen-encoding sequence. To date, however, only individual epitopes or short polypeptides have been either internally integrated or C-terminally fused to Gag carriers. These short, immunogenic fusions have included a Nef-derived epitope (Wagner *et al.*, 1994a; Wagner *et al.*, 1996c; Wagner *et al.*, 1994c), the gp120 V3 loop (Griffiths *et al.*, 1993; Luo *et al.*, 1992; Wagner *et al.*, 1993; Wagner *et al.*, 1996b; Wagner *et al.*, 1994a; Truong *et al.*, 1996; Brand *et al.*, 1995), tandem V3 repeats (Luo *et al.*, 1998) and CD4BD derivatives (Luo *et al.*, 1992; Truong *et al.*, 1996; Wagner *et al.*, 1994a; Wagner *et al.*, 1996b). With the exception of a single Nef-derived epitope (produced from recombinant vaccinia virus), all these reported fusion sequences are

derived from gp120 sequences, and all VLPs were produced in the baculovirus system (Table 1.1). In addition, a number of Gag constructs with V3 and CD4BD sequences within feasible replacement regions have failed to form VLPs in the baculovirus system (Table 1.2).

The available literature suggests that there is an upper limit of ~ 200 aa that can be fused in-frame to the C-terminus of Pr55^{gag} for correct assembly into VLPs (Deml *et al.*, 2005). However, no successful attempt to test this length limit has yet been reported. Furthermore, it would appear that the nature and composition of the fusion sequence can affect VLP formation (Tobin *et al.*, 1996a; Deml *et al.*, 2005), especially for longer fusions (Luo *et al.*, 1992), although the parameters of permissible fusions have not been defined.

Reported fusion positions

As illustrated in Figure 1.3, a number of regions within Gag are dispensable for VLP assembly and offer potential positions for replacement of Gag sequence with foreign sequence. To date, segments within the CA NTD and the p1 region (between the I- and L-domains) have been replaced by in-frame heterologous sequences to form chimaeric VLPs (Table 1.1). These two particular sites within the feasible replacement regions of Gag are thought to have the highest probability of being exposed near the VLP periphery (Wagner *et al.*, 1994a). Fusions to C-terminally truncated Gag have also been successful in producing VLPs, particularly for longer fusion sequences (Table 1.1).

Fusion position affects VLP formation

Two studies have indicated that the position of the in-frame fusion with Gag affects VLP formation (Luo *et al.*, 1992; Wagner *et al.*, 1994a). For example, Luo *et al.* incorporated HIV-1 V3 (91 aa) or HIV-2 V3 (90 aa) as either inserts within MA or C-terminal fusions to an HIV-2 Gag truncated in NC (Table 1.1). All four constructs expressed the chimaeric protein in recombinant baculovirus infected insect cells, but only the two C-terminal fusions produced VLPs (Luo *et al.*, 1992). This suggested that Gag without internal replacement sequences was a superior antigen carrier for these fusions, and that C-terminal fusions may be a preferable design strategy for type I in-frame VLPs.

TABLE 1.1 Chimaeric Gag constructs that have produced type I in-frame VLPs ¹

| Insert size ² (aa) | Fusion derivative | Gag carrier ⁴ | Fusion position in Gag (aa) | Reference |
|-------------------------------|-----------------------------------|---|-----------------------------|---|
| 11 | CD4BD | Pr55 ^{gag} | C-terminus | Tobin <i>et al.</i> , 1996a |
| 21 | Nef-derived CTL epitope | Gag(d211-241), Gag(d436-471), Pr55Gag | 211, 436, C-terminus | Wagner <i>et al.</i> , 1994a |
| 23 | CD4BD ³ | Gag(d211-241), Gag(d436-471), Pr55Gag | 211, 436, C-terminus | Wagner <i>et al.</i> , 1996b |
| 26 | V3 | Gag(d196-228) | 196 | Brand <i>et al.</i> , 1995 Truong <i>et al.</i> , 1996 |
| 36 | V3 ³ | Gag(d211-241), Gag(d436-471), Pr55 ^{gag} | 211, 436, C-terminus | Wagner <i>et al.</i> , 1996b |
| 40 | V3 | HIV-1 Gag (76 aa C-terminal truncation) | C-terminus | Griffiths <i>et al.</i> , 1993 |
| 54 | 3 tandem copies of consensus V3 | HIV-2 Gag (93 aa C-terminal deletion) | C-terminus | Luo <i>et al.</i> , 1998 |
| 55 | CD4BD | Gag(d211-241), Gag(d436-471), Pr55 ^{gag} | 211, 436, C-terminus | Wagner <i>et al.</i> , 1996b |
| 60 | 2 gp120 + 2 gp41 epitopes | HIV-2 Gag (93 aa C-terminal deletion) | C-terminus | Luo <i>et al.</i> , 1998 |
| 60 | V3 sequences from 4 HIV-1 strains | HIV-2 Gag (93 aa C-terminal deletion) | C-terminus | Luo <i>et al.</i> , 1998 |
| 90 | HIV-2 V3 | HIV-2 Gag (93 aa C-terminal deletion) | C-terminus | Luo <i>et al.</i> , 1992 |
| 91 | V3 | HIV-2 Gag (93 aa C-terminal deletion) | C-terminus | Luo <i>et al.</i> , 1992 |
| 92 | V3 (37 aa) + CD4BD (55 aa) | Gag(d211-241/d436-471) | 211 and 436 | Wagner <i>et al.</i> , 1996b |
| 198 | HIV-2 V3 + CD4BD | HIV-2 Gag (93 aa C-terminal deletion) | C-terminus | Luo <i>et al.</i> , 1992 |

¹ All proteins were expressed in the baculovirus system, except the Gag-Nef fusion, which was expressed by recombinant vaccinia virus. All polypeptides were derived from HIV-1 sequences unless otherwise indicated.

² Total number of aa if more than one fusion was present in the Gag carrier.

³ Analogous constructs also expressed by recombinant vaccinia virus (Wagner *et al.*, 1994a)

⁴ d = deleted for that amino acid region

TABLE 1.2 Chimaeric Gag constructs that did not produce type I in-frame VLPs ¹

| Insert size (aa) | Fusion derivative | Gag carriers ² | Fusion position in Gag (aa) | Reference |
|------------------|-------------------------|--|-----------------------------|------------------------------|
| 21 | Nef-derived CTL epitope | Gag(d99-154) | 99 | Wagner <i>et al.</i> , 1994a |
| 36 | V3 | Gag(d99-154) | 99 | Wagner <i>et al.</i> , 1994a |
| 45 | gp 41 fusion domain | Pr55 ^{gag} | C-terminus | Tobin <i>et al.</i> , 1996a |
| 59 | V3 | Pr55 ^{gag} | C-terminus | Tobin <i>et al.</i> , 1996a |
| 90 | HIV-2 V3 | HIV-2 Gag (93 aa C-terminal deletion) | 115 | Luo <i>et al.</i> , 1992 |
| 91 | V3 | HIV-2 Gag (93 aa C-terminal deletion) | 115 | Luo <i>et al.</i> , 1992 |
| 194 | V3 + CD4BD | HIV-2 Gag (93 aa C-terminal deletion) | C-terminus | Luo <i>et al.</i> , 1992 |

¹ All proteins were expressed in the baculovirus system, except the Gag(d99-154) carrier fusions, which were expressed by recombinant vaccinia virus. All polypeptides were derived from HIV-1 sequences unless otherwise indicated.

² d = deleted for that amino acid region

1.6.3.2 Morphology and yield of type I in-frame VLPs

Morphology

Reports on type I Gag-Env VLPs indicate that they usually resemble Gag-only VLPs by electron microscopy (EM), but were often larger in size (Luo *et al.*, 1992; Luo *et al.*, 1998; Wagner *et al.*, 1996b). In a study with V3- and CD4BD-derived sequences, all C-terminal fusions to Pr55^{gag} formed uniform VLPs (100-140 nm across), whereas some internal Gag fusions formed VLPs with irregular morphology and greater range in size (100-250 nm). Formation of large, heterologous particles was not limited to a specific fusion sequence or replacement position within Pr55^{gag} (Wagner *et al.*, 1996b).

Yield in baculovirus system

Type I in-frame VLPs expressed by recombinant baculovirus have been present in the culture medium at high yields, ranging from 5-20 mg/l depending on the fusion sequence and its position within Gag (Wagner *et al.*, 1996a; Wagner *et al.*, 1994c). However, internal fusions have given lower yields of VLPs than C-terminal fusions (Wagner *et al.*, 1996b), and increasing the size of the fusion has decreased VLP yield (Luo *et al.*, 1992).

1.6.3.3 Immune responses to type I VLPs

Position affects the strength of humoral but not cellular responses

Wagner *et al.* found that the magnitude of anti-V3 and anti-CD4BD humoral responses in rabbits was strictly dependent on the position of the replacements within Gag, whereas the CTL response magnitude in mice, for the same set of chimaeric VLPs, was not influenced by the replacement position (Wagner *et al.*, 1996b). Therefore, antigen processing for MHC class I epitope presentation is probably independent of the epitope position within the context of the Gag carrier. Although overall antibody responses were weak for these Env-derived epitopes, the highest antibody titres were detected for C-terminal fusions.

Effect of adjuvants on immune responses to type I VLPs

Although Gag-based VLPs can elicit immune responses in the complete absence of adjuvants (1.3.2), both aluminum hydroxide and Freund's incomplete adjuvant have been administered with type I in-frame VLPs in immunogenicity studies. Neither improved the V3 antibody titre or neutralizing ability in rats and rabbits (Griffiths *et al.*, 1993; Wagner *et al.*, 1996a; Wagner *et al.*, 1996b). In addition, both adjuvants have been shown to decrease the CTL response in mice (Griffiths *et al.*, 1993; Wagner *et al.*, 1996b). This down regulation of *in vivo* CTL responses in the presence of adjuvants has also been reported for hepatitis B virus surface antigen particles (Schirmbeck *et al.*, 1994).

Humoral responses to type I VLPs

Gag-V3 VLPs have elicited antisera in rabbits that neutralized homologous HIV infectivity where Gag-only VLPs did not, indicating that the neutralization activity was due to V3 specific antibodies (Luo *et al.*, 1992). However, similar experiments with other type I Gag-Env VLPs generated Env-specific antisera with only very weak neutralizing activity (Wagner *et al.*, 1996b; Luo *et al.*, 1998). Synergistic effects from VLPs containing both V3 and CD4BD fusions, or from combinations of different VLPs were not observed (Wagner *et al.*, 1996a).

Immunization of rats with Gag-V3 VLPs also produced weak V3 specific antibodies (Griffiths *et al.*, 1993), and in mice Gag-Env VLPs elicited weak anti-Env responses without neutralizing ability (Truong *et al.*, 1996). In the cases where low antibody responses to Env were observed, there was a strong anti-Gag antibody response (Griffiths *et al.*, 1993; Wagner *et al.*, 1996b; Wagner *et al.*, 1996a), and Gag may exert an immunodominant effect over Env. Alternatively, the heterologous fused epitopes were not sufficiently exposed to permit efficient B-cell interactions.

CTL responses to type I in-frame VLPs

Type I in-frame VLPs have elicited strong CTL responses in mice to V3 and CD4BD fusion sequences to Gag (Wagner *et al.*, 1996a; Wagner *et al.*, 1996b; Wagner *et al.*, 1993; Griffiths *et al.*, 1993; Schirmbeck *et al.*, 1995). Furthermore, CTL responses have been detected after a single, low dose (0.1 μ g) of purified particles (Wagner *et al.*, 1993), indicating the potential for CTL responses to be efficiently primed. Although some Gag-V3 VLPs have stimulated HIV-1 isolate specific CTL responses in mice (Griffiths *et al.*, 1993), VLPs carrying 3 tandem copies of a consensus V3 domain elicited a broad, cross-reactive CTL response in mice to four different strains of HIV-1 (Luo *et al.*, 1998), and this is promising for HIV vaccine development.

1.7 Study outline and objectives

The primary objective of this study was to make type I in-frame VLP analogues to the DNA vaccines pTHr.HIVA (Hanke & McMichael, 2000) and pTHr.grttnC (Burgers *et al.*, 2006), which could be used in future prime-boost vaccine strategies. These chimaeric Gag VLPs would be produced using the baculovirus system and characterized by western blot and electron microscopy. Both of these DNA vaccines express chimaeric Gag-derived proteins with C-terminal immunogenic fusions.

In the case of pTHr.HIVA, an exact analogue is impossible since the MA and CA domains in *gag* have been reversed in the DNA vaccine (Hanke & McMichael, 2000). Therefore, the closest VLP analogue would be an in-frame fusion of the HIV-1A CTL polypeptide (PE, 155 aa) to the C-terminus of a truncated Gag carrier molecule that is still capable of forming VLP. For pTHr.grttnC, an exact VLP analogue would necessitate fusing a 778 aa polyprotein (RTTN) to a p6-truncated Gag (Pr50^{gag}) carrier molecule. However, the use of a full-length Gag carrier (Pr55^{gag}) has the advantage of the inclusion of an increased number of potential Gag epitopes.

It has been suggested, but not demonstrated until this study, that the maximum length of antigen that can be incorporated into a type I in-frame VLP is at most around 200 aa (Deml *et al.*, 2005). Therefore, a broader objective of this study was to test the upper length limit of polypeptide that could be tolerated within a Gag-based VLP. To this end, PE and five sequences of differing length derived from RTTN were fused in-frame to the C-terminus of either Pr55^{gag} or Pr50^{gag}, and tested for VLP formation.

CHAPTER 2

Construction of recombinant Gag donor vectors

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2.1 INTRODUCTION

The overall objective of this study was to produce chimaeric Gag VLPs (to be tested as candidate HIV-1 vaccines) using a baculovirus system. HIV-1 subtypes A and C protein-encoding sequences were derived from the DNA vaccine plasmids pTHgagC (van Harmelen *et al.*, 2003), pTHr.grrtnC (Burgers *et al.*, 2006) and pTHr.HIVA (Hanke & McMichael, 2000). All coding sequences in these plasmids express proteins that have been functionally inactivated by mutation, as a safety precaution. A selection of gene fragments corresponding to additions of 113 – 778 aa were cloned as C-terminal fusions to either a full-length or p6-truncated *gag* gene in a donor vector (pFastBacTMDual). In total, 17 proteins (detailed in Fig. 2.10) would be expressed in insect cells by recombinant baculovirus to assess chimaeric Gag VLP formation.

C-terminal fusions to Gag were chosen over internal insertions since the longest successful fusions reported to date were achieved using this approach (Luo *et al.*, 1992), and a review of the available literature suggests that continuous Gag sequences (without internal sequence disruptions) may be superior antigen carriers (1.6.3.1). Furthermore, the magnitude of CTL responses was not affected by the position of a V3 sequence within a Pr55^{gag} carrier, whereas antibody responses were highest for C-terminal fusions (Wagner *et al.*, 1996b). Since the flanking residues in this case did not affect the exogenous antigen processing for MHC class-I presentation, it seems reasonable to assume this may extend to other antigenic inserts. C-terminal fusions also produced a higher yield of VLPs with less size variation than internal insertions (Wagner *et al.*, 1996b).

The inclusion of a p6-truncated *gag* was based on the hypothesis that a smaller Gag carrier moiety that forms VLPs by itself (Royer *et al.*, 1991) may be capable of tolerating larger fusions within the VLP structure than Pr55^{gag}. Other regions dispensable for VLP formation (Fig. 1.3) were not deleted so as to maintain maximum immunogenicity of the Gag carrier. Furthermore, minimal Gag constructs (1.5.3) often produced lower VLP yields than intact Gag sequences (Borsetti *et al.*, 1998; Accola *et al.*, 2000; Wang *et al.*, 1998).

The *gag* carrier genes contain the myristylation signal (ATGGGT, *myr*⁺) to facilitate Gag-only and chimaeric Gag VLP production, except for the negative controls for VLP formation, which

have an abrogated myristylation signal (ATGGCT, *myr*⁻). The corresponding myristylated and non-myristylated proteins are designated Myr⁺ and Myr⁻ respectively.

HIV codon usage differs substantially from that of most human genes and is characterized by a high AT content (Kypr & Mrazek, 1987). Human codon optimized (humanized) *gag* genes have shown 10-fold (Smith *et al.*, 2004), 20-fold (Kofman *et al.*, 2003) and 10³-fold (zur Megede *et al.*, 2000) increases in transient Gag expression relative to the native gene in human cells. Consequently, a number of HIV-1 DNA vaccines, including pTHgagC, pTHr.grttnC and pTHr.HIVA use humanized sequences (van Harmelen *et al.*, 2003; Burgers *et al.*, 2006; Hanke & McMichael, 2000). Therefore, humanized *gag*, with 76% nucleotide (nt) identity to wild-type (WT) *gag*, was used in all chimaeric constructs consistent with the vaccine fusion sequences. Although there are no reports in the literature on humanized *gag* expression in the baculovirus system, it has been shown in our laboratory that a humanized human papillomavirus type 16 L1 capsid protein gene expressed at significantly higher levels in insect cells than the non-optimized gene (A. Hopkins, unpublished).

2.1.1 Gag control constructs

The Gag controls included in this study were: wild-type Gag (WTgagC), full-length humanized Myr⁺ Gag (HMgagC = Pr55^{gag}), p6-truncated humanized Myr⁺ Gag (THMgagC = Pr50^{gag}) and Myr⁻ versions of both humanized Gag proteins (HAMgagC and THAMgagC). WTgagC was incorporated to compare expression with HMgagC, while THMgagC was included because 6 out of the 12 chimaeric Gag constructs contain this truncated form of Gag. It has been shown that Myr⁻ Gag does not form VLPs, irrespective of the presence of p6 (Royer *et al.*, 1991), and so HAMgagC and THAMgagC are the negative controls for VLP production in insect cells expressing Gag-based constructs.

2.1.2 Immunogenic components of Gag chimaeras from HIV-1 DNA vaccines

Sequences encoding portions of an HIV-1C polyprotein (GRTTN) and an artificial HIV-1A immunogen (HIVA) were chosen for inclusion in chimaeric VLPs. Expression of both GRTTN and HIVA from the respective DNA vaccines has generated encouraging immune responses (described below), justifying their inclusion in this study of chimaeric Gag VLP vaccine candidates.

2.1.2.1 RT-Tat-Nef (RTTN) derivatives

The candidate HIV-1C DNA vaccine pTHr.grttnC encodes GRTTN - a polyprotein comprising p6-truncated Gag (Myr⁻), inactivated reverse transcriptase (RT), shuffled Tat and inactivated Nef (TN), where the gene sequences were derived from the HIV-1C vaccine strains Du151 and Du422 (Burgers *et al.*, 2006). These strains were selected as having the closest amino acid similarity to a South African subtype C consensus sequence (Williamson *et al.*, 2003). The portion of the polyprotein C-terminal to p6-truncated Gag is collectively termed RTTN, and the components have been modified, at the genetic level, to be non-functional for stability and safety considerations (Burgers *et al.*, 2006). Briefly, the active site of RT was inactivated by mutation (YMDDL → YMAAL), Tat was inactivated through shuffling of the three gene regions known to be important to function (while maintaining all potential epitopes) and Nef was inactivated by the removal of 30 base pairs (bp) at the 5'-end. Vaccination of mice with pTHr.grttnC led to the induction of high CTL levels against multiple GRTTN epitopes, and strong IFN- γ responses to RT were stimulated (Burgers *et al.*, 2006).

2.1.2.2 HIV-1A CTL polyepitope (PE)

The HIVA immunogen (Hanke & McMichael, 2000) consists of subtype A consensus amino acid sequence of p24 and p17 (cloned in the reverse order to p17-p24 in native Gag) coupled to a string of overlapping CTL epitopes at the C-terminus. This HIV-1A CTL polyepitope (PE) was designed to induce CD8⁺ CTLs and includes 23 partially overlapping HIV-1A-derived CTL epitopes (8-11 aa) identified in Kenyan patients infected with HIV-1A. These epitopes, which are presented by 17 different HLA alleles (MHC class I restriction), were derived from Nef (8), Gag (7), Pol (6), and Env (4); many are conserved among HIV-1 subtypes or are immunodominant. Two non-HLA epitopes for SIV Gag and HIV Env, recognized by macaque and murine CTL respectively, were included for use in quality control and potency assays. Finally, the C-terminal residues IPNPLLGLD derived from the simian virus 5 (SV5) capsid protein are recognized by the SV5-Pk monoclonal antibody (mAb), which allows for detection and quantification of the full-length peptide (Hanke *et al.*, 1992). Expression of HIVA from the DNA vaccine pTHr.HIVA and a modified vaccinia virus Ankara (MVA) vector elicited HIV-specific CTL responses in mice (Hanke *et al.*, 2002), rhesus macaques (Wee *et al.*, 2002) and human volunteers in Phase I clinical trials (Mwau *et al.*, 2004; Goonetilleke *et al.*, 2006).

2.1.3 Donor vector: pFastBacTMDual

In this study, Gag and chimaeric Gag sequences to be expressed by recombinant baculovirus were assembled in the donor vector pFastBacTMDual (Appendix C). The baculovirus used was *Autographa californica* nuclear polyhedrosis virus (AcNPV), and the method used to generate recombinant AcNPV (rAcNPV) from a donor vector is detailed in Figure A.1 (A.5). Genes can be cloned into pFastBacTMDual to express proteins under the p10 promoter or the stronger polyhedron promoter (pPolh). Each promoter has a downstream multiple cloning site (MCS).

2.1.4 Chapter aims

The aim of the work reported in this chapter was to construct a set of donor vectors to generate rAcNPV expressing chimaeric HIV-1 Gag proteins of various lengths and sequence compositions, for subsequent evaluation of VLP formation.

2.2 MATERIALS AND METHODS

2.2.1 Site-directed mutagenesis and plasmid construction

The HIV-1 subtypes A and C DNA sequences used in this study were derived from plasmids shown in Table 2.1. The polymerase chain reaction (PCR) conditions and temperature profiles for site direct mutagenesis (Fig. 2.1 & 2.2) are given in Appendix B, while the associated primers are listed in Table 2.2. A number of sub-clones were constructed (Fig. 2.3 & 2.4) to facilitate the manipulation and cloning of *gag* sequences necessary for the construction of Gag and chimaeric Gag donor vectors (Fig. 2.5-2.9).

All DNA preparations and manipulations were carried out according to standard procedures (A.3). Cloning into pGEM[®] – T Easy was done according to manufacturer’s instructions. All pFastBac[™]Dual constructs contain HIV-1 protein encoding sequences cloned into the MCS of pPolh, where the complete protein-encoding sequences have start and stop codons.

TABLE 2.1. Parental plasmid, DNA vaccines and cloning vectors used in this study

| Plasmids ¹ | Sequence of interest / Reason for use | Source / Reference |
|----------------------------|--|-----------------------------------|
| pGEMgag10M1 | WT, Myr ⁺ HIV-1C Gag gene | A. Jaffray ² |
| pTHgagC | Full-length, humanized, Myr ⁻ HIV-1C Gag gene | van Harmelen <i>et al.</i> , 2003 |
| pTHgrttnC | Polygene encoding Myr ⁻ p6-truncated HIV-1C Gag, reverse transcriptase (RT), shuffled Tat and inactivated Nef (TN). | Burgers <i>et al.</i> , 2006 |
| pTH.HIVA | HIV-1A CTL polyepitope (PE) encoding sequence. | Hanke & McMichael, 2000 |
| pGEM [®] – T Easy | Used for cloning of PCR generated products. | Promega ³ |
| pFastBac [™] Dual | Used for cloning protein sequences to be transposed into baculovirus. | Invitrogen ⁴ |

¹ Both pTHgrttnC and pTH.HIVA contain the β -lactamase gene (conferring ampicillin resistance), which was removed when manufactured for clinical trial (designated pThr.grttnC and pThr.HIVA respectively)

² Department of Molecular and Cell Biology, University of Cape Town, Rondebosch 7701, Cape Town, South Africa.

³ Promega Corporation, Madison, USA.

⁴ Invitrogen Ltd, Paisley, UK.

TABLE 2.2. Oligonucleotide primers used in plasmid construction and DNA sequencing

| Primer ¹ | Nucleotide sequence ² | Use | Orientation |
|---------------------|--|----------------|-------------|
| HMgagF1 | 5'-CTT GCC ACC ATG GGT GCT CGC GCA TC-3' | PCR | Sense |
| HMgagR1 | 5'-GGT GTC CTC CCA CTG TTC AGC ATA GTG TTC-3' | PCR | Anti-sense |
| HMgagF2 | 5'-ATT AGG ATC CAA GCT TGC CAC CAT GGG TGC-3' | PCR | Sense |
| HMgagF3 | 5'-GCG AAG GCG CCA CTC CTC-3' | PCR | Sense |
| HMgagR2 | 5'-CTT GAA TTC TTG GCT GAG GGG GTC GCT AC-3' | PCR | Anti-sense |
| HMgagR3 | 5'- AGC GAA TTC <u>TTA</u> GCC AGG GCG GCC CTT ATG-3' | PCR | Anti-sense |
| FBDS1 | 5'-TAA AGG TCC GTA TAC TCC GG-3' | DNA sequencing | Sense |
| HMgagS1 | 5'-GTG CTT CAA TTG TGG CAA GGA GGG-3' | DNA sequencing | Sense |

¹ Oligonucleotide primers were synthesized by the DNA Synthesis Service, Department of Molecular and Cell Biology, University of Cape Town; with the exception of HMgagR1 (obtained from J. van Harmelen)

² Regions to introduce restriction enzyme sites and stop codons are in bold and underlined respectively.

2.2.2 Plasmid verification

2.2.2.1 DNA Sequencing

Inserts in all pGEM[®] – T Easy constructs were sequenced in both directions using a pUC/M13 sequencing primer set (Promega). All pFastBac[™] Dual constructs where blunting reactions were included in construction were sequenced in duplicate with the primer HMgagS1 to confirm the sequence near the blunting site. In addition, pFBD-HΔMgagC and pFBD-THΔMgagC were sequenced in duplicate with the primer FBDS1 to confirm the presence of the *myr*⁻ signal at the 5' end of *gag*. Details of sequencing reactions are supplied in Appendix A.4.

2.2.2.2 Restriction enzyme digest patterns

The veracity of each plasmid was confirmed by appropriate restriction enzyme digestion of small-scale DNA preparations (Appendix D). The banding patterns were visualized by electrophoresis through 0.8% agarose gels (Sambrook *et al.*, 2001) and compared to the expected fragment sizes based on restriction analysis using DNAMAN[®] Version 4.13 (Lynnon Biosoft).

2.3 RESULTS

Seventeen donor vectors to generate rAcNPV that express control and chimaeric Gag proteins (Fig. 2.10) in insect cells were constructed.

2.3.1 Site-directed mutagenesis

The WT HIV-1C *gag* in pGEMgag10M1 contains the *myr*⁺ signal whereas the humanized *gag* in pTHgagC does not. Site-directed mutagenesis restored the *myr*⁺ signal and inserted a *Bam*HI site at the 5'-end of humanized *gag* (Fig. 2.1). PCR amplifications (Fig. 2.2) were also used to insert a stop codon within *gag* to generate the 3'-end of p6-truncated *gag* (3'THgag), and to remove the stop codon at the 3'-end of full-length *gag* (3'HgagNS). DNA sequencing confirmed that the desired mutations were achieved without any errors.

2.3.2 Cloning strategies

2.3.2.1 Sub-clones

The PCR products 5'HMgag (Fig. 2.1B), 3'HgagNS (Fig. 2.2, lane 1) and 3'THgag (Fig. 2.2, lane 2) were cloned into pGEM[®] – T Easy for the construction of 3 further pFastBac[™]Dual sub-clones (Fig. 2.3 & 2.4).

2.3.2.2 Gag control donor vectors

The cloning strategies for the construction of the five Gag control donor vectors are shown in Figures 2.3-2.6. These donor vectors facilitate the generation of rAcNPV expressing 3 positive Gag VLP controls (WTgagC, HMgagC and THMgagC) and 2 negative Gag VLP controls (HΔMgagC and THΔMgagC).

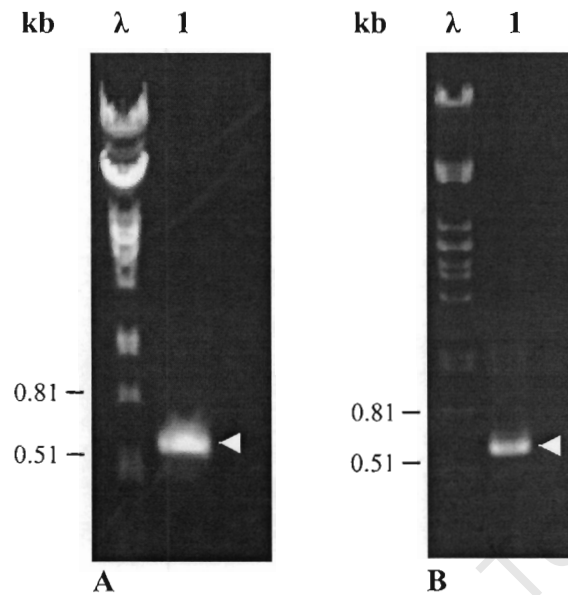


FIGURE 2.1. Site-directed mutagenesis to restore the *myr*⁺ signal and introduce a *Bam*HI cloning site upstream of *gag*. (A) PCR amplification of the 5' end of *gag* (5'HMgagI, 583 bp) in pTHgagC with primers HMgagF1 and HMgagR1 restored the *myr*⁺ signal (ATGGCT → ATGGGT) at the 5'-terminus of the gene. (B) PCR amplification of 5'HMgagI with primers HMgagF2 and HMgagR1 introduced a *Bam*HI cloning site upstream of the *gag* start codon (5'HMgag, 596 bp). λ = Lambda DNA-*Pst*I MW marker; kb = kilobase. Arrowheads indicate PCR products of the expected size.

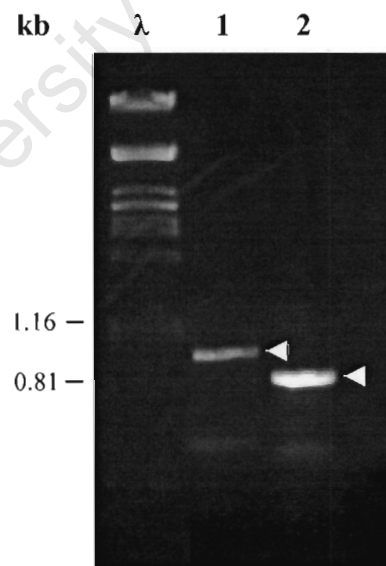


FIGURE 2.2. Site-directed mutagenesis to add and remove stop codons within *gag*. PCR amplification of the 3'-region of *gag* in pTHgagC with primers HMgagF3 and HMgagR2 removed the stop codon and introduced an *Eco*RI site (3'HgagNS, 968 bp), lane 1. A stop codon (TAA) and an *Eco*RI site were inserted within a 3'gag region upstream of the p6 encoding sequence using primers HMgagF3 and HMgagR3 (3'THgag, 821 bp), lane 2. λ = Lambda DNA-*Pst*I MW marker; kb = kilobase. Arrowheads indicate PCR products of the expected size.

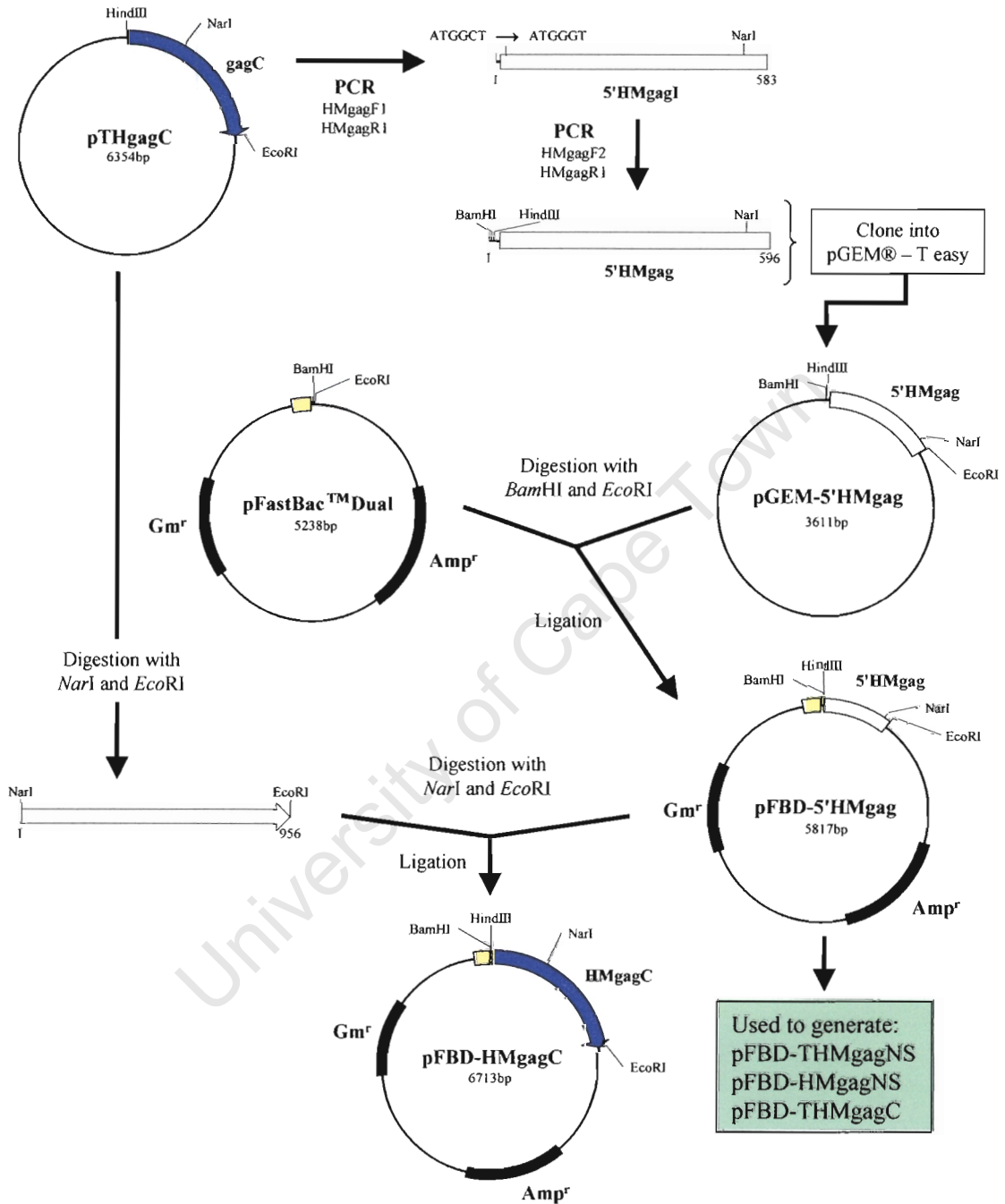


FIGURE 2.3. Construction of a donor vector to generate rAcNPV expressing full-length humanized Gag. The HIV-1C humanized, *myr*^r gag gene (HMgagC) was assembled into the pFastBac™Dual donor vector under pPolh (yellow box); black boxes represent relevant antibiotic resistance genes. Genes expressing Gag are shown as blue arrows. The sub-clone pFBD-5'HMgag was used in the construction of 3 other vectors (Fig. 2.4).

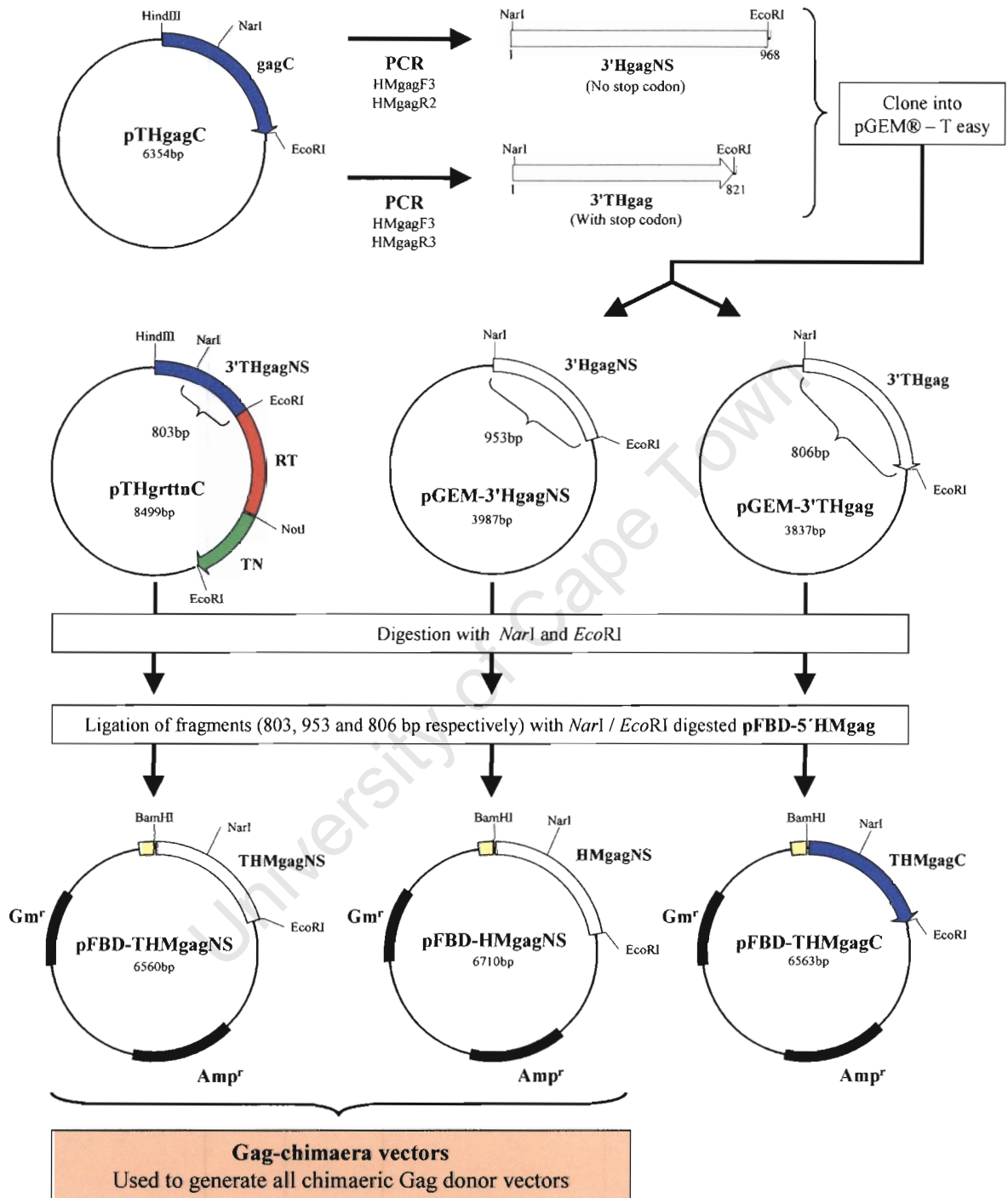


FIGURE 2.4. Construction of Gag-chimaera vectors and a donor vector to generate rAcNPV expressing THMgagC. The HIV-1C p6-truncated, humanized, *myr⁺* gag gene (THMgagC) was assembled into the pFastBac™ Dual donor vector under the pPolh (yellow box); black boxes represent relevant antibiotic resistance genes. Genes expressing Gag, RT, TN are blue, red and green respectively. The Gag-chimaera vectors harboring gag sequences without stop codons (NS) were used in the construction of the 12 chimaeric Gag donor plasmids (Fig. 2.7-2.9).

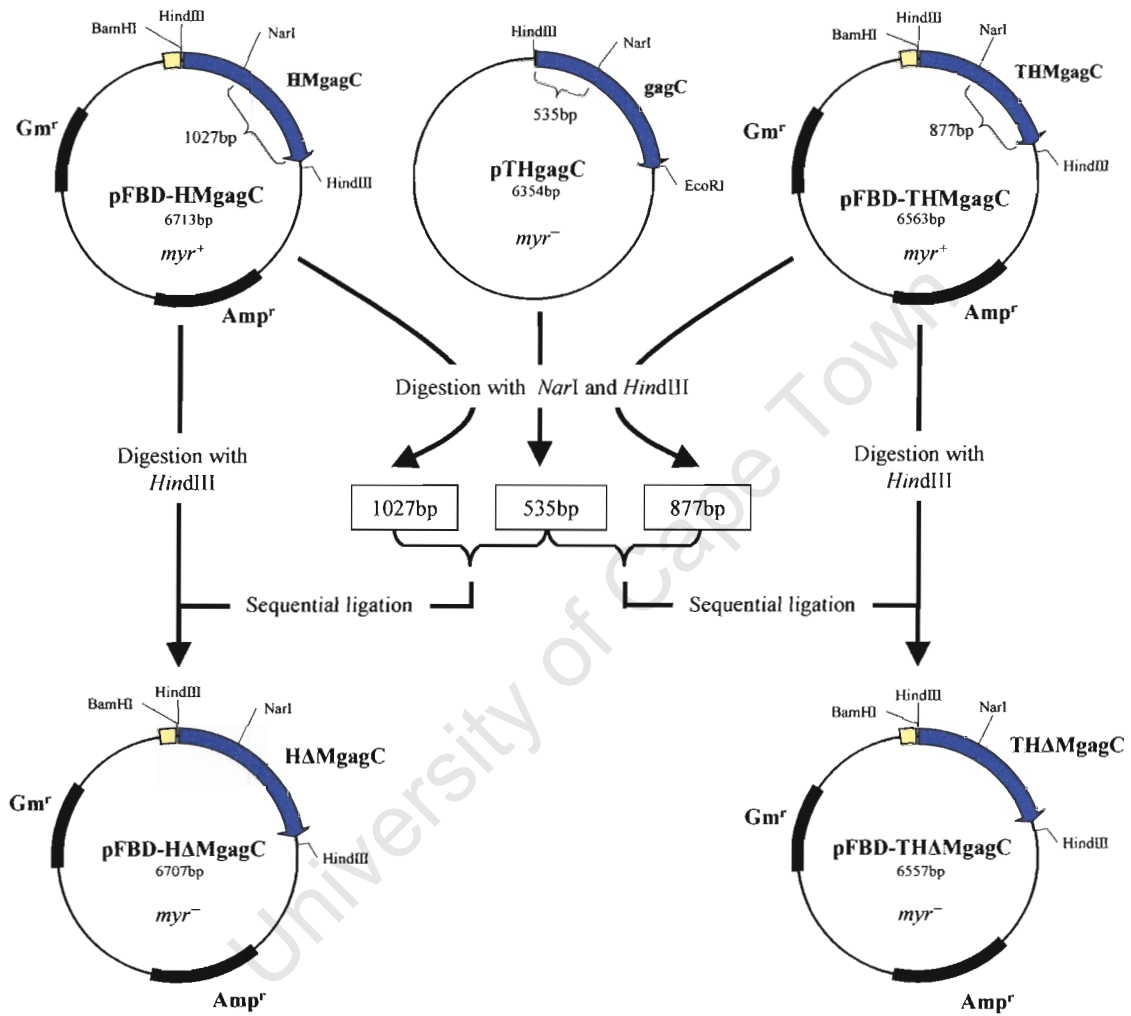


FIGURE 2.5. Construction of donor vectors to generate rAcNPV expressing Myr⁻ Gag proteins. The HIV-1C full-length and p6-truncated, humanized, myr⁻ gag genes (HΔMgagC and TΔMgagC) were assembled into pFastBacTMDual under pPolh (yellow box); black boxes represent relevant antibiotic resistance genes. Genes expressing Gag proteins are shown as blue arrows.

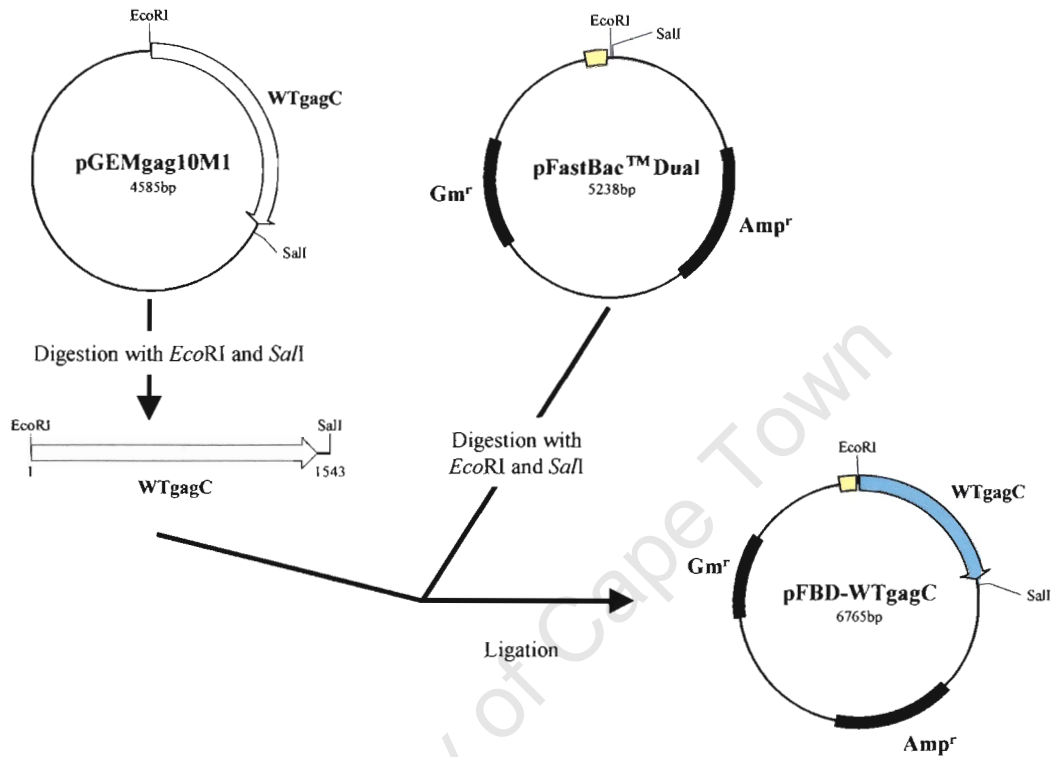


FIGURE 2.6. Construction of a donor vector to generate rAcNPV expressing WT Gag. The HIV-1C *myr⁺ WT gag* gene (white arrow) was cloned into the pFastBac™Dual donor vector under pPolh (yellow box), black boxes represent relevant antibiotic resistance genes. The functional gene (in the donor vector) expressing Myr⁺ WT Gag (WTgagC) is shown as the light blue arrow.

2.3.2.3 Chimaeric Gag donor vectors

All 12 donor vectors for the generation of rAcNPV expressing chimaeric Gag proteins were constructed by cloning selected in-frame HIV-1 DNA sequences into the Gag-chimaera vectors (Fig. 2.7-2.9). These donor vectors represent 10 RTTN-derived Gag fusion constructs and two Gag-PE constructs, nomenclature for the encoded proteins is given in Figure 2.10.

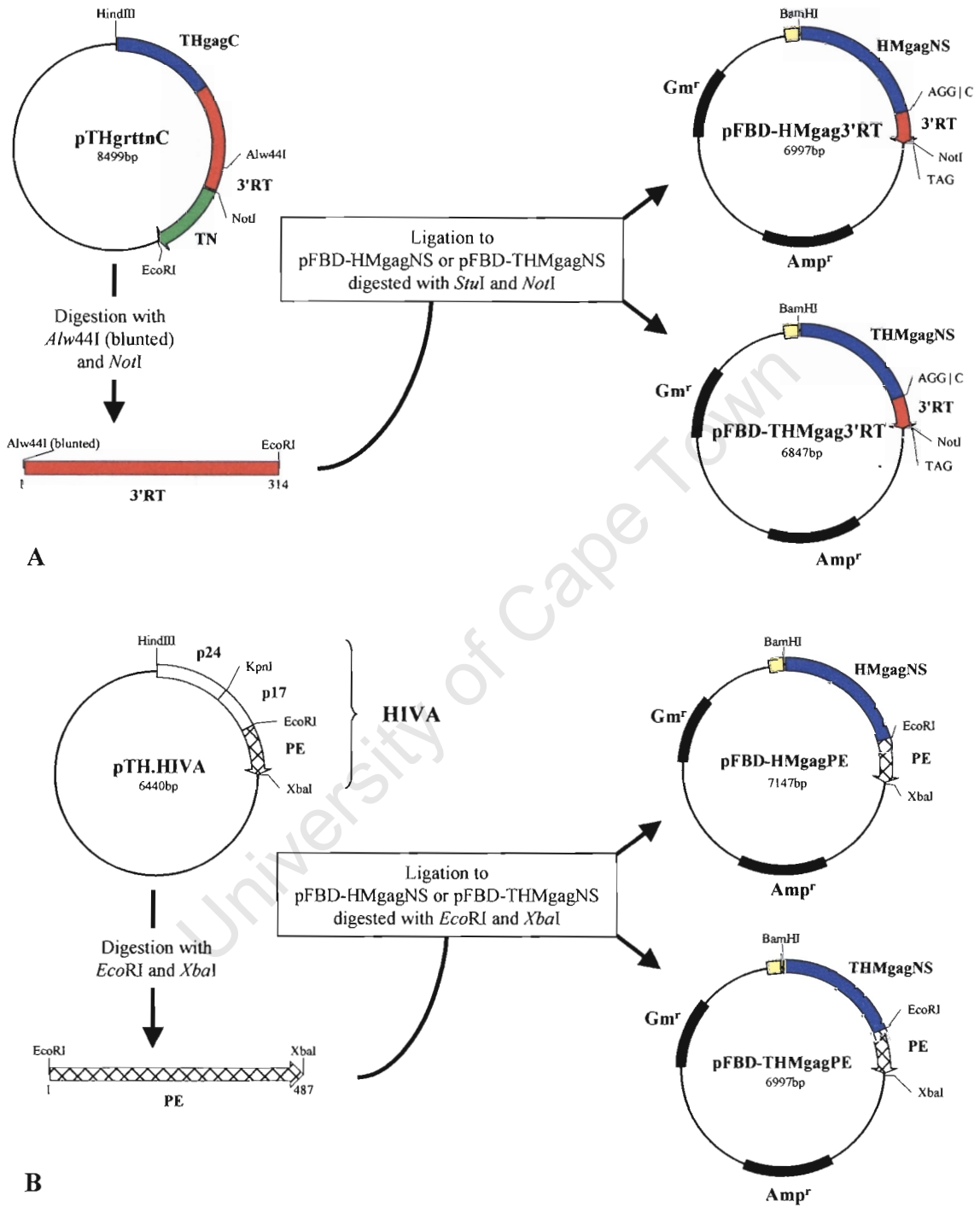


FIGURE 2.7. Construction of donor vectors to generate rAcNPV expressing Gag-3'RT and Gag-PE chimaeras. The 3'-end of the RT gene and the PE encoding sequence from the DNA vaccines pTHgrttnC and pTH.HIVA were cloned into the Gag-chimaera vectors under pPolh (yellow box); black boxes represent relevant antibiotic resistance genes. 3'RT was blunted with mung bean nuclease. Genes expressing 3'RT and PE are shown as red and cross-hatched arrows respectively.

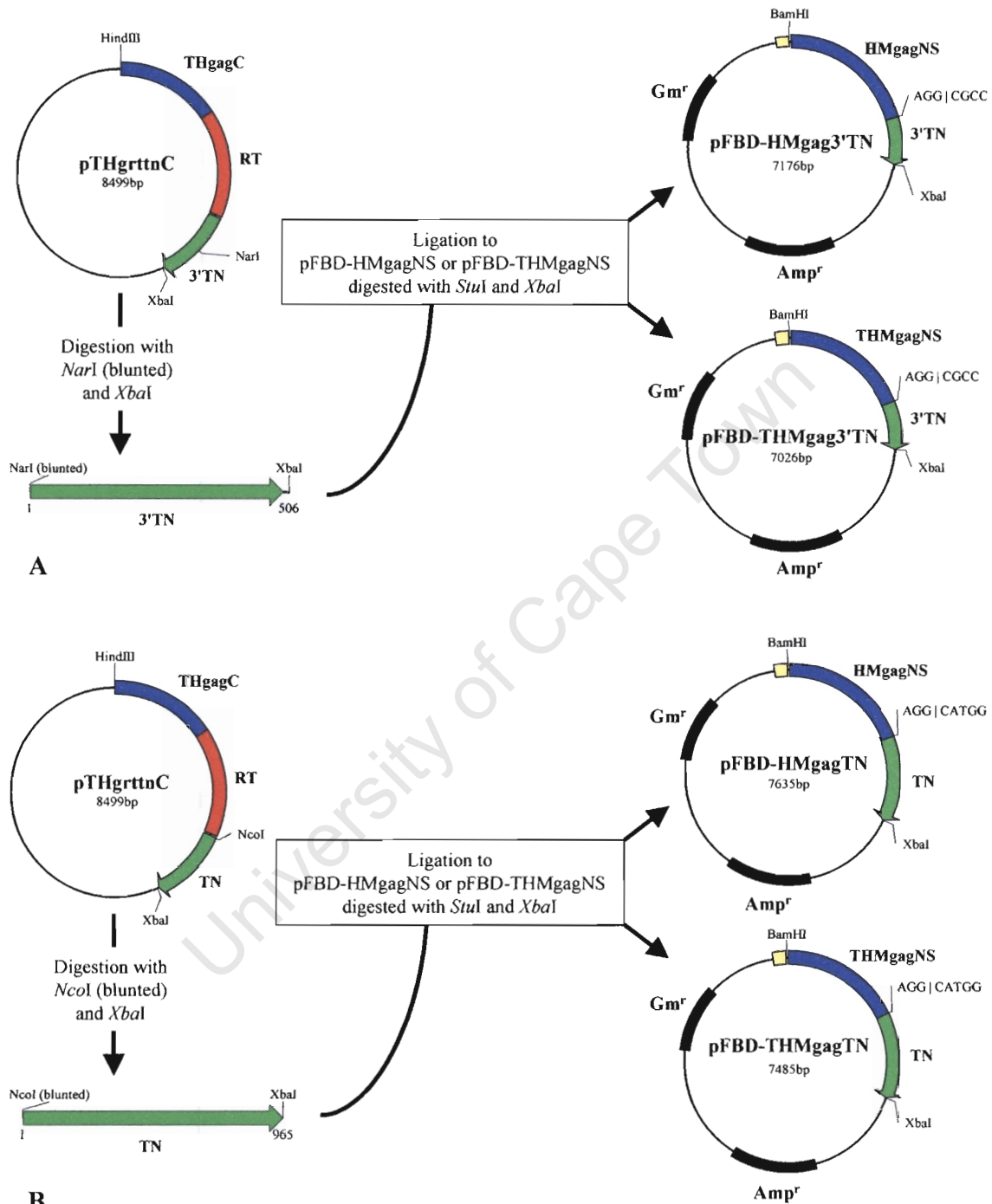


FIGURE 2.8. Construction of donor vectors to generate rAcNPV expressing Gag-3'TN and Gag-TN chimaeras. The 3'-end of the TN gene (encoding only Nef sequence) and the full TN gene from pTHgrrtnC were cloned into the Gag-chimaera vectors under pPolh (yellow box); black boxes represent relevant antibiotic resistance genes. 3'TN and TN were blunted with Klenow enzyme. Genes expressing 3'TN and TN are shown as green arrows.

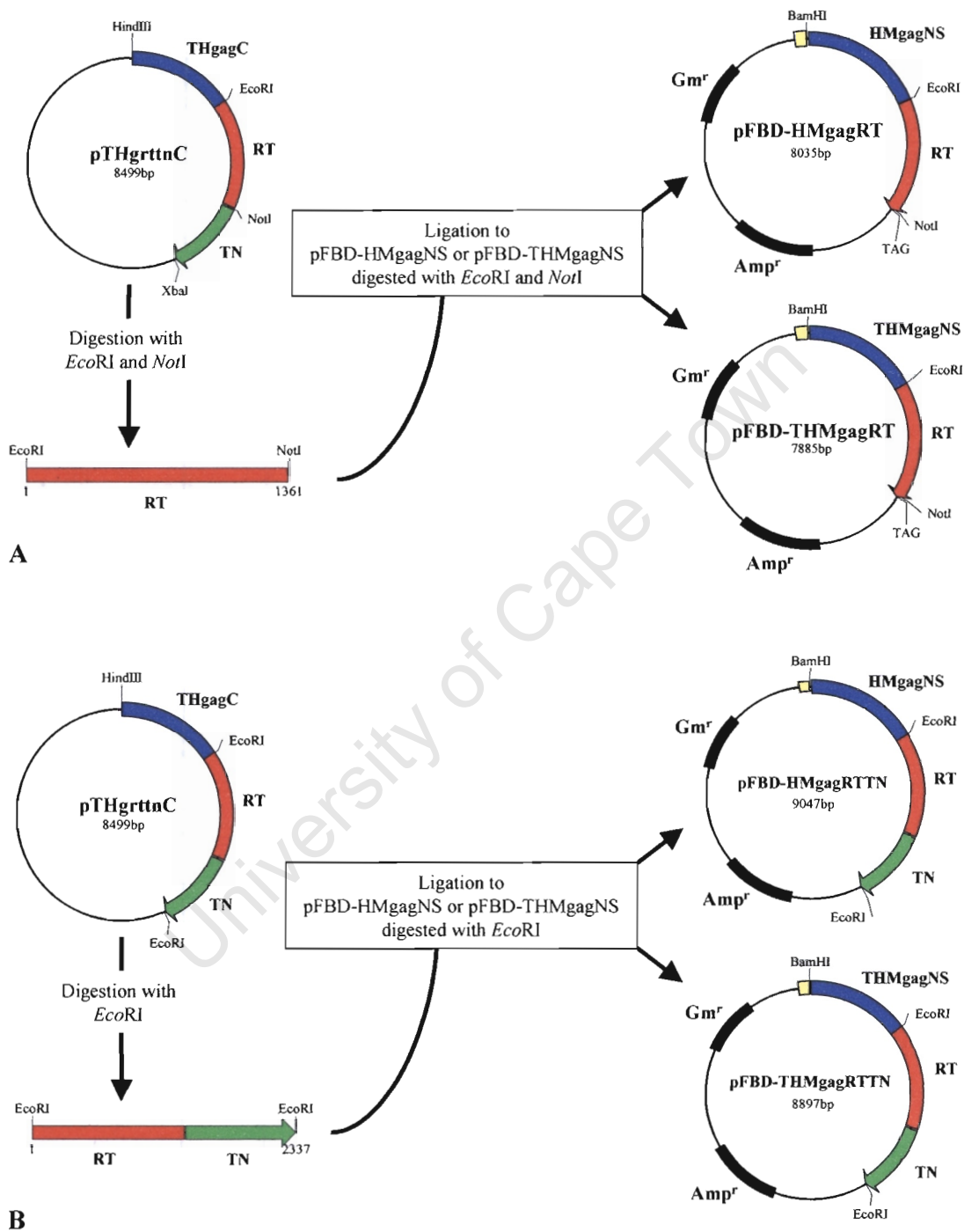


FIGURE 2.9. Construction of donor vectors to generate rAcNPV expressing Gag-RT and Gag-RTTN chimaeras. The RT gene and the concatenated RT and TN genes from pTHgrttnC were cloned into the Gag-chimaera vectors under pPolh (yellow box); black boxes represent relevant antibiotic resistance genes. Genes expressing RT and TN are shown in red and green respectively.

2.3.3 Control and chimaeric Gag constructs

The 17 donor vectors (2.3.2) each contain regions encoding either a Gag control protein or a chimaeric Gag fusion. The expected molecular weights of these full-length proteins and the number of amino acid residues in C-terminal fusions are summarized in Figure 2.10.

2.4 DISCUSSION

The recombinant Gag donor vectors constructed in this study allow for the possible expression of 17 different Gag-based proteins in insect cells by rAcNPV. The principle function of this vector set was to generate potential HIV-1 vaccine candidates and simultaneously test the size limit (up to 778 aa) of C-terminal fusions to Gag that still permit VLP formation. To this end, fusion sequences from two existing HIV-1 DNA vaccines were chosen, so that any chimaeric VLPs produced would potentially be superior to Gag-only VLPs in terms of antigenicity. Furthermore, particulate protein analogues to these DNA vaccines could be used in prime-boost strategies to enhance immune responses.

Apart from providing internal experimental controls, the Gag control donor vector set allows for a number of features of Gag expression in the baculovirus system to be investigated, which includes the following: Evaluation of the effect of human codon optimization on Gag expression by quantitative comparison of WTgagC and HMgagC levels in cell lysate and culture supernatant. Determination of the influence of p6 on Gag expression, VLP production and particle morphology by analysis of HMgagC and THMgagC expressing cells. Determining the expression levels, cellular distribution and structures formed by Myr⁻ Gag relative Myr⁺ Gag. Inclusion of Pr55^{gag} and Pr50^{gag} Myr⁻ versions may elucidate whether the role of p6 changes in the absence of a *myr*⁺ signal.

Furthermore, all the pFastBacTMDual donor vectors contain protein-encoding sequence under the control of pPolh, leaving the p10 promoter available for insert cloning. An application of this would be to express HIV-1 Env (or a suitable Env-derivative) under p10, thereby allowing type I in-frame VLPs that also incorporate Env components in the VLP membrane to be produced from a single donor vector. This would effectively be a combination of a type I and II VLP.

However, it may still prove preferable to co-express Env under pPolh in a separate donor vector, since pPolh is a stronger promoter than p10 and the optimal infection conditions of each corresponding recombinant baculovirus could be determined separately.

Finally, the Gag-chimaera vectors constructed in this study provide a general backbone for constructing donor plasmids encoding C-terminal Myr⁺ Gag fusions. Genes of interest can be cloned in-frame into the pPolh MCS between the *EcoRI* and *HindIII* restriction enzyme sites to express chimaeric Gag proteins. In addition to antigenic sequences for candidate Gag-based VLP vaccines, reporter genes or fluorescent markers could be inserted to investigate biochemical properties of Gag and Gag-VLPs in insect cells.

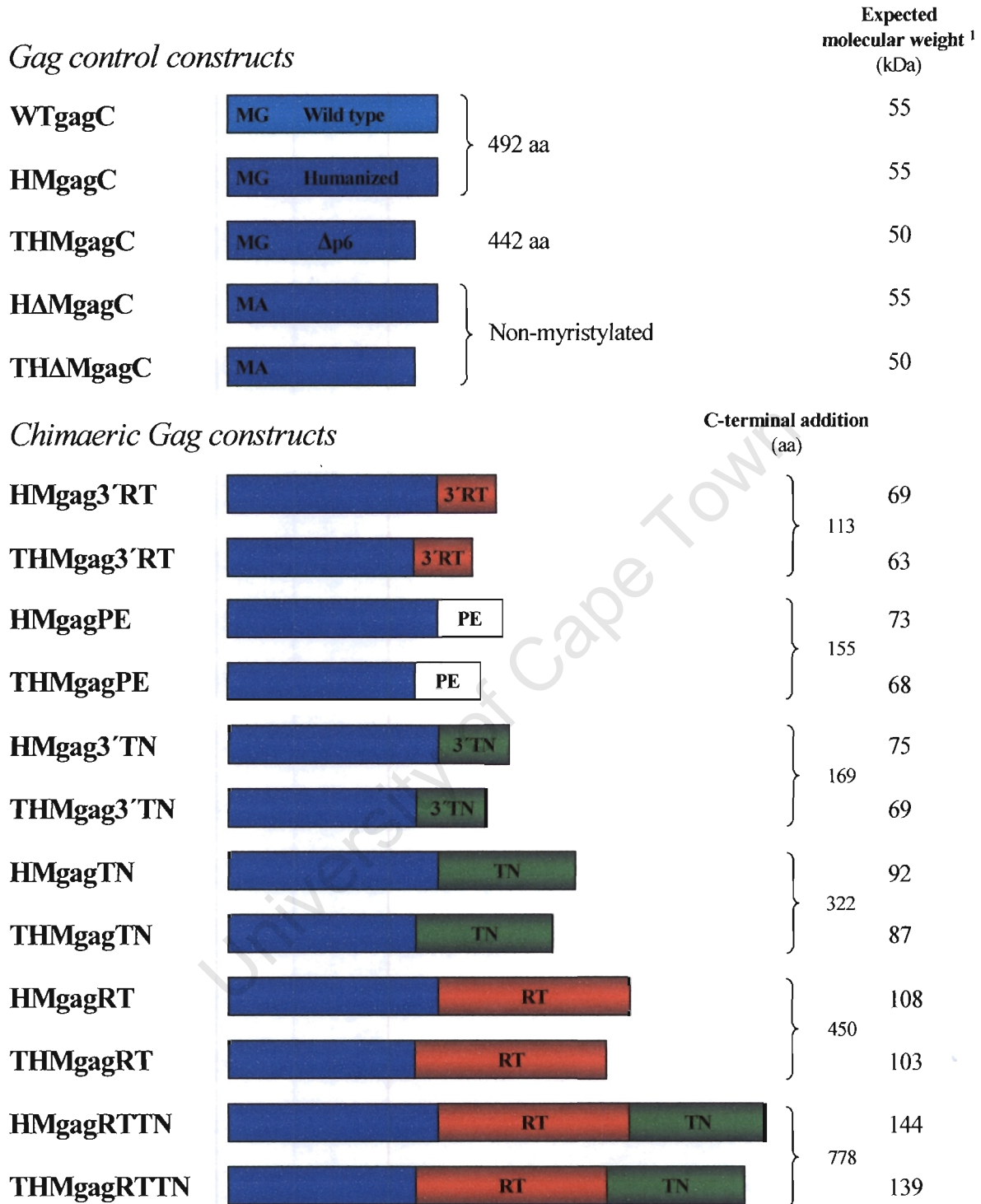


FIGURE 2.10. Schematic representation of control and chimaeric Gag constructs.

Each Gag and Gag-fusion encoding DNA sequence was cloned into pFastBacTMDual under pPolH. The corresponding full-length proteins to be expressed by rAcNPV are shown with the N-terminus on the left. Chimaeric Gag constructs are arranged in ascending order of C-terminal fusion size.

Construct nomenclature: WT = wild-type, T = p6-truncated (Δp6) Gag, H = humanized, M = Myr⁺ (gly2), ΔM = Myr⁻ (ala2), C = HIV-1C derived, PE = HIV-1A CTL polyepitope, RT = HIV-1C reverse transcriptase with mutated active site, TN = HIV-1C epitope - shuffled Tat and inactivated Nef, 3'RT = product from 3' end of RT gene, 3'TN = product from 3' end of TN gene, containing only Nef sequence (nomenclature for 3'TN reflects the method of construction and is consistent with that for RT and 3'RT).

Legend: aa, amino acid; kDa, kilo Dalton; MG, met1gly2; MA, met1ala2.

¹ Rounded off to nearest kDa.

CHAPTER 3

Expression of chimaeric Gag proteins

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3.1 INTRODUCTION

The baculovirus system (1.4.1) has a number of advantages over other heterologous protein expression systems (Kost *et al.*, 2005; Hu, 2005). These include high protein yields, potential for large gene insertions, biosafety (non-infectious to humans), the capacity to produce complex quaternary structures and the ability to perform some higher eukaryotic post-translational modifications - including phosphorylation and myristylation. These two modifications in particular are important in the context of HIV Gag expression for VLP production. A number of VLPs from other viruses, including human papillomavirus, hepatitis C virus, parvovirus, polyomavirus, herpes simplex virus and severe acute respiratory syndrome coronavirus have also been produced using recombinant baculovirus (Hu, 2005).

3.1.1 Assessing VLP formation

Gag assembly domains and the production of Gag-based VLPs have both been extensively studied in insect cells using baculovirus expressed proteins (1.4.1). In this system, the presence of Gag-based proteins in the culture supernatant, following recombinant baculovirus infection is a necessary but not sufficient criterion for determining whether extracellular VLPs have been formed. Although myristylation of Gag is essential for VLP budding and release from insect cells (Wagner *et al.*, 1992; Gheysen *et al.*, 1989; Royer *et al.*, 1991), Myr⁺ Gag can be released from these cells in a soluble, non-particulate form (Royer *et al.*, 1992). Furthermore, even Myr⁻ Gag is released from insect cells in this soluble form (Royer *et al.*, 1992) and cell lysis (increased by baculovirus infection) causes the further release of both intracellular protein aggregates and soluble proteins into the culture medium. The localization of Gag-based proteins to the cell membrane is a necessary but not sufficient condition for extracellular VLP formation. Although stable membrane binding of Myr⁺ Gag to the cell membrane is essential for VLP assembly (Fig. 1.2), some Myr⁺ Gag truncations (Gheysen *et al.*, 1989) and in-frame Myr⁺ Gag-V3 fusions (Tobin *et al.*, 1996a) have localized to insect cell membranes and not formed VLPs.

3.1.2 Expression and proteolysis of Gag proteins in insect cells

The expression of Gag proteins in recombinant baculovirus-infected insect cells is regulated primarily at the level of transcription (Royer *et al.*, 1992). Post-translational events including VLP assembly and extracellular protein release were not found to influence the level of Gag synthesis in the baculovirus system (Royer *et al.*, 1992)

During HIV virion maturation, Pr55^{gag} is cleaved into the mature Gag proteins (MA, CA, NC, p6) and two smaller peptides (p1 and p2) by the viral PR ((Freed, 1998). However, insect cell and baculovirus proteases also process Pr55^{gag} (Wagner *et al.*, 1992) and chimaeric Gag proteins (Wagner *et al.*, 1996b). A number of specific cleavage products including p49 (MA-CA-p2-NC), p41 (MA-CA), p25 (CA-p2) and p24 (CA) have been observed in cell lysates and culture supernatants from insect cells expressing Pr55^{gag} (Wagner *et al.*, 1992) or chimaeric Gag proteins (Tobin *et al.*, 1996a). This processing is normally partial, and the full-length proteins can still be detected by western blot. The presence of p41 in the culture supernatant has also been attributed in part to a premature translational stop signal near the C-terminus of CA (Gowda *et al.*, 1989). Degradation of Pr55^{gag} VLPs in the culture medium also occurs, and this has been linked to cell lysis which releases proteolytic enzymes (Cruz *et al.*, 1999). In most cases this VLP particle degradation can be minimized by process optimization.

3.1.3 Bac-to-Bac[®] Baculovirus Expression System

In this study, the Bac-to-Bac[®] Baculovirus Expression System (A.5) was chosen to express Gag-based proteins in insect cells. The recombinant Gag donor vectors described in Chapter 2 were used to generate the corresponding rAcNPV. A quantitative comparison of protein expression levels in this system requires the standardization of the multiplicity of infection (MOI), where infections are conducted at a defined virus:cell ratio. A prerequisite to this is the accurate determination of the viral titre of amplified rAcNPV stocks by plaque assays, where the numbers of plaque forming units in serial dilutions of rAcNPV stock are counted in monolayer infections.

3.1.4 Chapter aims

The main aims of the work reported in this chapter were to:

1. Generate rAcNPV from the 17 Gag donor vectors described in Chapter 2.
2. Determine whether the expected Gag and Gag fusion proteins (Fig. 2.10) are expressed in rAcNPV infected insect cells and whether the same proteins are present in the culture supernatant, using western blot analysis.
3. Investigate whether the chimaeric Gag proteins are localized at the cell membrane using indirect immunofluorescence.
4. Compare humanized and WT Gag expression levels.

3.2 MATERIALS AND METHODS

3.2.1 Insect cell culture.

The *Spodoptera frugiperda* cell lines, Sf9 and Sf21, and the *Trichoplusia ni* cell line High Five™ (Invitrogen) were maintained in sterile tissue culture wells or flasks as recommended by the supplier. For infection with rAcNPV, Sf9 and Sf21 cells were grown in monolayer culture at 27°C in TC-100 Insect medium (Sigma) supplemented with: 10% (v/v) foetal bovine serum (FBS, containing up to 50 mg/ml serum proteins, Gibco), 50 µg/ml neomycin, 69.2 µg/ml penicillin G and, 100 µg/ml streptomycin. For expression of proteins in serum-free medium, High Five™ cells were grown in Express Five® medium (Gibco) with 10 µg/ml gentamycin and 18 mM L-glutamine.

3.2.2 Generation of recombinant baculovirus.

Recombinant baculoviruses were generated using the Bac-to-Bac® Baculovirus Expression System (Invitrogen, A.5). Briefly, recombinant pFastBac™Dual constructs were used individually to transform competent *Escherichia coli* DH10Bac™ cells (Invitrogen) by standard heat shock transformation (Sambrook *et al.*, 2001) to allow transposition of *gag* and chimaeric *gag* DNA constructs into the resident bacmid. The recombinant bacmid DNA was extracted by a modified alkaline lysis method according to the Bac-to-Bac® protocol.

Sf21 cells (1×10^6) were seeded in 2 ml supplemented medium (3.2.1) into sterile 35 mm tissue culture wells and transfected with 10 µl of a 1:1 ratio of recombinant bacmid mini-prep DNA and Cellfectin™ (Invitrogen) according to the Bac-to-Bac® protocol. The rAcNPV stocks were amplified in 10 ml volumes over 3 passages (according to the Bac-to-Bac® protocol), stored at 4°C, and subsequently used to infect Sf9, Sf21 and High Five™ cells for protein expression in monolayer culture.

3.2.3 Plaque assays.

Sf9 cells (1.5×10^6) were seeded in 2 ml supplemented medium (3.2.1) into sterile 35 mm tissue culture wells and allowed to attach for 1 hr with gentle horizontal agitation (to ensure an even monolayer, confirmed by microscopy). The culture medium was removed and cells were infected (in triplicate wells) with 1 ml of a log₁₀ serial dilution series of rAcNPV stock, from 10⁻⁵ to 10⁻⁸ dilution.

After a 2 hr incubation period at 27°C, infectant was removed and cells were overlaid with 3 ml Plaquing mix (Graces Insect Plaquing medium (Gibco), 4% SeaPlaque Agarose (Adcock Ingram) and water in a 2:1:1 ratio). Cells were stained 4 days post infection (dpi) with 100 µg/ml Neutral red dye (Sigma) for 5 hr, and incubated for another day at 27°C before plaques were counted.

3.2.4 Protein samples and SDS-PAGE.

Sf21 and High FiveTM cells (1×10^6) were seeded in 2 ml supplemented medium (3.2.1) in 35 mm tissue culture wells, infected with 20µl rAcNPV stock and protein samples were collected at 3 dpi. Infected culture supernatant was removed from the tissue culture well, cleared by centrifugation at 2600 x g in a benchtop centrifuge for 10 min and diluted in a quarter volume of 5x sample loading buffer (A.2). The infected monolayer cells were washed twice in 1 ml phosphate buffered saline (PBS, A.2) and resuspended in 1 ml PBS. Cells were lysed by the addition of loading buffer and heating at 90°C for 5 min, as reported previously (Luo *et al.*, 1992). The samples were then either stored at -20°C for later use or separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% denaturing gels (Sambrook *et al.*, 2001). The positive control proteins used in SDS-PAGE are listed in Table 3.1, and a pre-stained molecular weight (MW) marker (PageRulerTM, Fermentas) was used on all blots.

3.2.5 Western blotting.

For western blotting, proteins were transferred from the SDS-PAGE gels onto nitrocellulose membranes (NitroBind, Osmonics Inc.) in transfer buffer (A.2) using a Trans Blot[®] semi-dry transfer cell (Bio-Rad) at 15 V for 2 hr. Membranes were then pre-incubated in blocking buffer (A.2) overnight and probed with appropriate primary antibodies (Table 3.2) for 2 hr in blocking buffer at ambient temperature. Following 3 sets of washes in washing buffer (A.2) for 10 min each, blots were hybridised with the appropriate secondary antibody (Table 3.3) for 1 hr in blocking buffer at ambient temperature. After a second set of washes, blots were developed with Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche).

TABLE 3.1. Positive control proteins used in SDS-PAGE and western blotting

| Protein | Details | Size (kDa) | Designation | Source |
|---------|--|------------|-------------|--------------------------|
| Gag | HIV-1 BH10 Pr55 ^{gag} | 55 | 303058 | Commercial ¹ |
| RT | HIV-1 _{HXB2} Reverse Transcriptase dimer (<i>E. coli</i>) | 51, 66 | 2897 | S. Le Grice ² |
| Nef | Recombinant HIV-1 Nef (<i>E. coli</i>) | 27 | EVA650 | V. Erfle ³ |

¹ Quality Biological, Inc, Gaithersburg, USA.

² NIH AIDS Research & Reference Reagent Program, McKesson BioServices Corporation, Germantown, USA.

³ National Institute for Biological Standards and Control (NIBSC), Centralised Facility for AIDS Reagents, Medical Research Council (MRC), United Kingdom (UK).

TABLE 3.2. Primary antibodies used in western blotting

| Antigen | Host | Dilution | Details | Designation | Source |
|---------|--------|----------|---|-------------|--------------------------|
| p24 | Rabbit | 1:2000 | Polyclonal antiserum to recombinant HIV-1 _{HXB2} p24 GST (<i>E. coli</i>). | ARP432 | G. Reid ¹ |
| p24 | Mouse | 1:500 | Monoclonal antibody to HIV-1 p24/p55. Mapped to RMYSP | ARP319 | L. Maaheim ¹ |
| RT | Sheep | 1:2000 | Polyclonal antiserum to Recombinant HIV-1 LAV Reverse Transcriptase | ARP428 | M. Page ¹ |
| Nef | Mouse | 1:1000 | Monoclonal antibody (IgG1) to HIV-1 Nef. Mapped to VEEANK | 01-003 | FIT Biotech ² |
| SV5-Pk | Mouse | 1:1000 | Monoclonal antibody to the SV5-Pk tag. Mapped to IPNPLLGLD | EVA3066 | R. Randall ¹ |

¹ National Institute for Biological Standards and Control (NIBSC), Centralised Facility for AIDS Reagents, Medical Research Council (MRC), United Kingdom (UK).

² FIT Biotech Oyj Plc, Tampere Finland.

TABLE 3.3. Secondary antibodies used in western blotting

| Antigen | Host | Dilution | Details | Source |
|------------|------|----------|--|--------------------|
| Rabbit IgG | Goat | 1:5000 | Affinity purified alkaline phosphatase conjugate | Sigma ¹ |
| Sheep IgG | Goat | 1:5000 | Affinity purified alkaline phosphatase conjugate | Sigma ¹ |
| Mouse IgG | Goat | 1:5000 | Affinity purified alkaline phosphatase conjugate | Sigma ¹ |

¹ Sigma, Atlasville, South Africa

3.2.6 Indirect immunofluorescence (IIF)

Sf21 cells (1×10^6) were seeded in 2 ml supplemented medium (3.2.1) onto sterile 400 mm² glass cover slips in 35 mm tissue culture wells, left to settle for 30 mins, and infected with 20 μ l recombinant baculovirus stock. At 3 dpi, cells were fixed and permeabilised *in situ* with 100% methanol at -20°C for 20 min. The rest of the procedure was conducted at ambient temperature. Cover slips were washed (3 times for 10 min each) in PBS-IIF (A.2) and then incubated in blocking buffer (A.2) for 2 hr. After washing, cover slips were probed with the appropriate primary antibody (Table 3.4) in blocking buffer for 3 hr. Following 3 more washes (PBS-IIF), cover slips were reacted in the dark with fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (1:500, Sigma) in blocking buffer for 1 hr, and washed. Nuclei and cell membranes were stained for 1 hr in the dark with 5 μ g/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) and 0.01% Evans Blue (BDH Chemicals Ltd.) in wash buffer respectively, with washing in between. Cover slips were then mounted on glass slides in Gel Mount™ (Sigma), sealed, and stored in the dark at 4°C. Cellular fluorescence was viewed with a Zeiss AxioVert 200M fluorescent light microscope (Faculty of Health Sciences, University of Cape Town). Photographs were obtained using a Zeiss AxioCam MRm digital camera and AxioVision 4.0 software.

TABLE 3.4. Primary antibodies used in IIF

| Antigen | Host | Dilution | Details | Designation | Source |
|---------|-------|----------|--|-------------|--|
| RT | Mouse | 1:500 | Monoclonal antibody to HIV-1 RT. Mapped to PLTEEALELELAENREILKEPVHGVY | EVA3018 | D. Helland and A. Szilvay ¹ |
| Nef | Mouse | 1:500 | Monoclonal antibody (IgG ₁) to HIV-1 Nef. Mapped to VEEANK | 01-003 | FIT Biotech ² |
| SV5 | Mouse | 1:500 | Monoclonal antibody to the SV5-Pk tag. Mapped to IPNPLLGLD | EVA3066 | R. Randall ¹ |

¹ National Institute for Biological Standards and Control (NIBSC), Centralised Facility for AIDS Reagents, Medical Research Council (MRC), United Kingdom (UK).

² FIT Biotech Oyj Plc, Tampere Finland.

3.3 RESULTS

3.3.1 Plaque assays.

Plaque assays were used to determine the viral titre of rAcNPV stocks in the Bac-to-Bac[®] Baculovirus Expression System (A.5). The protocol was optimized as far as possible, and plaque counts from individual assays were made. However, in replicate assays at the same dilution, the number of plaques would often vary by more than 50-fold. For example, plaque counts from equal volumes of a 10^{-5} dilution of rAc-WTgagC stock would range from less than 5 to more than 250. Similar counts were recorded for rAc-HMgagC stocks. All attempts to develop a reproducible plaque assay were unsuccessful.

In the absence of accurate rAcNPV stock titres, the MOI could not be standardized in experimental sets. Therefore, quantitative analysis of protein expression between different constructs was not completed. To facilitate at least semi-quantitative comparisons, experimental sets of infections were standardized as much as possible. Essentially, equal volumes of bacmid DNA preparations were used in all the initial transfections and subsequent viral amplifications were conducted uniformly using the same insect cell starter cultures. An equal volume of each rAcNPV stock was then used for each infection within a single set of experiments.

3.3.2 Western blot analysis

Initially, all Gag-based constructs were expressed in Sf21 cell monolayers. However, the high concentration of serum proteins present in the medium obscured protein bands in the ~50-75 kDa range on western blots of culture supernatant samples. Therefore, all Gag-based constructs were also expressed in High Five[™] cell monolayers adapted to serum-free medium. This circumvented the problem of serum proteins in the culture supernatant samples, but protein yields were consistently lower compared to Sf21 cells (as estimated from band intensity on western blot). Therefore, both cell lines were used in the analysis of protein expression.

3.3.2.1 Gag control constructs

The five Gag control constructs (Fig. 2.10) each expressed an intracellular protein of the expected size (50 or 55 kDa) in Sf21 cells, which reacted with p24-specific antiserum (Fig. 3.1). There was no substantial difference in WTgagC and HMgagC band intensity, and analogous blots from infected High FiveTM cells were comparable (data not shown). Expression of the four humanized Gag control constructs in High FiveTM cells allowed for culture supernatant proteins to be observed (Fig. 3.2). As expected, bands in the culture supernatant corresponding to HMgagC and THMgagC reacted strongly with p24-specific antiserum. Although the HΔMgagC and THΔMgagC bands in the culture supernatant were less intense than the corresponding Myr⁺ analogues, they were still detectable with p24-specific antiserum (Fig. 3.2). Bands in the expected positions for p41 and p25/24 were evident in the culture supernatant for each Gag control construct (Fig. 3.2). Since bands at the expected p25 and p24 positions were not always clearly distinguishable, they are indicated here as p24 only.

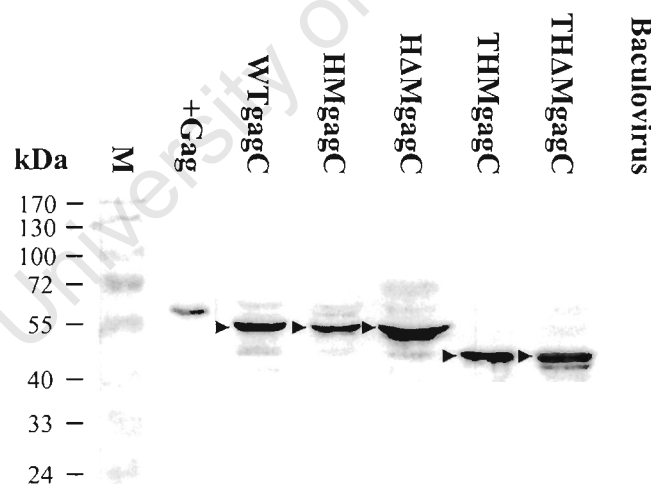


FIGURE 3.1. Expression of control Gag proteins in Sf21 cells.

Sf21 cells were infected with AcNPV (baculovirus) or rAcNPV expressing one of the 5 Gag control proteins indicated. Cells were harvested at 3 dpi and 10 μ l of cell lysate was loaded in each lane. Membranes were probed with p24-specific antiserum, +Gag = 50 ng. Arrowheads indicate the full-length and truncated Gag protein bands. M = MW marker

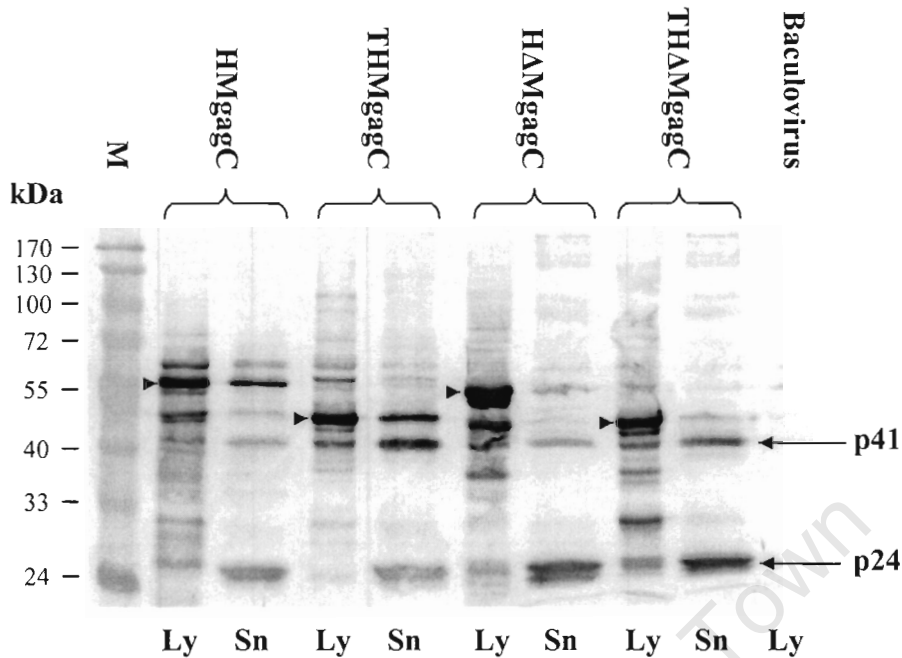


FIGURE 3.2. Comparison of Myr⁺ and Myr⁻ Gag expression in High FiveTM cell lysates and culture supernatants. High FiveTM cells were infected with rAcNPV expressing one of the 4 control Gag proteins indicated. Cells were harvested at 3 dpi and 10 μ l of cell lysate (Ly) or 30 μ l culture supernatant (Sn) was loaded in each lane. Membranes were probed with p24-specific antiserum. Arrowheads indicate the full-length and p6-truncated Gag protein bands. Arrows indicate supernatant bands corresponding to p41 and p24 positions. M = MW marker.

3.3.2.2 RTTN-derived constructs

Cell lysates

Each of the 10 RTTN-derived chimaeric constructs (Fig. 2.10) expressed a full-length intracellular protein of the expected size that was detected with p24-specific antiserum (Fig. 3.3A, B) and anti-p24 mAb (Fig. 3.4A, B) in High FiveTM and Sf21 cells. A number of other bands were detected with p24-specific antiserum, but for the fusions containing TN moieties (-3'TN, -TN and -RTTN) there were 1 or 2 prominent bands (Fig. 3.3A, B). Those in -3'TN and -RTTN fusions were also detectable with anti-p24 mAb (Fig. 3.4A, B). Detection with RT-specific antiserum confirmed that HMgag and THMgag-fusions with -3'RT, -RT and -RTTN carried RT protein (Fig. 3.5A). Similarly, probing with anti-Nef mAb (mapped to the C-terminal half of Nef) demonstrated that all fusions with -3'TN, -TN and -RTTN carried Nef protein (Fig. 3.5B). As Nef is C-terminal to the shuffled Tat in -TN and -RTTN fusions, the presence of Tat in these constructs was inferred from the positive reaction of the anti-Nef mAb with the TN-containing Gag fusion proteins, together with protein band sizing by western blot. The prominent Gag containing bands of lower MW for fusions with TN moieties (Fig. 3.3A,B & 3.4A,B) were not bound by anti-Nef mAb (Fig. 3.5B).

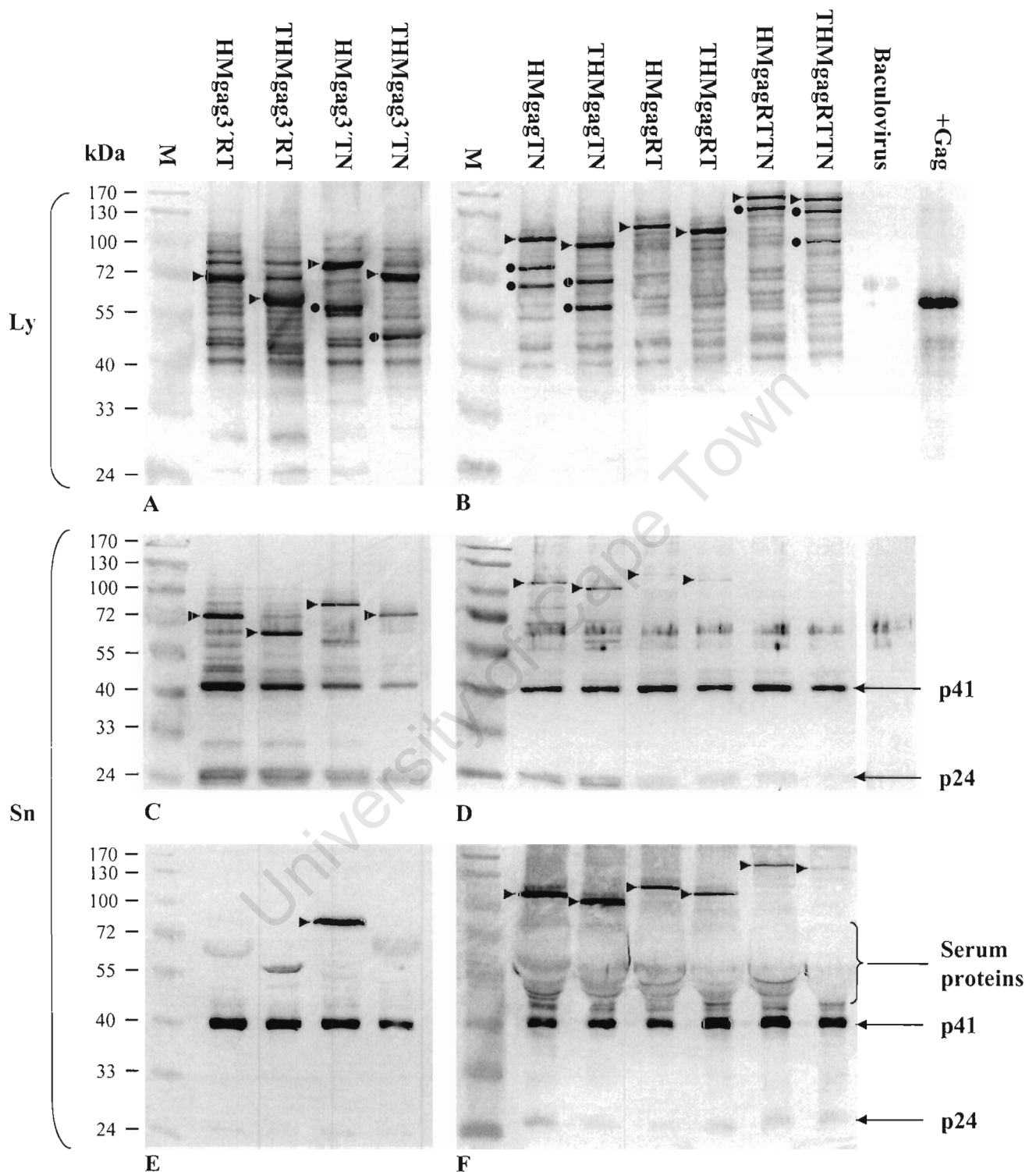


FIGURE 3.3. Expression of RTTN-derived chimaeric Gag proteins in insect cell lysates and culture supernatants. High Five™ (panels A-D) and Sf21 (panels E, F) cells were infected with rAcNPV expressing one of 10 RTTN-derived chimaeric Gag proteins. Cells were harvested at 3 dpi and 10 μ l of cell lysate (Ly) or 30 μ l of culture supernatant (Sn) was loaded in each lane. Membranes were probed with p24-specific antiserum, +Gag = 100 ng. Arrowheads indicate the full-length chimaeric Gag protein bands; dots indicate distinct bands of lower MW. Arrows indicate supernatant bands corresponding to p41 and p24. The blotting disruption due to serum proteins is indicated for Sf21 supernatants. M = MW marker.

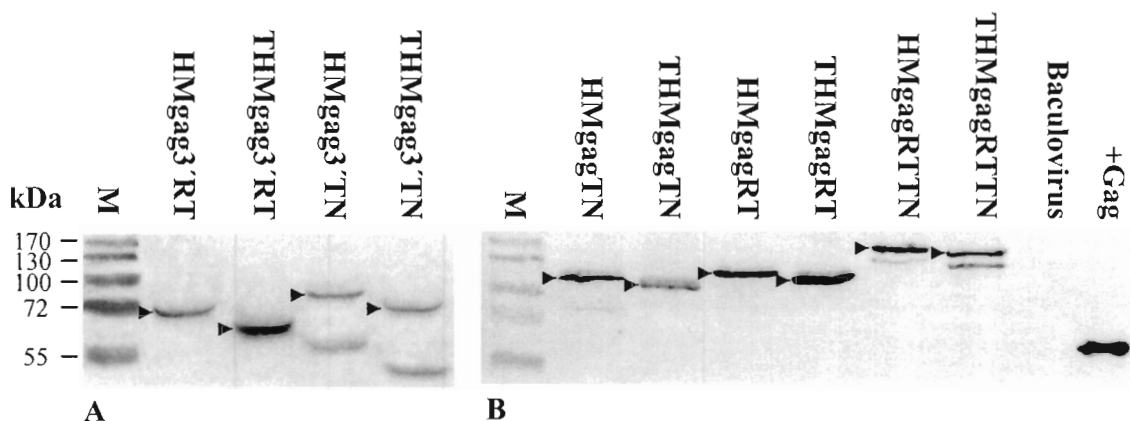


FIGURE 3.4. **Detection of RTTN-derived chimaeric Gag proteins in insect cell lysates with anti-p24 mAb.** High FiveTM (A) and Sf21 (B) cells were infected with rAcNPV expressing one of the 10 RTTN-derived chimaeric Gag proteins. Cells were harvested at 3 dpi and 30 μ l of cell lysate was loaded in each lane. Membranes were probed with anti-p24 mAb, +Gag = 100 ng. Arrowheads indicate the full-length chimaeric Gag protein bands. M = MW marker.

Culture supernatant

In High FiveTM cells, all RTTN-derived chimaeras (except -RTTN fusions) were detected in the culture supernatant with p24-specific antiserum (Fig. 3.3C, D), although the -RT fusion bands were only just visible (Fig. 3.3D). In Sf21 cells, 7 of the 10 RTTN-derived chimaeric proteins were detected in the culture supernatant with p24-specific antiserum (Fig. 3.3E, F).

The three that could not be detected (HMgag3'RT, THMgag3'RT and THMgag3'TN) as well as the prominent bands of lower MW for the fusions with TN moieties are within the MW range that was obscured by the medium serum proteins. The cleavage products p41, and to a lesser extent p24, were also evident in culture supernatants. For both cell lines, the intensity of the chimaeric protein bands relative to p41 appears to decrease with an increase in MW of the protein chimaera (e.g. Fig. 3.3F). Under improved expression conditions, that were not part of these controlled experimental sets, culture supernatant levels of Sf21 expressed -RTTN fusions were higher than those presented in Figure 3.3F, as estimated from band intensity (data not shown).

Four of the culture supernatant samples from Sf21 cells (Fig. 3.3C) and 6 from High FiveTM cells (Fig. 3.3F) were also run on gels with the corresponding cell lysates in adjacent lanes, confirming that it is the same band present in both samples (data not shown).

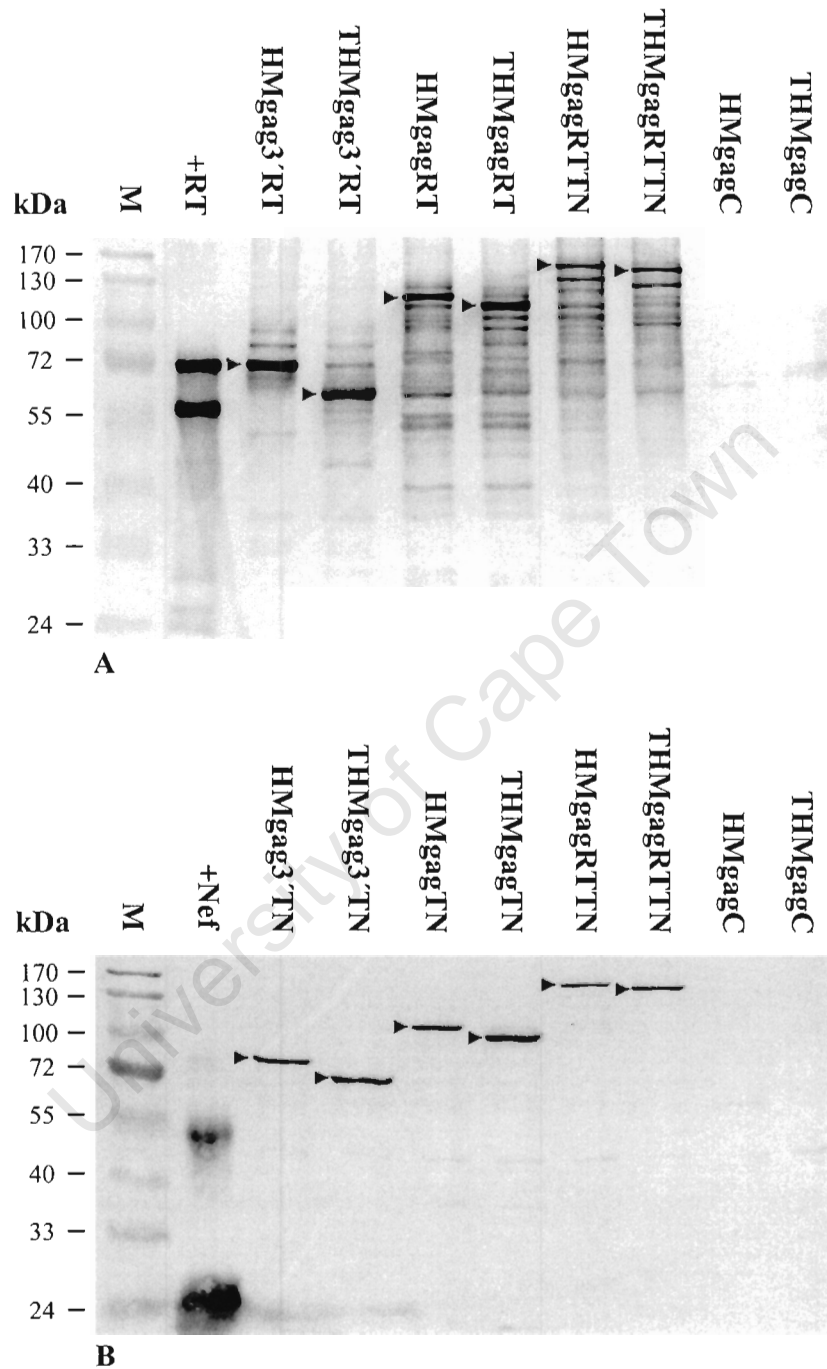


FIGURE 3.5. Detection of RTTN-derived chimaeric Gag proteins in High FiveTM cells with anti-RT antiserum and anti-Nef mAb. High FiveTM cells were infected with rAcNPV expressing one of 10 RTTN-derived chimaeric Gag proteins. Cells were harvested at 3 dpi and 10 μ l (A) or 30 μ l (B) of cell lysate was loaded in each lane. Membranes were probed with RT-specific antiserum (A) and anti-Nef mAb (B). Control proteins: +RT (100 ng) is a heterodimer, +Nef (50 ng) is partly present as a homodimer (~50 kDa). Arrowheads indicate the full-length chimaeric Gag protein bands. M = MW marker.

3.3.2.3 Gag-PE constructs

Cell lysates

Both Gag-PE constructs expressed an intracellular protein of the expected MW that was detected with p24-specific antiserum (Fig. 3.6A). These Gag-PE proteins each carry the SV5 murine mAb epitope (IPNPLLGLD) on their C-termini, and reactivity with anti-SV5-Pk mAb confirmed that these fusions contained full-length PE (Fig. 3.6B).

Culture supernatants

Unlike the shortest two RTTN-derived fusions (Gag-3'RT and Gag-3'TN, Fig. 3.3C), the Gag-PE proteins could not be detected in High FiveTM cell culture supernatant with p24-specific antiserum (Fig. 3.6C), but p41 and p24 bands were visible. Gag-PE proteins were not detectable in Sf21 cell culture supernatants (data not shown), but these proteins are within the MW range that is obscured on western blots by serum proteins in the culture medium.

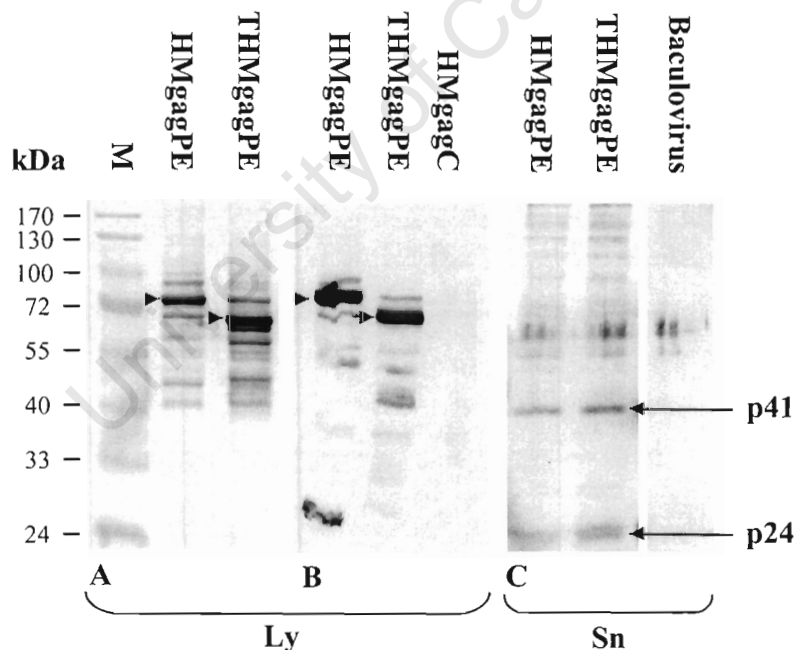


FIGURE 3.6. Expression of Gag-PE proteins in High FiveTM cell lysates and supernatants. High FiveTM cells were infected with rAcNPV expressing one of the Gag-PE proteins. Cells were harvested at 3 dpi and 10 μ l (A), 5 μ l (B) of cell lysate (Ly) or 30 μ l culture supernatant (Sn) was loaded in each lane. Membranes were probed with p24-specific antiserum (A, C), and anti-SV5-Pk mAb (B). Arrowheads indicate the full-length chimaeric Gag protein bands. Arrows indicate supernatant bands corresponding to p41 and p24. Controls: +Gag and baculovirus (Ly) reactivity with p24-specific antiserum as for Figure 3.3. The SV5-Pk tag (IPNPLLGLD) is located at the C-terminus of the Gag-PE proteins. M = MW marker.

3.3.3 Indirect immunofluorescence

The cellular localization of Gag-based proteins in Sf21 cells was investigated by indirect immunofluorescence (IIF), to ascertain whether these proteins were localized at the cell membrane.

3.3.3.1 Gag control constructs

Cells infected with one of the 4 humanized Gag control constructs (HMgagC, THMgagC, HΔMgagC and THΔMgagC) or an AcNPV negative control were probed 3 dpi with one of 5 different Gag antibodies (E.1) and a suitable fluorescent conjugate. In each case IIF staining was either absent or non-specific (E.1).

3.3.3.2 RTTN-derived constructs

With the exception of the -3'RT fusions, all RTTN-derived protein chimeras were visualized in Sf21 cells by IIF staining of the fusion component within the chimaeric protein (Fig. 3.7-3.9). The Gag-3'RT constructs contain the C-terminal 113 aa of RT, and an anti-RT mAb (ARP384, E.1) mapped to the C-terminal 100 aa of RT was available. Although Gag-3'RT and Gag-RT were detected with ARP384 by western blot, IIF staining was absent in cells expressing these proteins that were probed with ARP384 (data not shown). Anti-Nef mAb was used to detect -3'TN (Fig. 3.7A, B), -TN (Fig. 3.8A, B) and -RTTN (Fig. 3.8C, D) fusions, while anti-RT mAb (EVA3018, 3.2.6) was used to detect Gag-RT (Fig. 3.9A, B). For each construct, proteins could be detected in the cytoplasm within some cells (e.g. Fig. 3.9A), and DAPI staining of cell nuclei (data not shown) confirmed these proteins were external to the nucleus. However, in all cells that showed IIF staining (including those with additional IIF staining in the cytoplasm), proteins were detected as a distinct layer (Fig. 3.7-3.9, white arrowheads) or in concentrated sections (e.g. Fig. 3.8C) at the cell periphery. Staining of cellular membranes with Evans Blue dye confirmed that this green fluorescent layer was not within the cytoplasm, and provides evidence for the co-localization of these proteins with the cell membrane itself. In addition, patches of protein localized to the exterior cell surface were visualized at appropriate focal planes (e.g. Fig. 3.7B & 3.9B, yellow arrowheads).

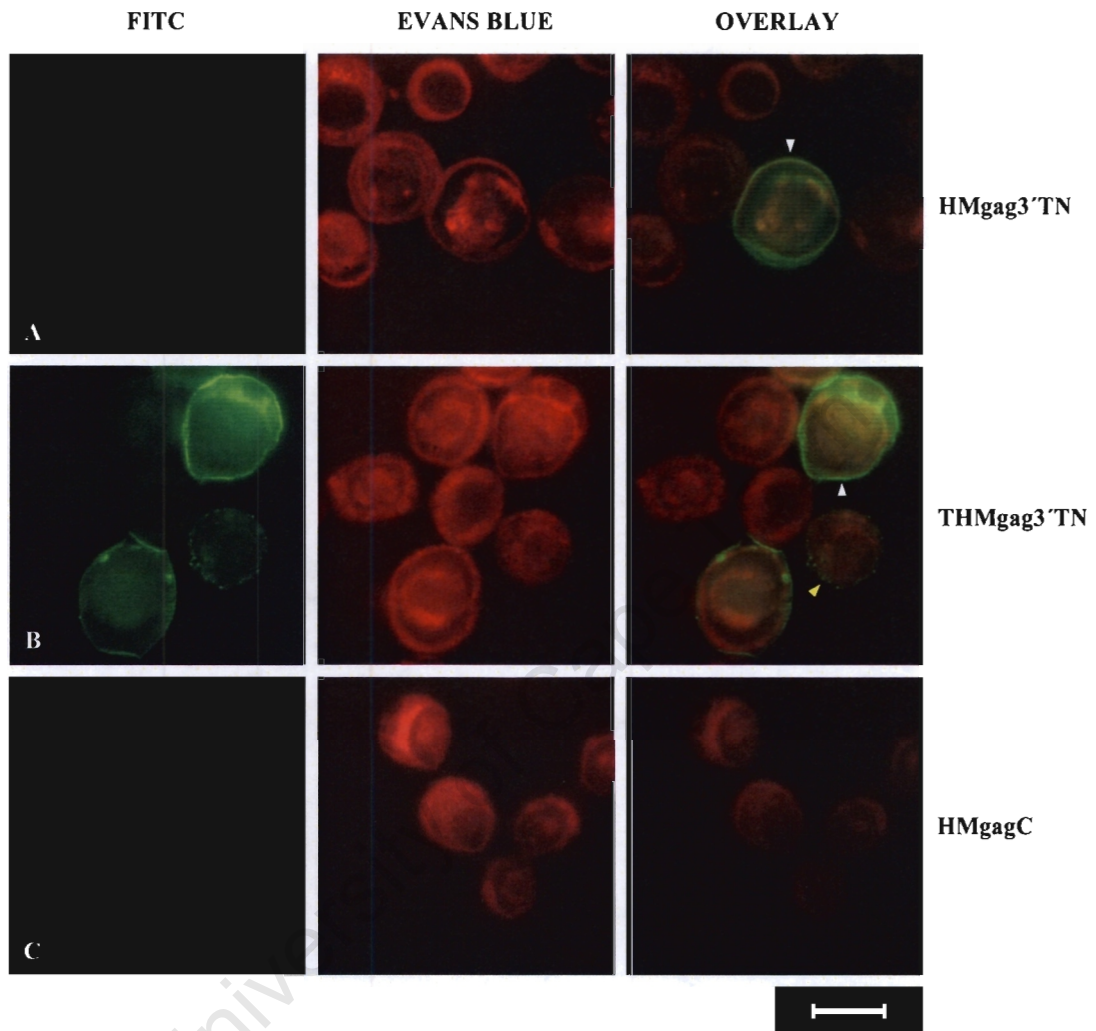


FIGURE 3.7. Evidence for the localization of Gag-3'TN chimaeras to the Sf21 cell membrane. Sf21 cells infected with rAcNPV (expressing the indicated proteins) were labeled with anti-Nef mAb and detected with secondary FITC-conjugated anti-mouse IgG₁ (green fluorescence). Cellular membranes were stained with Evans Blue dye (red fluorescence) and the images were merged in the overlay panels. Negative control: rAc-HMgagC infected cells. White arrowheads indicate IIF staining at the cell membrane while the yellow arrowhead indicates an example of isolated fluorescent IIF staining patches at the cell surface. Bar = 20 μ m, all photographs at the same scale.

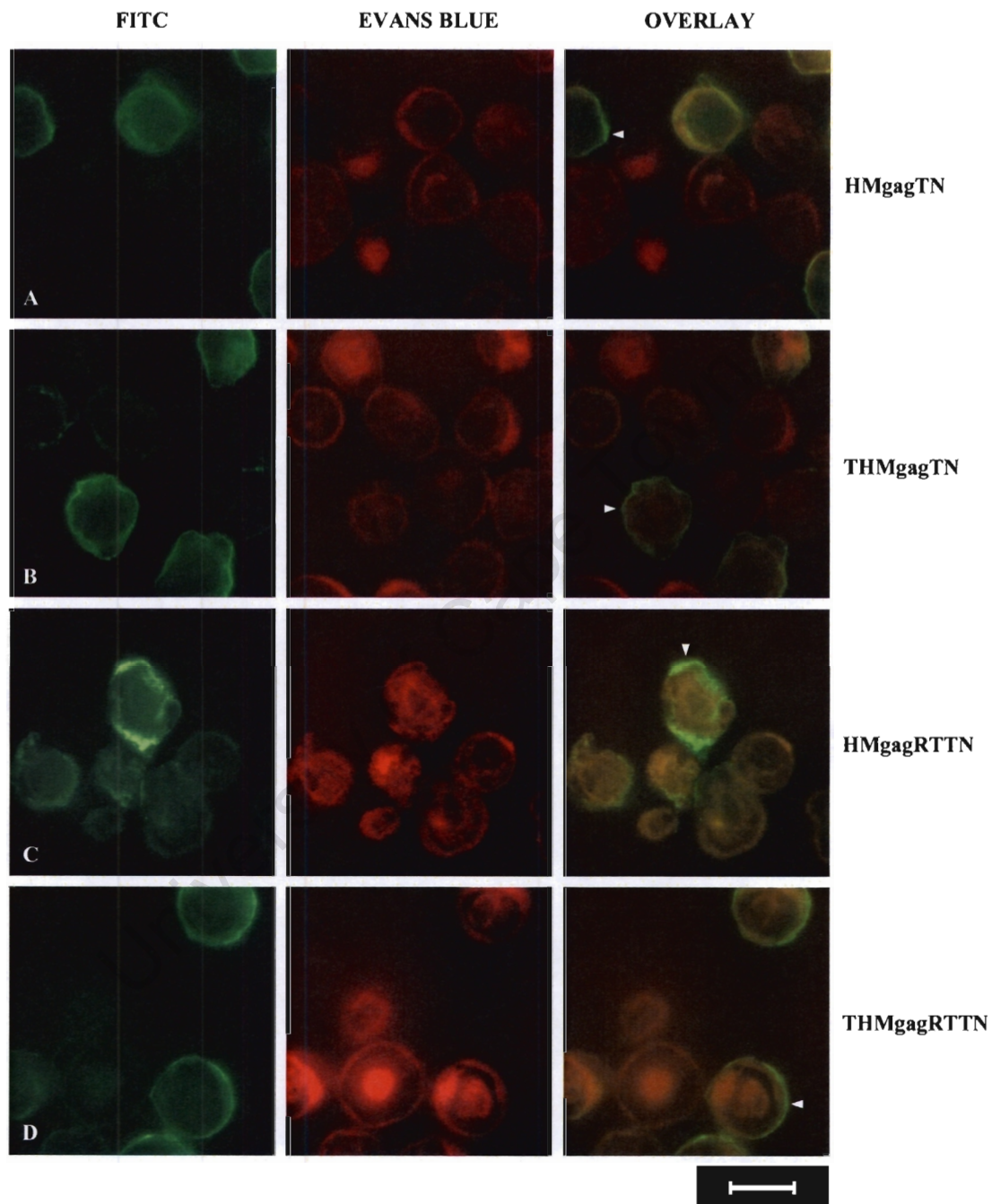


FIGURE 3.8. Evidence for the localization of Gag-TN and Gag-RTTN chimaeras to the Sf21 cell membrane. Sf21 cells infected with rAcNPV (expressing the indicated proteins) were labeled with anti-Nef mAb and detected with secondary FITC-conjugated anti-mouse IgG₁ (green fluorescence). Cellular membranes were stained with Evans Blue dye (red fluorescence) and the images were merged in the overlay panels. Negative control (rAc-HMgagC infected cells) as for Figure 3.7. White arrowheads indicate IIF staining at the cell membrane. Bar = 20 μ m, all photographs at the same scale.

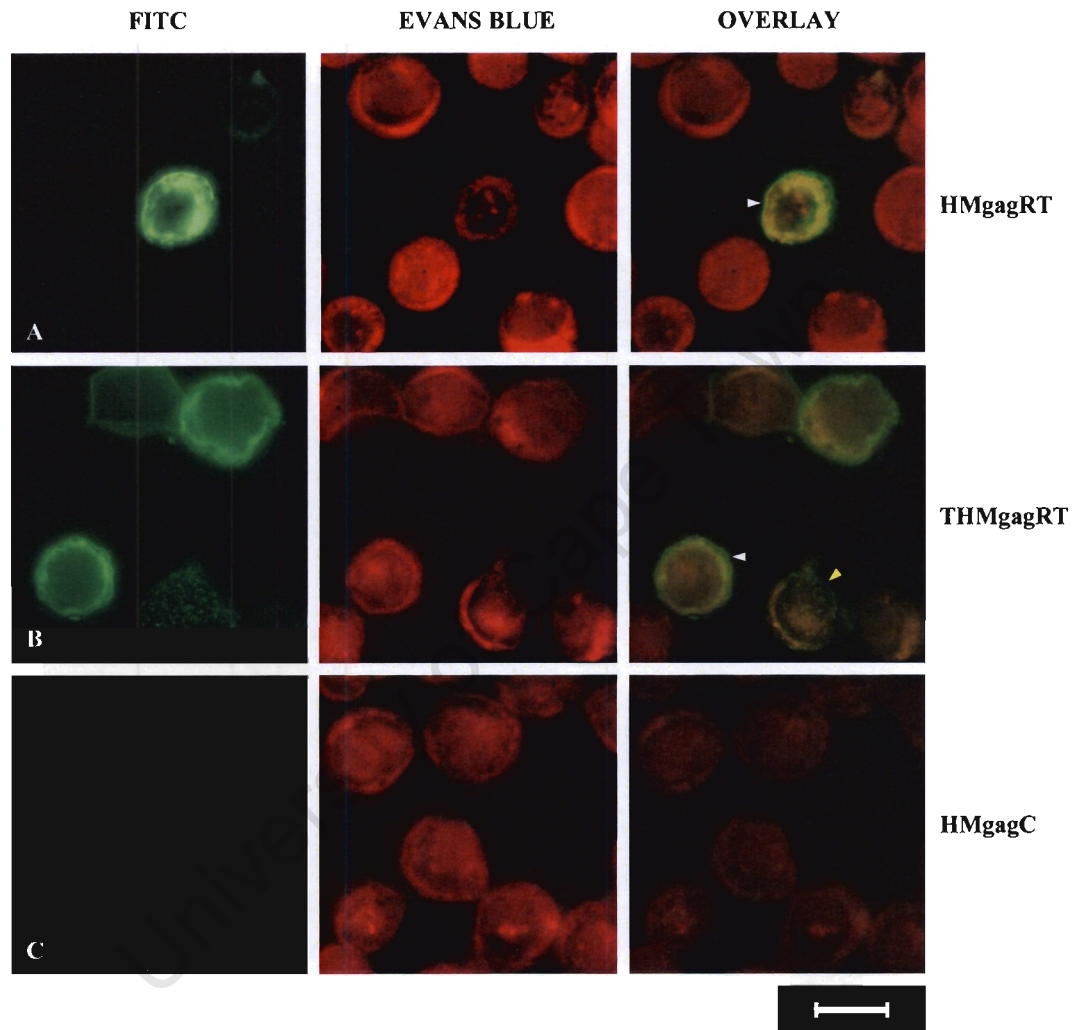


FIGURE 3.9. Evidence for the localization of Gag-RT chimaeras to the Sf21 cell membrane. Sf21 cells infected with rAcNPV (expressing the indicated proteins) were labeled with anti-RT mAb and detected with secondary FITC-conjugated anti-mouse IgG (green fluorescence). Cellular membranes were stained with Evans Blue dye (red fluorescence) and the images were merged in the overlay panels. Negative control: rAc-HMgagC infected cells. White arrowheads indicate IIF staining at the cell membrane while the yellow arrowhead shows an example of isolated fluorescent IIF staining patches at the cell surface. Bar = 20 μ m, all photographs at the same scale.

3.3.3.3 Gag-PE constructs

The Gag-PE proteins were detected in Sf21 cells probed with anti-SV5-Pk mAb by IIF staining (Fig. 3.10A, B). In both cases, protein was detected throughout the cytoplasm, but not in the nucleus (no co-localization of IIF staining with DAPI stained nuclei, data not shown). In contrast to the RTTN-derived Gag chimaeras, no distinct protein concentration was visible at the cell periphery. However, since the IIF staining of the cytoplasmic proteins extended completely to the cell boundary, some membrane localization could have occurred. However, these data were inconclusive in demonstrating membrane localization of Gag-PE chimaeras.

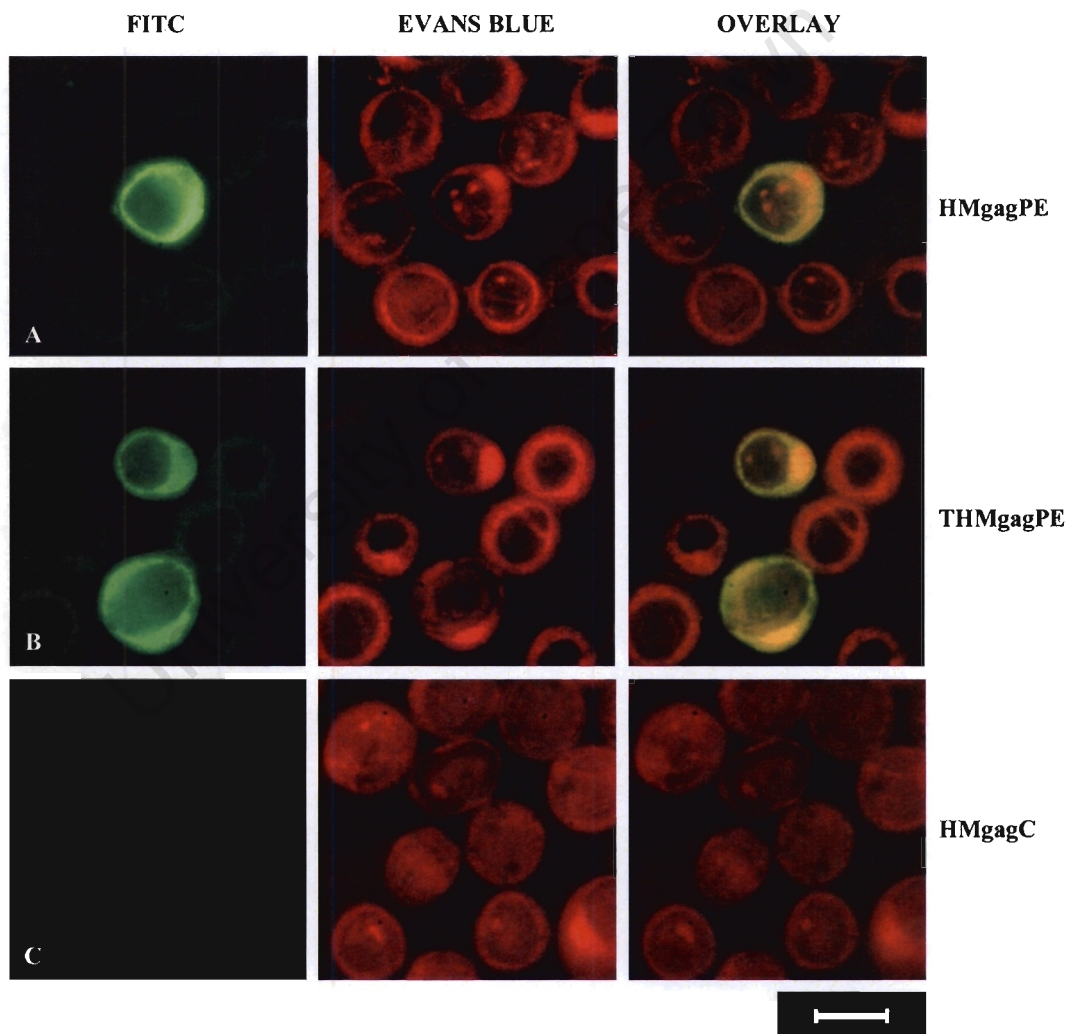


FIGURE 3.10. Localization of Gag-PE chimaeras within Sf21 cells.

Sf21 cells infected with rAcNPV (expressing the indicated Gag-PE fusions) were labeled with anti-SV5-Pk mAb and detected with secondary FITC-conjugated anti-mouse IgG (green fluorescence). Cellular membranes were stained with Evans Blue dye (red fluorescence) and the images were merged in the overlay panels. Negative control: rAc-HMgagC infected cells. Bar = 20 μ m, all photographs at the same scale.

3.4 DISCUSSION

In an attempt to determine the viral titre of each rAcNPV stock, plaque assays were conducted. Plaque counts from rAc-WTgagC and rAc-HMgagC stocks indicated that accurate titre determinations could not be obtained with this method under the conditions used, and consequently the MOI could not be set. Although infection conditions were standardized as far as possible (3.3.1), without a standardized MOI the differences in protein expression could not be quantified. Therefore, any change in Gag expression level due to human codon optimization, myristylation, p6-truncation or the fusion component could not be evaluated. To conduct such studies on protein expression in the future, either a reproducible plaque assay for our group's laboratory conditions will need to be developed, or another method to titre baculoviral stock (e.g. TCID₅₀ assay) will need to be pursued. However, the main objectives of this section of the study were to confirm expression of the correct proteins, determine whether full-length chimaeric proteins were present in the culture supernatant and examine intracellular protein localization. None of these analyses were precluded by the infection conditions used, and the relative levels of intra- and extracellular proteins for each construct could still be observed by western blot.

There was an obvious difference in protein composition between cell lysate and culture supernatant for each of the four humanized Gag controls (Fig. 3.2). This indicated that the full-length proteins (50 or 55 kDa), p41 and p24 were not present in the culture supernatant due to cell lysis alone. Precursor proteins may be released from the cell and then partly cleaved into p41, p24 and other breakdown products, or this partial cleavage may occur intracellularly prior to protein release. These explanations are not mutually exclusive and evidence in the literature (Wagner *et al.*, 1992; Cruz *et al.*, 1999) suggests that both processes do occur. The predominance of Myr⁺ Gag (HMgagC and THMgagC) in the culture supernatant (relative to p41 and p24) compared to Myr⁻ Gag (HAMgagC and THAMgagC) may be explained by the finding that Myr⁺ Gag is released from rAcNPV infected insect cells as VLPs and soluble protein, whereas Myr⁻ Gag is only released in the soluble form (Royer *et al.*, 1992), but this would require further investigation.

The expression of each of the 10 RTTN-derived Gag chimaeras (Fig. 2.10) was confirmed by western blot of cell lysates, where a band of the expected MW was detected with p24-specific antiserum and either anti-Nef mAb or RT-specific antiserum.

Cell lysates of cells expressing fusions containing TN moieties (-3TN, -TN and -RTTN) had 1 or 2 distinct bands of lower MW (Fig. 3.3A, B). Since these bands did not react with anti-Nef mAb (Fig. 3.5B), these products may have resulted from specific proteolytic cleavage within the TN moiety, or from premature termination of translation at a defined point in the C-terminal fusion.

Each RTTN-derived Gag chimaera was shown to be present in the culture supernatant (for one or both cell types), and for the same reasons as for the Gag controls, these proteins were probably selectively released from the cell. It appears that smaller fusions may be released more efficiently. For example, the difference in band intensity for HMgag3^{RT} between the cell lysate and culture supernatant (Fig. 3.3A, C) is qualitatively less than for HMgagRT (Fig. 3.3B, D). Alternatively, larger fusions may be degraded at a higher rate in the culture supernatant. When p41 is expressed alone, it is efficiently released from insect cells (Royer *et al.*, 1992), therefore the p41 present in the culture supernatant (Fig. 3.2 & 3.3C-F) may originate from intra- or extracellular cleavage of chimaera Gag precursors, or from a premature translation stop signal (3.1). Chimaeras with longer fusions showed qualitatively less full-length chimaera relative to p41 in the culture supernatant than shorter fusions (e.g. compare HMgagTN and HMgagRTTN lanes, Fig. 3.3F). However, from these data it is not clear whether these differences were as a result of lower synthesis, lower release or higher cleavage levels of the larger fusion proteins.

Unlike the RTTN-derived chimaeras, neither of the Gag-PE constructs was detected in the culture supernatant with p24-specific antiserum (Fig. 3.6B). Either the -PE fusions were not released from the cell, or the levels were below the detection limit. Complete degradation of HMgagPE or THMgagPE in the culture supernatant is unlikely since p41 had not been fully processed.

The cellular localization of Gag control proteins could not be visualized by IIF with the five anti-Gag antibodies available in this study. Therefore, the localization of chimaeric Gag proteins within the cell was evaluated without comparison to positive controls for membrane localization (HMgagC and THMgagC). As such, the distinct protein layers (or concentrations) at the cell periphery for the -3TN, -TN, -RT and -RTTN fusions must be regarded as supportive rather than direct evidence for membrane localization. Moreover, the distinct patches of protein detected on the exterior cell surface (e.g. Fig. 3.9B), are only suggestive of budded particle formation without

a comparative IIF stained positive VLP control. No results were obtained for IIF staining of -3'RT fusions, but the available mAb (ARP384) to this portion of RT was unable to show IIF staining of cells expressing -RT fusions either. Since both -3'RT and -RT fusions were detected by ARP384 in western blot, the epitope recognized by the mAb is probably not accessible to antibody binding in the native chimaeric proteins. IIF staining results for cells expressing -PE fusions were inconclusive regarding membrane localization, but the even distribution of Gag-PE chimaeras in the cytoplasm suggested that even if membrane targeting did occur for these proteins, it was not efficient.

The movement of RTTN-derived fusion proteins into the culture supernatant, in conjunction with the apparent localization of these chimaeras to the cell membrane (not demonstrated for the -3'RT fusions), provided supportive evidence for VLP formation. Yet the release of these constructs from the cell as soluble proteins, or as other budding structures via the cell membrane cannot be excluded.

Based on the available literature on tolerable fusion size, VLP formation from the -3'RT, -PE and -3'TN constructs could reasonably be expected, but not for -TN, -RT and -RTTN fusions (Deml *et al.*, 2005). Therefore, only the 6 smallest chimaeric Gag constructs (Fig. 2.10) would be expected to be readily detectable at high levels in the culture supernatant. The results presented here are in contrast to this prediction, and supported further investigation of all 10 RTTN-derived constructs for VLP production. Given the anticipated immunogenicity of the Gag-PE chimaeras, and their unexpected expression properties described here, these constructs also warranted further characterization, to clarify why these chimaeras displayed different expression patterns compared to the -3'RT and -3'TN fusions.

CHAPTER 4

Characterization of chimaeric Gag particles

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4.1 INTRODUCTION

The potential use of Gag and chimaeric Gag VLPs as candidate HIV vaccines has led to the refinement of protocols to purify these particles from heterologous expression systems for biochemical and immunological analysis. VLPs produced in the baculovirus expression system are typically harvested from clarified culture supernatant by ultracentrifugation through a sucrose density gradient ranging from between 10% and 70% in PBS. Preparations of VLPs by these methods are stable and generally have a purity of greater than 80% (Deml *et al.*, 2005; Doan *et al.*, 2005). VLP identity, composition and morphology are typically investigated by western blotting and electron microscopy, and these techniques are suitable for determining whether chimaeric Gag proteins form type I VLPs.

4.1.1 Sucrose density gradient centrifugation

Pr55^{gag} VLPs have a sedimentation coefficient of ~600S (Svedberg units) in sucrose density gradients (Royer *et al.*, 1992) and a density of 1.15-1.20 g/ml (Wagner *et al.*, 1992). Type I chimaeric Gag-V3 and Gag-CD4BD VLPs also have densities in this range (Wagner *et al.*, 1996b; Wagner *et al.*, 1994a). Sucrose sedimentation analysis has shown that fractions in the gradient with the highest p24 content (antigenic peaks) overlap with the fractions containing Pr55^{gag} VLPs (Wagner *et al.*, 1992). The particle-containing fractions generally correspond to the ~40-50% sucrose region of the gradient, and the Gag particle band in this region can be visualized by white-light *trans*-illumination. Soluble proteins such as p41 and serum proteins in the culture medium have much lower sedimentation coefficients, and remain at the top of the gradient (Royer *et al.*, 1992). A disadvantage of these sucrose density gradient centrifugation strategies is that baculovirus cannot be completely separated from the VLPs produced in the baculovirus system. Although this is undesirable for clinical VLP preparations, these contaminating baculoviruses have been implicated in enhancing immune responses by acting as danger signals to stimulate the innate immune system (1.4.2).

4.1.2 Transmission electron microscopy (TEM)

The two broad applications of TEM in this context are to visualize particles in VLP preparations and to examine budding and internal VLP structure in ultrathin sections. Pr55^{gag} VLPs produced by rAcNPV in insect cells were indistinguishable from those produced in mammalian cells by recombinant vaccinia virus (Wagner *et al.*, 1992), and were on average 100-120 nm across

(Gheysen *et al.*, 1989; Jaffray *et al.*, 2004). Larger particles (160 nm) and oligomeric structures resulting from incompletely dissociated complexes or fused particles were also evident (Wagner *et al.*, 1992). Type I VLPs have shown larger particles with less homogenous morphology (1.6.3.2). Immunoelectron microscopy (IEM) has been used to confirm the presence of Gag and fusion components within type I in-frame VLPs by immunogold labeling of ultrathin sections.

4.1.3 Chapter aims

To ascertain which chimaeric Gag constructs formed VLPs, the main aims of the work reported in this chapter were to:

1. Perform sucrose density gradient centrifugation analysis on the culture supernatant of rAcNPV infected insect cells to partly purify any VLPs or budded particles produced.
2. Analyze the protein content of the VLP preparations by western blot and characterize any VLPs present in the preparations by TEM.
3. Analyze the budding and internal ultrastructure of VLPs in ultrathin sections by TEM.
4. Confirm the presence of the fusion components of the chimaeric Gag proteins within VLPs by IEM.

4.2 MATERIALS AND METHODS

4.2.1 Insect cell culture

Sf21 cells were cultured as a monolayer in supplemented medium (3.2.1) and 10 ml volumes were infected with 100 μ l rAcNPV stock. For suspension culture of Sf9 cells, 500 ml glass flasks were prepared by coating with Repelcote (VWR International Ltd), washing, autoclaving and baking at 200°C for 2 hr. Sf9 cells were grown in supplemented medium with the addition of 0.1% Pluronic[®] F-68 (a surfactant to decrease membrane shearing in suspension culture, Sigma). Culture flasks were incubated at 27°C in a rotary shaker at 120 rpm.

4.2.2 Budded particle purification methods

4.2.2.1 Sucrose cushion centrifugation (SCC)

Culture supernatant (20 ml) from pooled Sf21 monolayer infections was collected 3 dpi and cleared by centrifugation at 3800 x g in a Beckman JA-14 rotor for 10 min at 25°C. Clarified supernatant was layered onto a sucrose cushion (7 ml 30% sucrose above 2 ml 70% sucrose in PBS in Ultra-Clear[™] tubes, Beckman) and centrifuged at 120 000 x g in a Beckman SW 28 rotor

for 90 min at 4°C. The particulate material at the interface between the sucrose concentrations was syringe-extracted (by side puncturing tube), resuspended in 10 ml PBS, and centrifuged at 110 000 x g for 30 min in a Beckman SW 55 Ti rotor at 4°C. Pelleted material was resuspended in 500 µl PBS with Complete Protease Inhibitor Cocktail[®] (Roche), and stored at -20°C.

4.2.2.2 Sucrose cushion and density gradient centrifugation (SXC)

Alternatively, particulate material in 40 ml infected Sf21 culture supernatant extracted from a sucrose cushion, was loaded onto a continuous 30-70% sucrose density gradient and centrifuged in a Beckman SW 28 rotor at 120 000 x g for 90 min at 4°C. The particle-containing band was visualized and syringe-extracted under white-light *trans*-illumination and then pelleted as described (4.2.2.1).

4.2.2.3 Sucrose density gradient centrifugation (SGC)

Culture supernatant from 60ml Sf9 suspension culture infected with 600µl of rAcNPV stock was harvested at 3 dpi and cleared by centrifugation at 3800 x g in a Beckman JA-14 rotor for 10 min at 25°C. Clarified supernatant was centrifuged in a Beckman type 35 rotor at 105 000 x g for 3 hr at 4°C. The pellet was resuspended in 1 ml PBS and sedimented through a continuous 30-70% sucrose density gradient at 120 000 x g in a Beckman SW 28 rotor for 2 hr at 4°C. The VLP-containing band was syringe-extracted under white-light *trans*-illumination, resuspended in 40 ml PBS, and re-centrifuged at 120 000 x g for 1 hr. Purified VLPs were resuspended in 500 µl PBS with Complete Protease Inhibitor Cocktail[®] (Roche), and stored at -20°C.

4.2.3 Western blotting

Samples from SCC, SXC and SGC preparations were diluted in 5x sample loading buffer (A.2), separated by SDS-PAGE and analyzed by western blot (3.2.5).

4.2.4 Transmission electron microscopy (TEM)

4.2.4.1 Adsorbed particle TEM (apTEM)

SCC, SXC and SGC sample preparations were adsorbed onto carbon coated copper grids for 20 min at ambient temperature, rinsed twice in distilled, de-ionized water (ddH₂O) and stained with 2% (w/v) uranyl acetate for 2 min. Grids were then re-rinsed with ddH₂O and air dried.

4.2.4.2 Thin section TEM (tsTEM)

Sample preparation

Sf21 cells (1×10^6) were seeded in 2 ml supplemented medium into 35 mm tissue culture wells and infected with 20 μ l rAcNPV stock. At 3 dpi cells were fixed overnight *in situ* with 2.5% (w/v) glutaraldehyde in PBS at 4°C in the dark. Fixed cells were gently resuspended in 1 ml PBS and pelleted at 2600 x g in a benchtop centrifuge, washed twice in PBS and resuspended in 2% (w/v) low melting point agarose in PBS at 37°C. Samples were post-fixed with 1% (w/v) osmium tetroxide in PBS, washed twice in PBS and twice in ddH₂O, dehydrated through an ethanol series (30, 50, 70, 80, 90, 95, 100%, 5 min each) and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections, ~100 nm thick, were cut with a glass knife using a Leica Reichert Ultracuts microtome and mounted on copper grids. Sections were stained with 2% uranyl acetate for 10 min at ambient temperature, rinsed 5 times with ddH₂O (1 min each), post-stained with Reynold's lead citrate (Reynolds, 1963) for 10 min, re-rinsed with ddH₂O and air dried.

4.2.4.3 Immunoelectron microscopy (IEM)

Unstained ultrathin sections, mounted on duplicate Formvar-coated nickel grids, were floated section side downward on PBS containing 1% (w/v) bovine serum albumen (BSA) for 5 min, then on PBS containing 0.02 M glycine for 3 min, and subsequently washed twice in PBS-BSA for 1 min. Duplicate grids were floated overnight at ambient temperature on p24-specific antiserum (Table 3.1, 1:100 dilution in PBS-BSA), washed 5 times in PBS-BSA with 0.1% (v/v) Tween 20 (PBS-BSA-T) and 3 times in PBS-BSA. Grids were then floated on anti-rabbit 10 nm colloidal gold conjugate (Sigma, 1:50 dilution in PBS-BSA) for 2 h at ambient temperature and washed as above for primary antiserum. Conjugate labeled complexes were fixed in 1% glutaraldehyde in PBS for 3 min, washed 5 times with ddH₂O, stained in 2% uranyl acetate for 10 min, washed 5 times with ddH₂O and air dried.

All apTEM, tsTEM and IEM samples were visualized with a LEO 912 transmission electron microscope (EM Unit, University of Cape Town).

4.3 RESULTS

Two variations of a VLP purification strategy (Nermut *et al.*, 1994), SCC and SXC, were tested for the purification of Gag and chimaeric Gag particles expressed in rAcNPV infected insect cells. The resultant preparations were examined by western blot and apTEM, but results were inconclusive for VLP formation. Therefore, tsTEM and IEM were conducted, and larger scale VLP purifications were carried out using SGC.

4.3.1 Purifying particles from Sf21 culture supernatants

4.3.1.1 SCC of Sf21 culture supernatant

Initially a sucrose cushion was used to purify particles from 20 ml Sf21 culture supernatant. WTgagC and HMgagC SCC samples were comparable by western blot (Fig. 4.1A), as expected from the cell lysate expression profiles (Fig 3.1). In THMgagC SCC samples there was a protein band of ~115 kDa (that was not present in the culture supernatant) in addition to THMgagC (Fig 4.1B). This band of higher MW may represent partly denatured protein in the concentrated preparation.

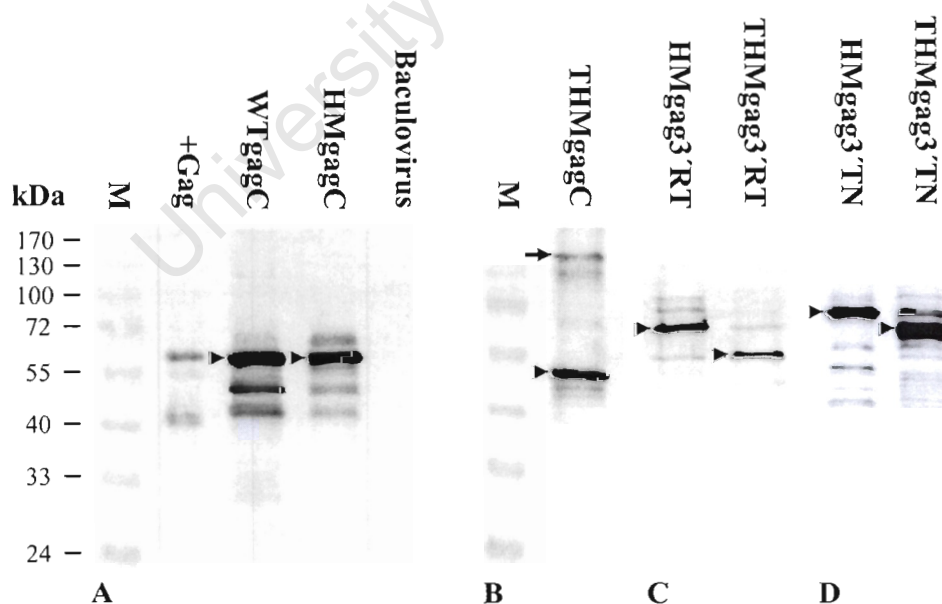


FIGURE 4.1. Control and chimaeric Gag proteins are detectable in some SCC samples.

Sf21 cells were infected with rAcNPV expressing one of indicated constructs. Each lane was loaded with 25 μ l of a SCC sample, where (A-D) represent blots from separate gels. Membranes were probed with p24-specific antiserum, +Gag = 50 ng. Arrowheads indicate the full-length Gag and chimaeric Gag protein bands. The arrow indicates a band of higher MW consistently present in THMgagC samples. M = MW marker.

A band of the expected size was visible for the -3'RT and -3'TN fusion proteins (Fig. 4.1C & D), which was shown to be the same band present in the High FiveTM culture supernatant (3.3.2.2) by comparison of samples run in adjacent lanes on the same gel (data not shown). In preparations where the protein yield was higher, such as the Gag-3'TN examples shown (Fig. 4.1D), there were an increased number of detectable bands, including p41 and p24. This may indicate preparations of lower purity or co-purification of soluble proteins. By this purification method, the -PE, -TN, -RT and -RTTN Gag chimaeras were not reproducibly detectable in SCC samples by western blot, and therefore SXC was investigated.

4.3.1.2 SXC of Sf21 culture supernatant

In an effort to detect particles from -PE, -TN, -RT and -RTTN chimaeras, and improve preparation purity, the particulate material from 40 ml of culture supernatant extracted by SCC was then centrifuged through a 30-70% sucrose density gradient. The particle-containing band in the sucrose density gradient (e.g. Fig. 4.2A) was shown by western blot to contain chimaeric protein for Gag-3'RT, -3'TN and -TN constructs (e.g. Fig. 4.2B).

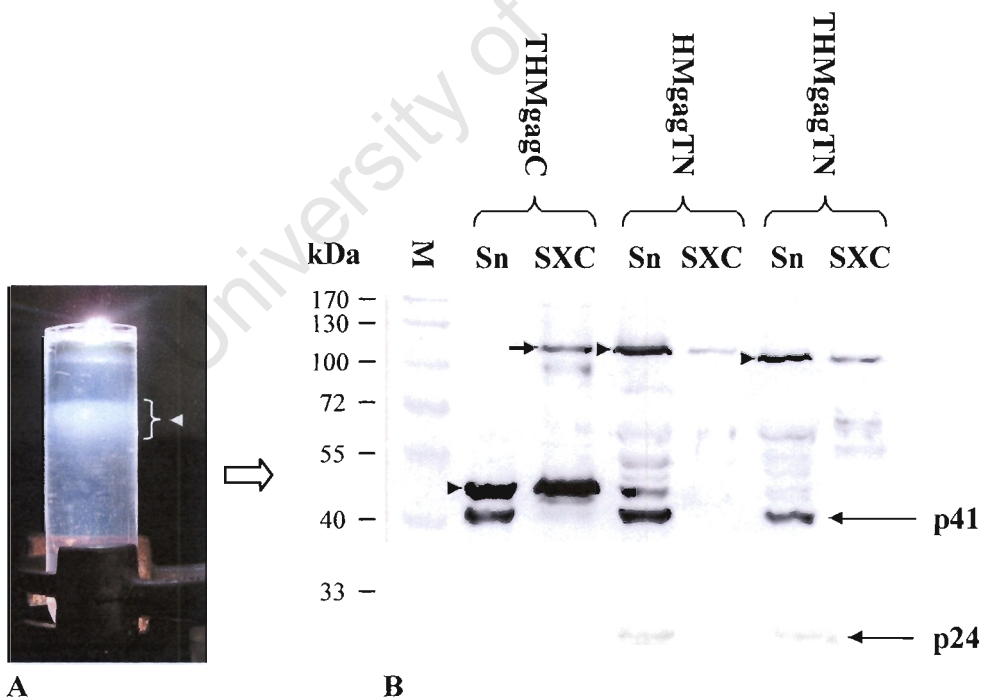


FIGURE 4.2. SXC samples contain full-length proteins but not p41.

(A) Example of a particulate band (THMgagC, SXC purification) in a sucrose gradient visible by white light *trans*-illumination (white arrowhead). (B) Particles were purified from culture supernatants of Sf21 cells expressing the indicated constructs, and 10 μ l of Sn or 25 μ l of SXC sample was loaded in each lane. The membrane was probed with p24-specific antiserum. Arrowheads indicate the full-length protein bands. The arrow indicates a band of higher molecular weight present in the THMgagC sample (same as Fig. 4.1). M = MW marker, Sn = supernatant.

These examples demonstrate that p41 in the culture supernatant is excluded from the particle purifications by SXC (compare p41 levels in Sn and SXC lanes, Fig. 4.2B). Gag-RT, Gag-RTTN and surprisingly Gag-PE constructs were only just detectable in SXC samples by western blot (data not shown).

4.3.2 apTEM of SCC and SXC samples

All SCC samples were analyzed by apTEM in an attempt to ascertain whether VLPs were present in the samples. The HMgagC and THMgagC samples (Fig. 4.3A, B) contained typical VLPs (100-150 nm) based on previously characterized morphological features (Luo *et al.*, 1990; Jaffray *et al.*, 2004). Structures resembling VLPs in overall morphology, were found in all chimaeric Gag SCC samples (e.g. Fig. 4.3C-E), but the size of these putative chimaeric VLPs ranged from ~150-300 nm. However, structures of very similar appearance could be found in the negative Gag control (AcNPV infection, Fig. 4.3F), and the negative VLP controls (Myr⁻ Gag, data not shown), albeit at lower concentrations on the grid. Membrane-bound, folded baculovirus could be differentiated from Gag-only VLPs (compare Fig. 4.4A & B), but for the -PE, -RT and -RTTN SCC samples in particular, this distinction was not always clear since the putative VLPs were of similar size to the membrane-bound folded baculovirus (compare examples in Fig. 4.4C & D). apTEM of SXC samples gave similar results for all constructs (data not shown). Essentially, apTEM was insufficiently objective under these conditions for conclusively determining if VLPs were present in SCC and SXC samples.

4.3.3 tsTEM

The inadequacy of apTEM in this study for confirming VLP production (or characterizing the particles) prompted the use of tsTEM to analyze cells expressing chimaeric Gag proteins. It was reasoned that if budding of particles at the cell surface could be observed, this would be compelling evidence for the production of chimaeric Gag VLPs. In uninfected Sf21 cells, membrane protrusions (Fig. 4.5B) and cellular debris (Fig. 4.5C) were sometimes associated with the cell surface, but these were easily distinguished from Gag VLPs (compare to Fig. 1.2). Similar membrane protrusions were visible for AcNPV- and all rAcNPV-infected cells (data not shown). Single baculovirus particles were also visible budding from infected cells at the cell surface (Fig. 4.6A, inset). In these cases the internal rod-shaped virion is clearly identifiable, and the whole structure does not resemble a VLP.

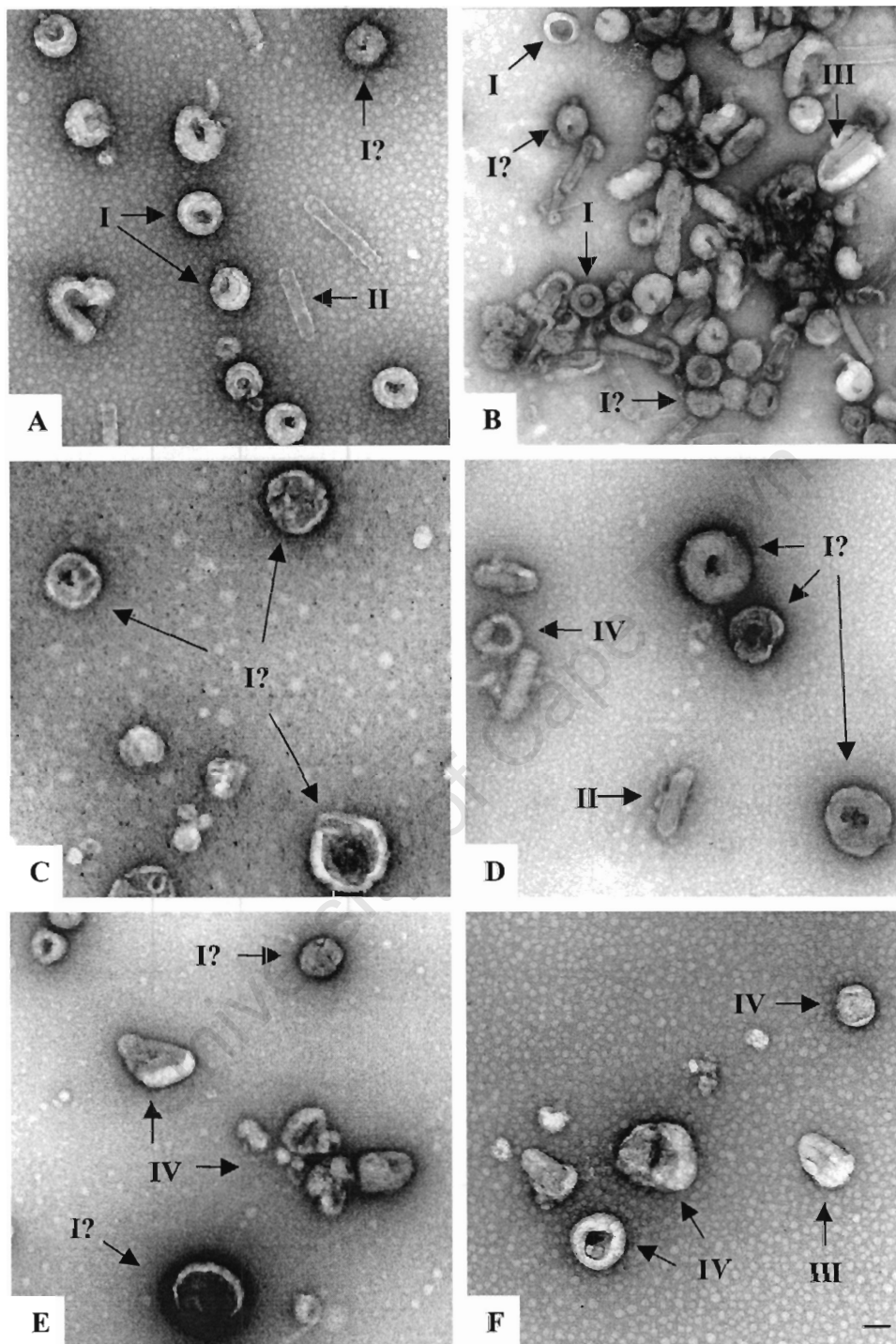


FIGURE 4.3. apTEM unable to confirm the presence of VLPs in SCC samples.

Sf21 cells were infected with rAcNPV expressing one of the following constructs: HMgagC (A), THMgagC (B), HMgag3'RT (C), HMgag3'TN (D), HMgagPE (E), baculovirus (F). At 3 dpi SCP samples were prepared and analyzed by apTEM. I = VLP, identification based on distinctive morphology, II = co-purified baculovirus, III = membrane enclosed baculovirus (folded), IV = unknown structure. Bar = 100 nm, all micrographs at the same scale.

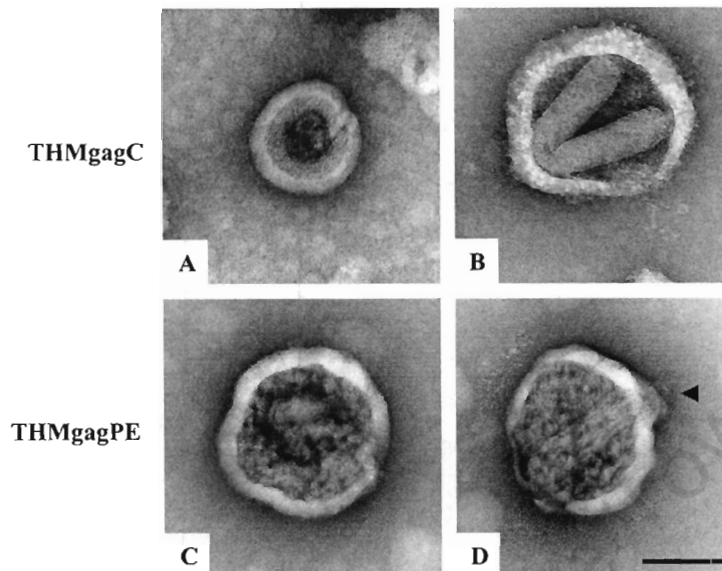


FIGURE 4.4. Membrane bound, folded baculovirus could be misidentified as a large VLP by apTEM. Examples of structures in SCC samples with shared morphological features that could lead to misidentification. Representatives are shown from THMgagC (A, B) and THMgagPE (C, D) expressing cells. Arrowhead indicates a presumed baculovirus within a membrane. Bar = 100 nm, all micrographs at the same scale.

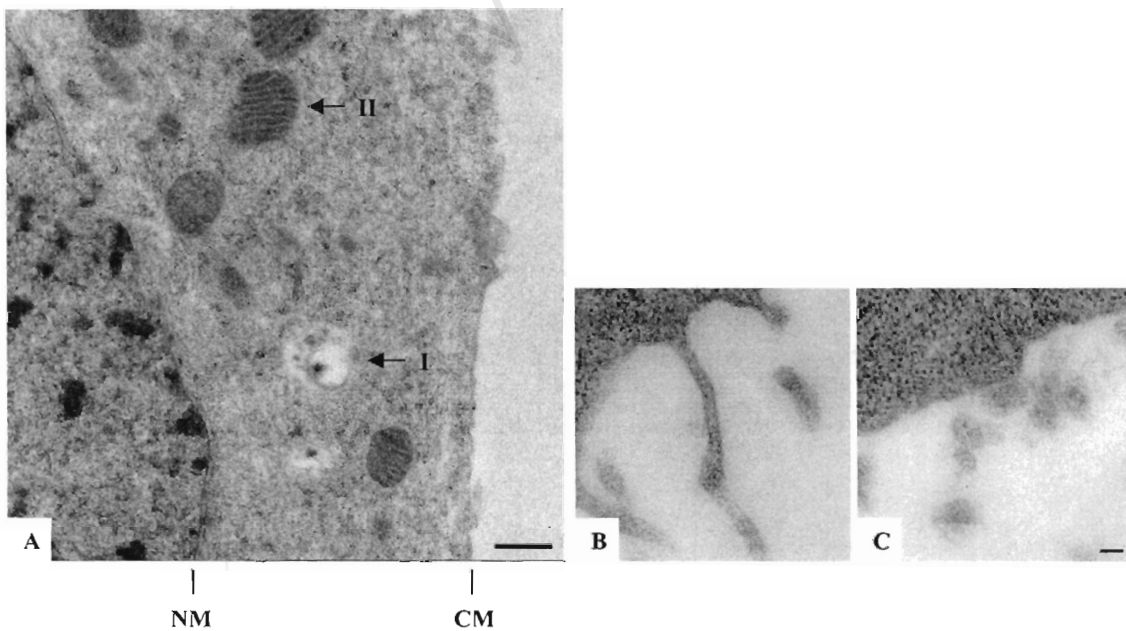


FIGURE 4.5. Uninfected Sf21 cell morphology.

(A) Sf21 cell not infected with baculovirus: I, intracellular vesicle and II, mitochondria. Cell surface features observed were: (B) cell membrane protrusions and (C) external cellular debris. NM, nuclear membrane; CM, cell membrane. Bar = 500 nm (A), 100 nm (B, C)

As a result of baculovirus infection, numerous intracellular vesicles (20-400 nm across) were formed (Fig. 4.6B, C) and released from the cell upon lysis (Fig. 4.6D). This observation is significant to this study, since some of these vesicles may have a buoyant density equivalent to VLPs and would therefore be co-purified with VLPs by either SCC or SXC. This may in part account for the structures visible by apTEM from AcNPV-infected cells (Fig. 4.3F).

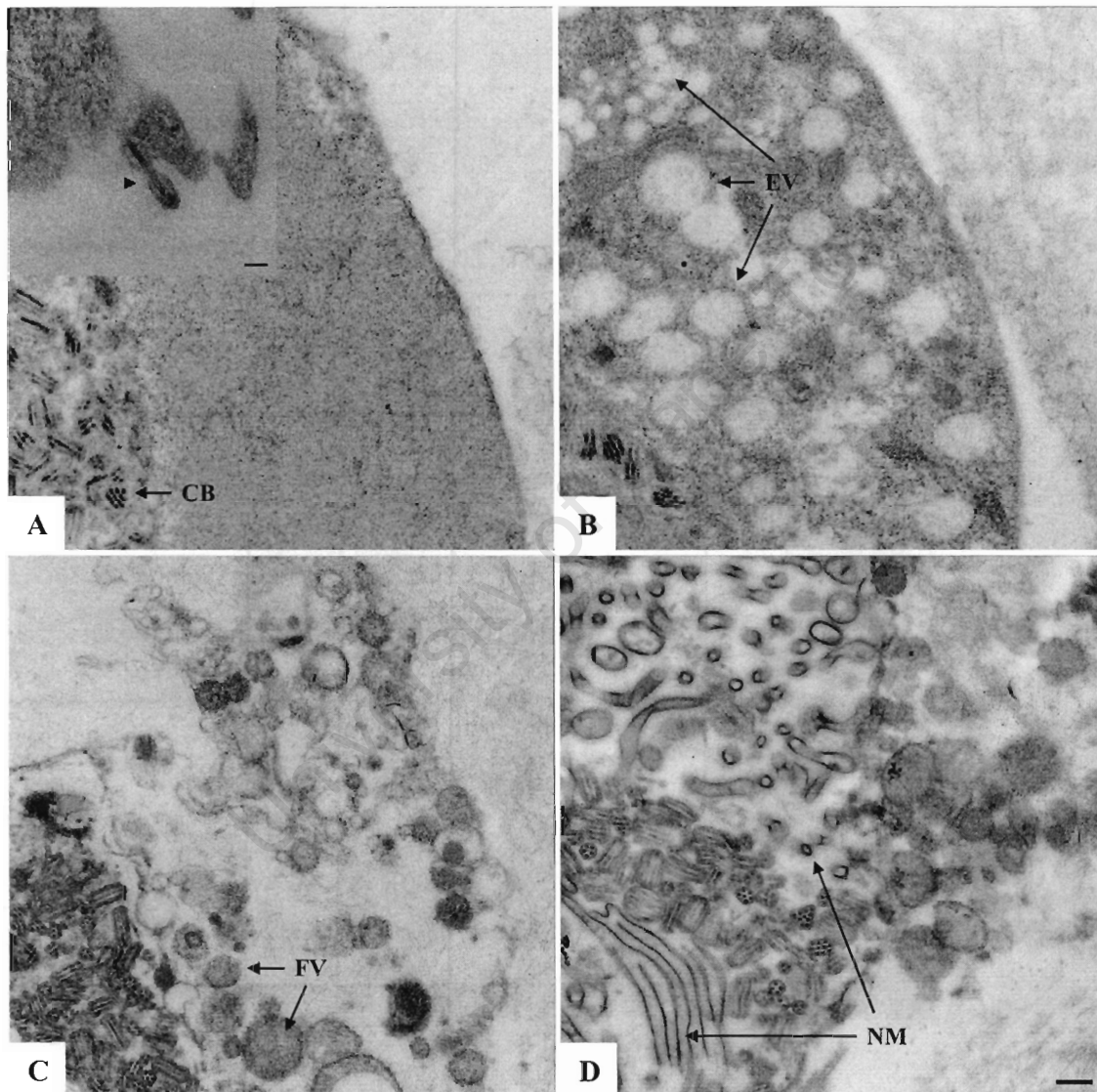


FIGURE 4.6. Effects of baculovirus infection on Sf21 cell morphology.

Morphological features visible during baculovirus infection in Sf21 cells include: (A) Condensed baculovirus (CB) predominated in the nucleus, while the cytoplasm showed reduced numbers of organelles. Inset: baculovirus budding from the cell indicated by the arrowhead. (B) Extensive empty vesicular structures (EV) in the cytoplasm. (C) Numerous filled vesicular structures (FV) visible between the intact nuclear- and cell membranes. (D) Cell lysis, nuclear membrane (NM) formed lamella and tubular structures while FVs were released. Bar = 500 nm (A-D), 100 nm (panel A, inset).

To illustrate that these vesicles could not be mistaken for budding particles by tsTEM, two low magnification examples of rAcNPV-infected cells are provided (Fig. 4.7A & B). The particles from THMgagRT (Fig. 4.7A), and to a lesser degree THMgagC (Fig. 4.7B), resemble some of the cytoplasmic vesicles in cross section, but were clearly extracellular with the cell membrane still intact. These micrographs also illustrate that the particles produced by THMgagRT (as well as HMgagRT and Gag-RTTN fusion proteins, described in section 4.3.3.3) differed significantly from typical Gag VLPs (e.g. THMgagC, Fig. 4.7B) in several areas of ultrastructure. For this reason, these larger, less compact and heterogeneous particles are termed here as virus-derived particles (VDPs).

4.3.3.1 Myr⁻ Gag controls

The expression of Myr⁻ Gag proteins did not lead to the budding of VLPs from the cell, nor was there any noticeable increase in electron density at the cell membrane (data not shown), which is potentially indicative of protein localization. There was one distinct difference between HΔMgagC and THΔMgagC expression: structurally ordered electron dense aggregates were visible in the nucleus for both constructs (Fig. 4.8A & 4.9A) but isolated structures in the cytoplasm were only visible for THΔMgagC (Fig. 4.9 A). The nuclear aggregates for both Myr⁻ Gag constructs were composed of individual 120 - 200 nm structures with 2 - 4 defined layers (Fig. 4.8B, C & 4.9D, E), which were always approximately circular in cross-section, and are therefore probably spherical rather than tubular structures. The cytoplasmic units from THΔMgagC resemble Gag VLPs (Fig. 1.2) but were often composed of 2 - 3 layers with less distinct electron dense rings (Fig. 4.9C). Immunogold labeling within the layers of all these structures confirmed they contain p24 (Fig. 4.10A-G). AcNPV-infected cells showed no immunogold labeling (data not shown).

4.3.3.2 Myr⁺ Gag controls

The expression of both WTgagC and HMgagC was associated with the budding and release of VLPs (~100 nm in diameter) at the cell membrane (Fig. 4.11A, B). Likewise, THMgagC produced abundant VLPs ~100 nm across (Fig. 4.12A-E). VLPs showed an outer shell (~15 nm) surrounding an electron dense ring (Fig. 4.11A, inset ii). The center of the VLP was often translucent and immunogold labeling confirmed the presence of p24 (Fig. 4.13A), which was concentrated at the electron dense ring within the VLP (Fig. 4.13B, C).

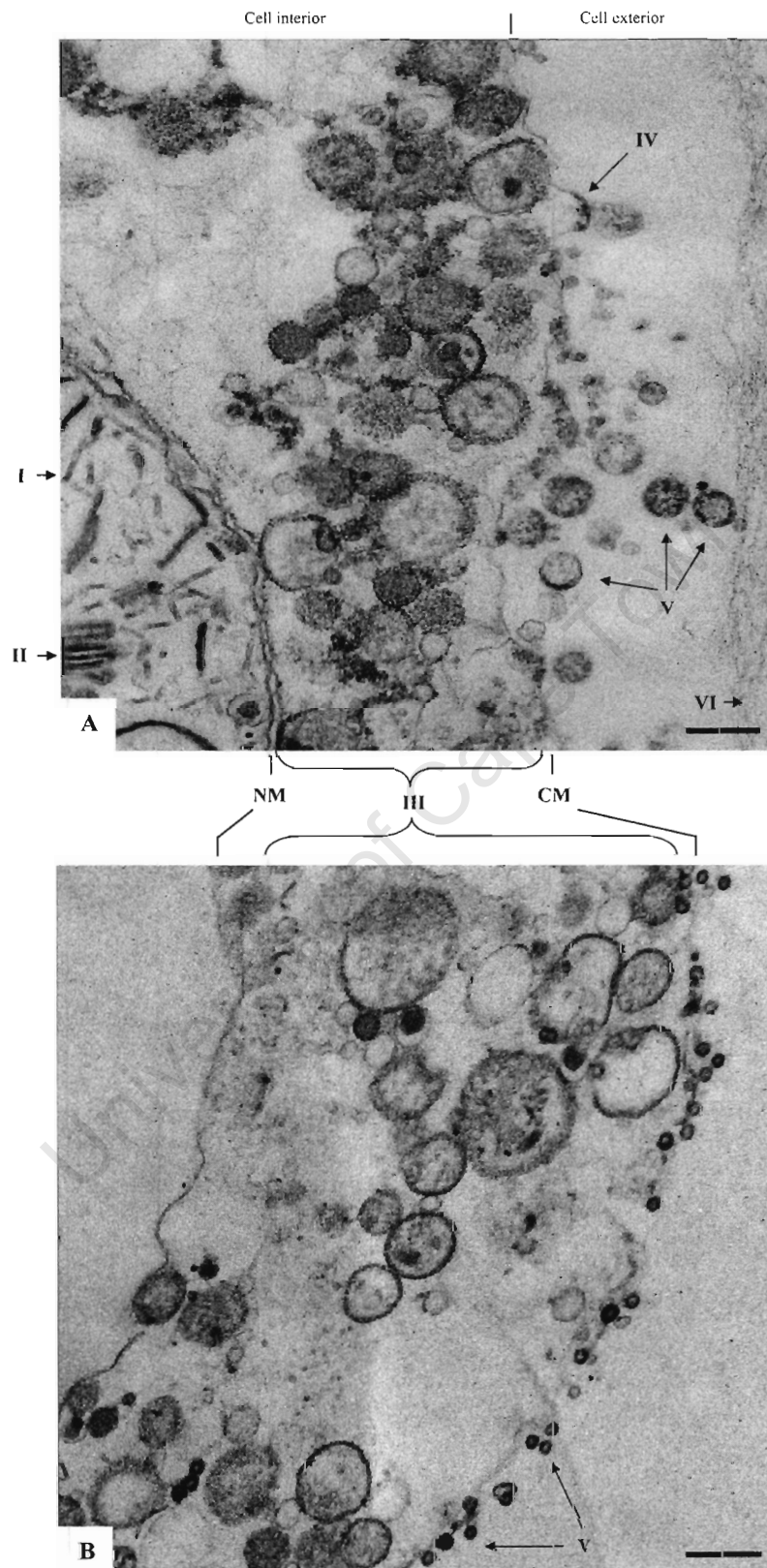


FIGURE 4.7. Budded particles are distinguishable from cytoplasmic vesicular structures before cell lysis in Sf21 cells. Sf21 cells infected with rAc-THMgagRT (A) and rAc-THMgagC (B) are shown as examples. Budded particles at the cell surface were separated from cytoplasmic vesicles by the cell membrane. I, single baculovirus; II, condensed baculoviruses; III, vesicular structures in cytoplasm; IV, budding; V, VLPs and VDPs; VI, low melting point agarose used in embedding process; NM, nuclear membrane; CM, cell membrane. Bar = 500 nm

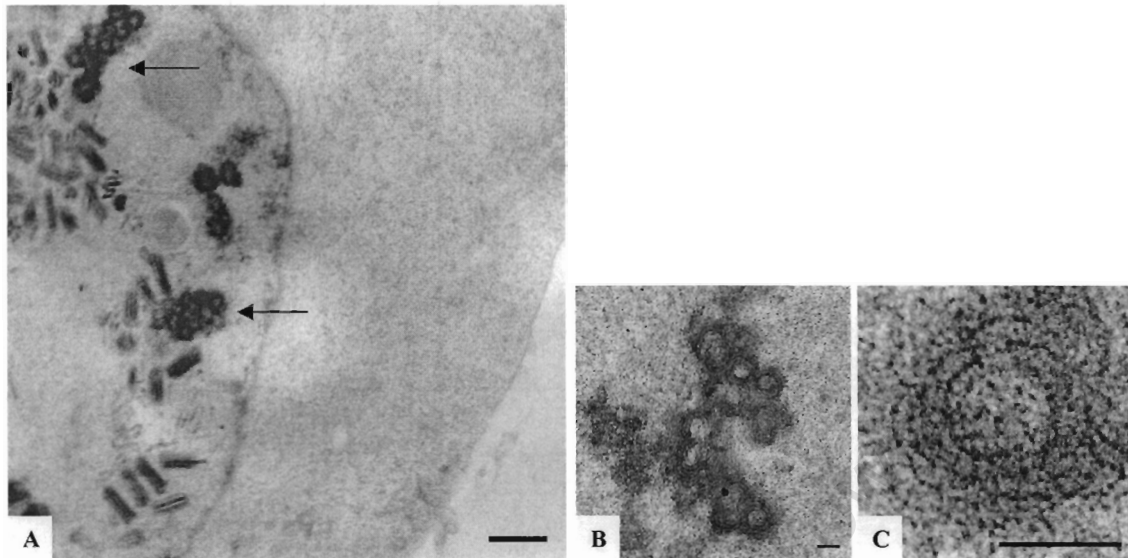


FIGURE 4.8. **Accumulation of H Δ MgagC structures in the nucleus.**

Sf21 cells infected with rAc-H Δ MgagC showed ordered structures present only in the nucleus, indicated by the arrows (A). Magnifications of these multilayered aggregations are shown in (B) and (C). Bar = 500 nm (A), 100 nm (B, C)

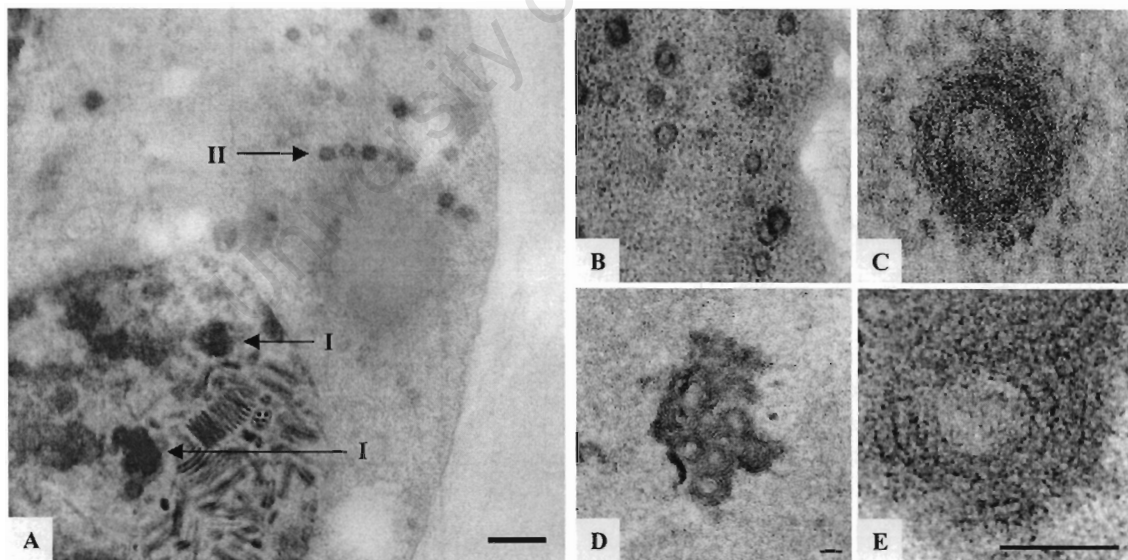


FIGURE 4.9. **Accumulation of TH Δ MgagC in the nucleus and cytoplasm.**

Sf21 cells infected with rAc-TH Δ MgagC showed ordered structures present in both the nucleus and cytoplasm, indicated by I and II respectively (A). Magnifications of cytoplasmic structures (B, C) and multilayered nuclear aggregations (D, E) are shown. Bar = 500 nm (A), 100 nm (B, D), 100 nm (C, E)

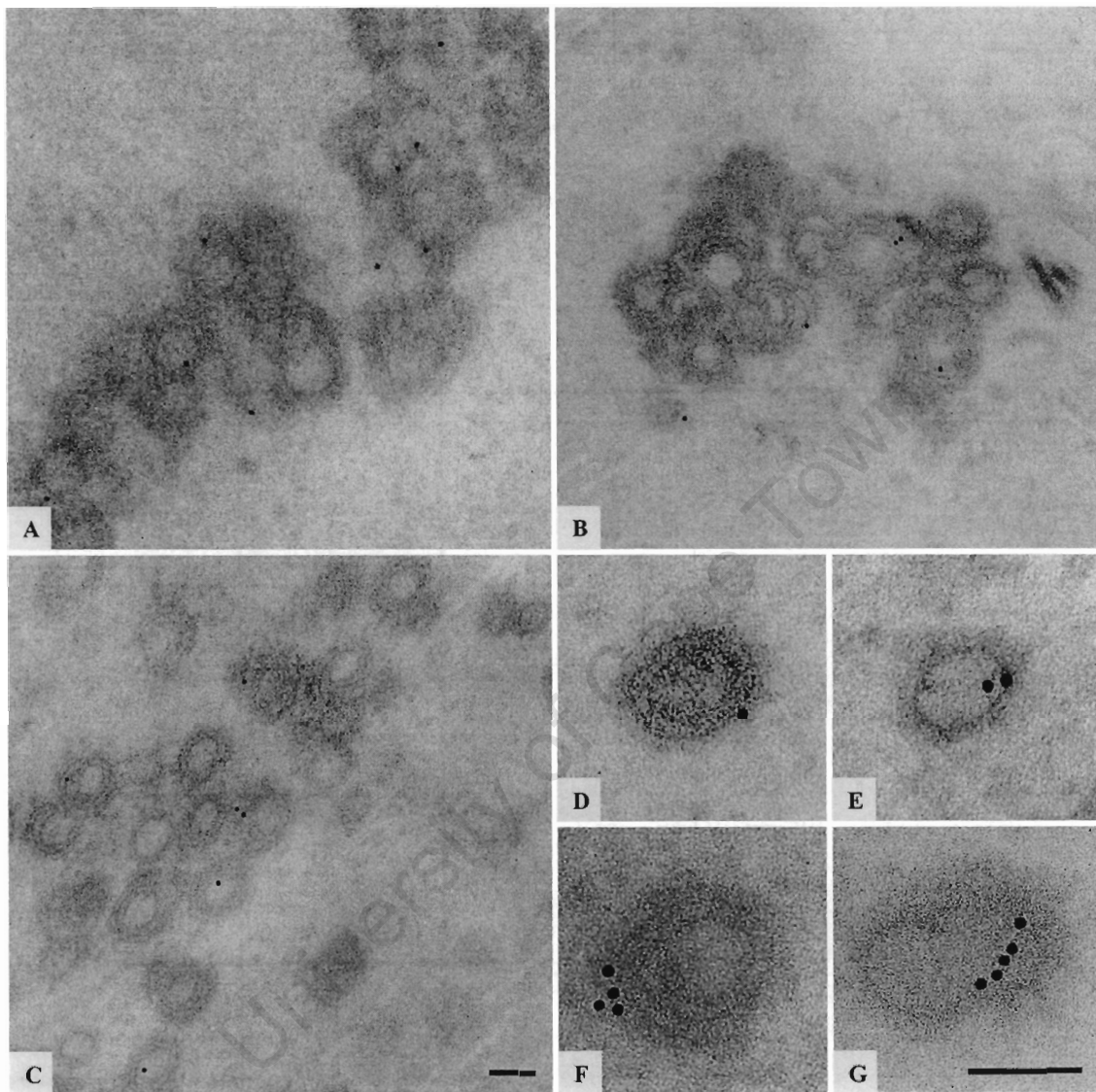


FIGURE 4.10. Immunogold labelling of structures in cells expressing H Δ MgagC and TH Δ MgagC. Ultrathin unstained sections were probed with p24 antiserum and labelled with 10 nm colloidal gold conjugate. H Δ MgagC (A) and TH Δ MgagC (B) structured aggregates from nucleus. TH Δ MgagC cytoplasmic structures (C) with magnifications (D-G). Bar = 100 nm (A-C), 100 nm (D-G).

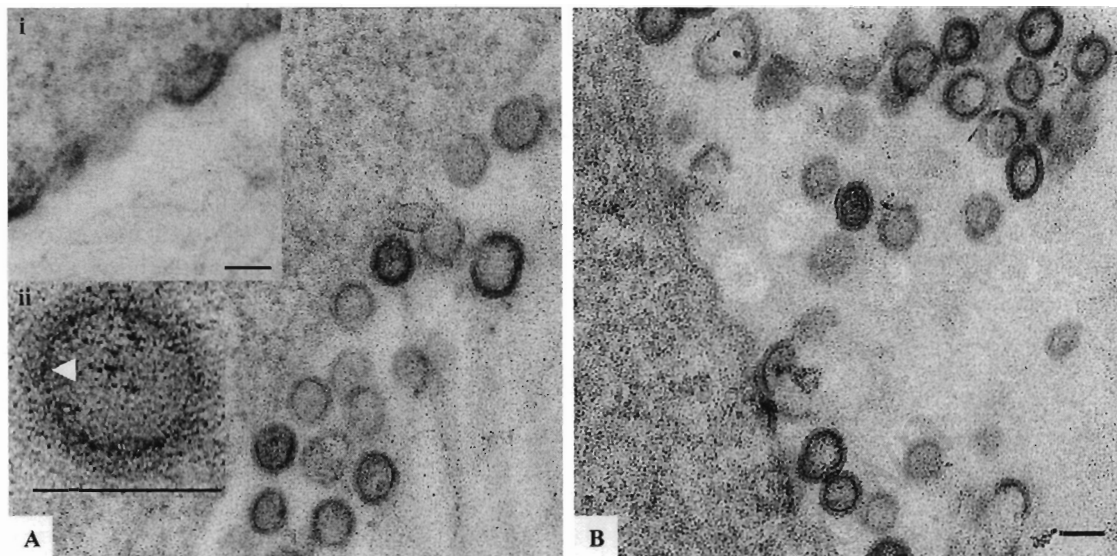


FIGURE 4.11. **WTgagC and HMgagC VLPs.**

(A) WTgagC VLPs. Insets indicate (i) membrane curvature associated with budding (ii) the characteristic electron dense ring (white arrowhead) below the VLP periphery. (B) HMgagC VLPs. Bars = 100 nm (insets), 100 nm (A, B).

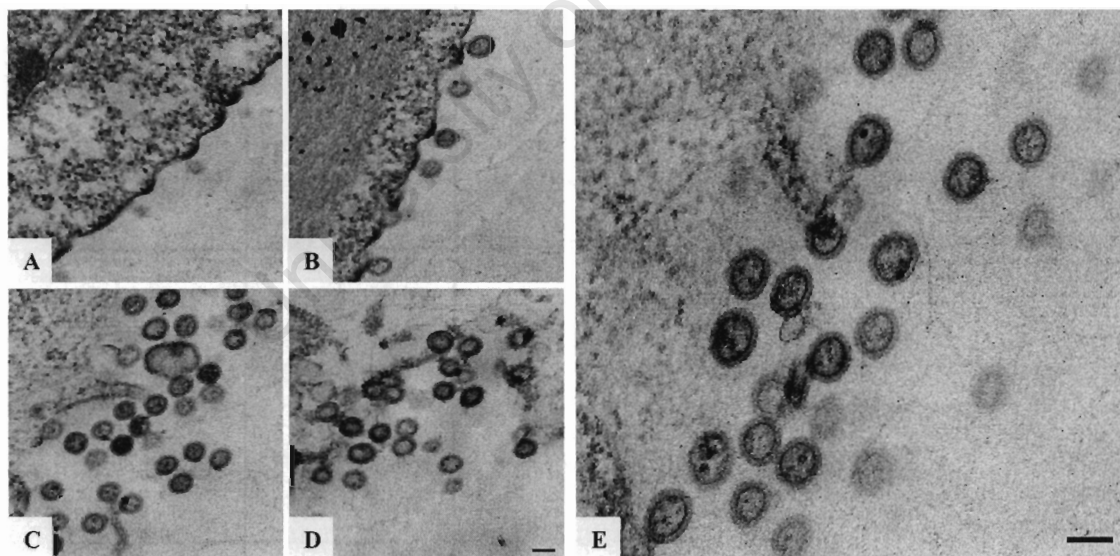


FIGURE 4.12. **THMgagC VLPs.**

Budding and release of extracellular VLPs from cells infected with rAc-THMgagC (A-E). Bars = 100 nm (A-D), 100 nm (E).

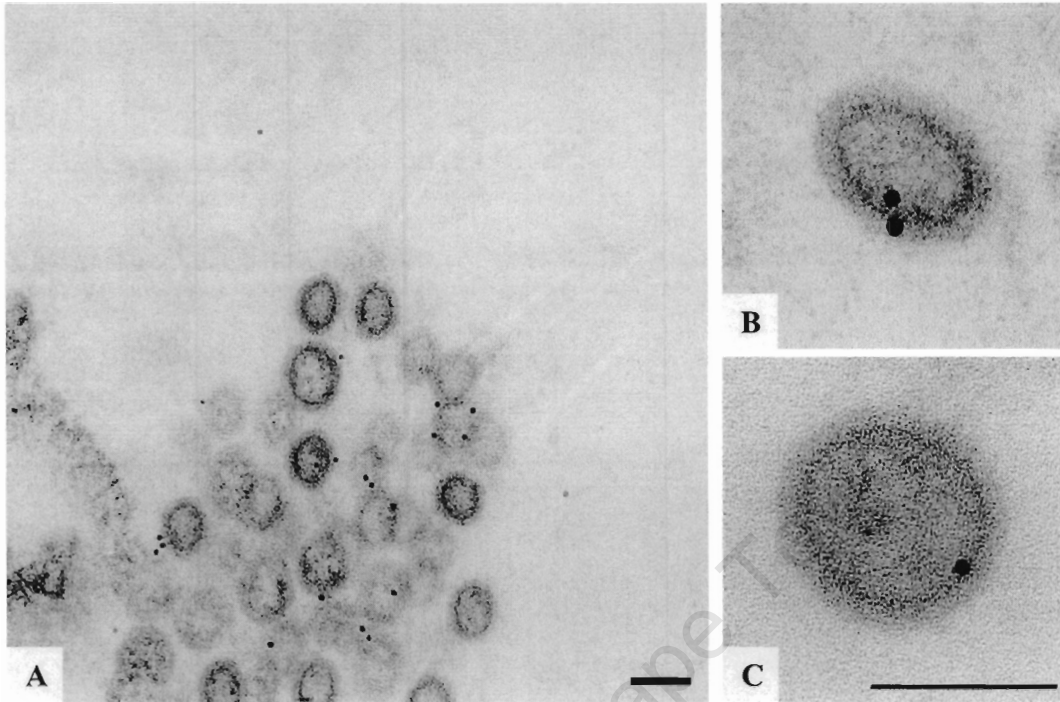


FIGURE 4.13. **Immunogold labelling of HMgagC and THMgagC VLPs.**

Ultrathin unstained sections were probed with p24 antiserum and labelled with 10 nm colloidal gold conjugate. (A) THMgagC VLPs at the cell surface and specific labelling of the electron dense ring in a THMgagC VLP (B) and a HMgagC VLP (C). Bar = 100 nm (A), 100 nm (B, C).

4.3.3.3 RTTN-derived Gag chimaeras

For each specific C-terminal fusion sequence (e.g. TN), the appearance of the budded particles was largely identical between HMgag- and THMgag-based chimaeras. However, the appearance of the particles varied significantly between chimaeras with different C-terminal fusion sequences. There seemed to be an overall trend that as the protein fusion length increased (from 113 – 778 aa), the particle size apparently increased but the average number of VLPs observed budding per cell decreased.

HMgag3'RT and THMgag3'RT budded off compact, uniform VLPs with diameters of ~120 nm and ~100 nm respectively (Fig. 4.14A-J). Both were comparable in structure to Gag-only VLPs (compare to Fig 4.11 and 4.12). HMgag3'TN and THMgag3'TN budded off slightly larger VLPs with considerable size heterogeneity compared to Gag-only VLPs, ranging in diameter from 120 - 210 nm (Fig. 4.15A-J). The HMgag3'TN VLPs in particular were often observed as disintegrated or incompletely formed VLPs (Fig. 4.15C, E).

Interestingly, the constructs carrying the substantially longer TN fusion, generated similar sized VLPs to the Gag-3'TN constructs, ranging from 130-190 nm in diameter (Fig. 4.16A-J). In addition, the majority of these VLPs were observed intact, in contrast to the HMgag3'TN VLPs.

HMgagRT and THMgagRT budded off even larger, less compact particles, ranging in diameter from 180 - 330 nm and 180 - 410 nm respectively (Fig. 4.17A-J). In this case, the THMgagRT VDPs were generally larger and more variable in size than those of HMgagRT. Both chimaeric VDPs often showed granular interiors with electron dense patches around the particle periphery as opposed to a defined electron dense ring. Some budded structures showed only a membrane in sections of the VDP periphery (e.g. Fig. 4.17A, inset), and were up to 400 nm across for HMgagRT (Fig. 4.17B). This phenomenon of localized absence of Gag at the particle periphery has been observed in type I in-frame VLPs previously (Wagner *et al.*, 1994a). The constructs carrying the longest fusion (778 aa), HMgagRTTN and THMgagRTTN, also budded off large VDPs with diameters of 170 - 320 nm for both (Fig. 4.18A-J). These Gag-RTTN VDPs exhibited a similar overall appearance to the Gag-RT VDPs.

In a few cells, budding occurred into intracellular compartments (in addition to extracellular VLP formation), and a particularly striking example is provided in Figure 4.19. This phenomenon was observed in some cells expressing each construct, but was the exception rather than the rule.

Ultrathin sections of cells expressing each RTTN-derived construct were reacted in separate experiments with 4 anti-Nef and 3 anti-RT antisera/antibodies under a range of conditions (E.2). In each case immunogold labeling was either absent or non-specific (binding to sectioned HMgagC VLPs). The use of LR WhiteTM Resin (London Resin Company), which is formulated for use in immunocytochemistry, did not improve immunogold labeling compared to Spurr's resin with tested antisera/antibodies (E.2).

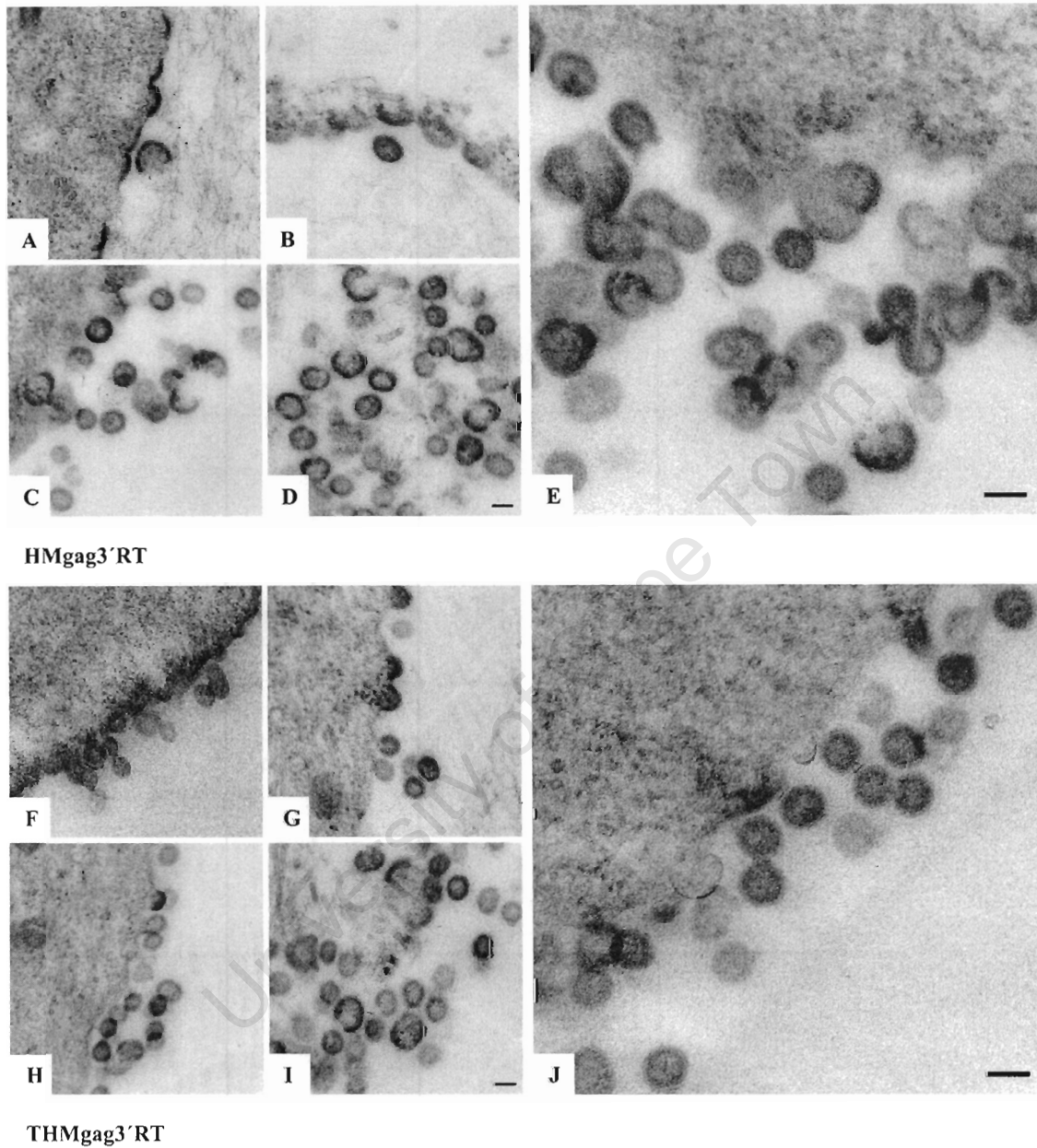


FIGURE 4.14. **HMgag3'RT and THMgag3'RT VLPs.**

Budding and release of extracellular VLPs from rAc-HMgag3'RT and rAc-THMgag3'RT infected cells. Bars = 100 nm (A-D, F-I), 100 nm (E, J).

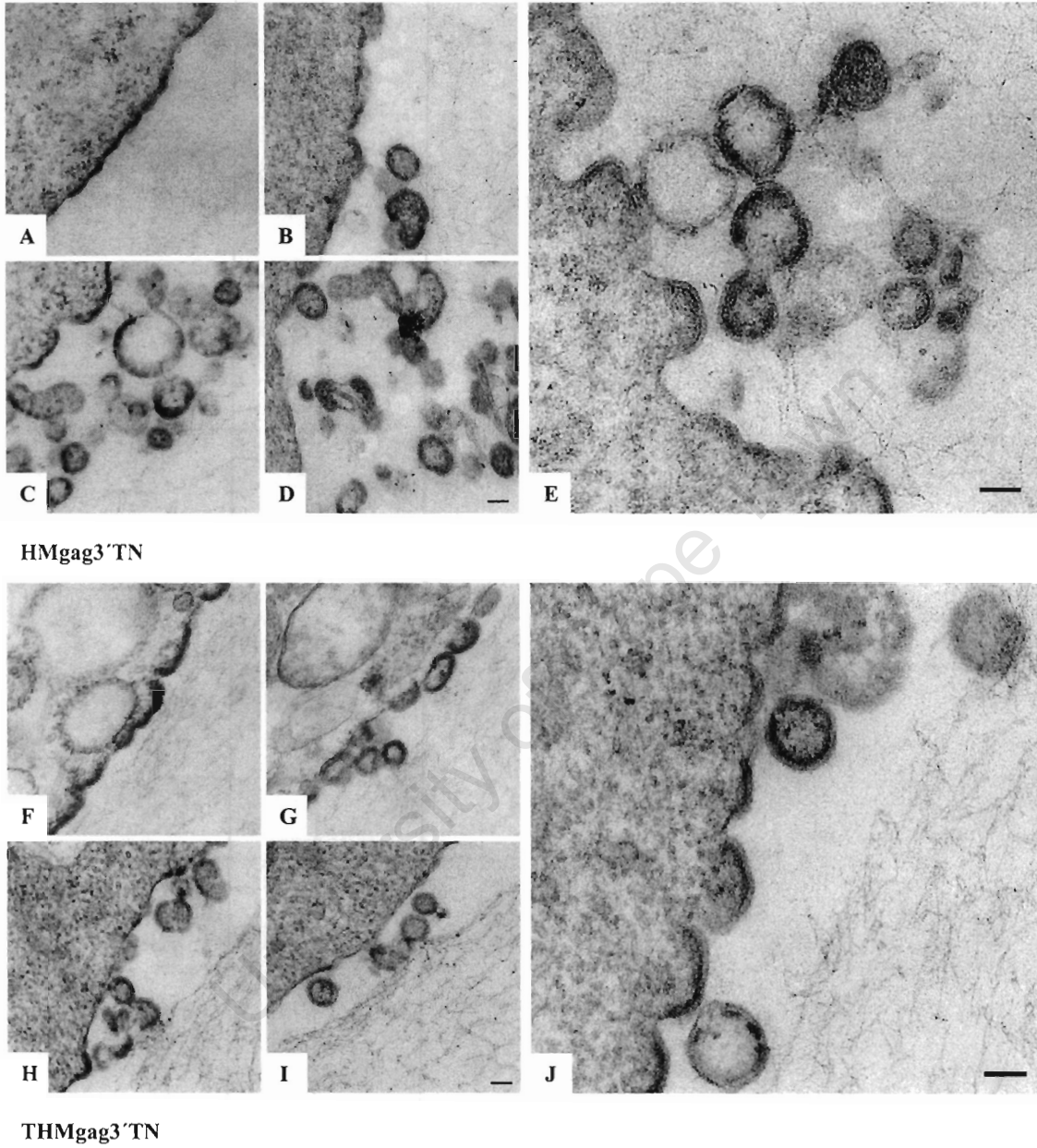


FIGURE 4.15. HMgag3'TN and THMgag3'TN VLPs.
 Budding and release of extracellular VLPs from rAc-HMgag3'TN and rAc-THMgag3'TN infected cells. Bars = 100 nm (A-D, F-I), 100 nm (E, J).

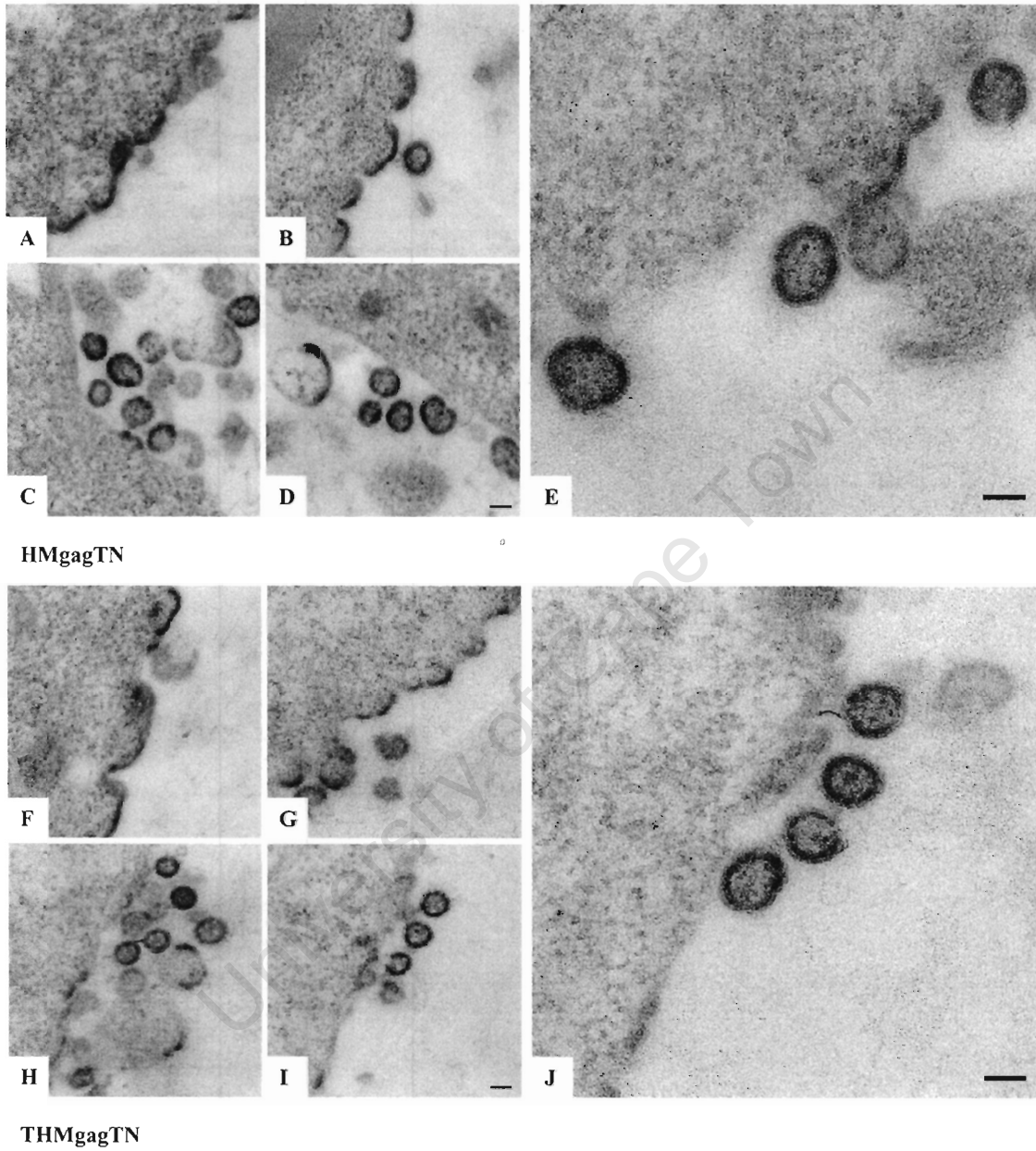


FIGURE 4.16. HMgagTN and THMgagTN VLPs.
 Budding and release of extracellular VLPs from rAc-HMgagTN and rAc-THMgagTN infected cells.
 Bars = 100 nm (A-D, F-I), 100 nm (E, J).

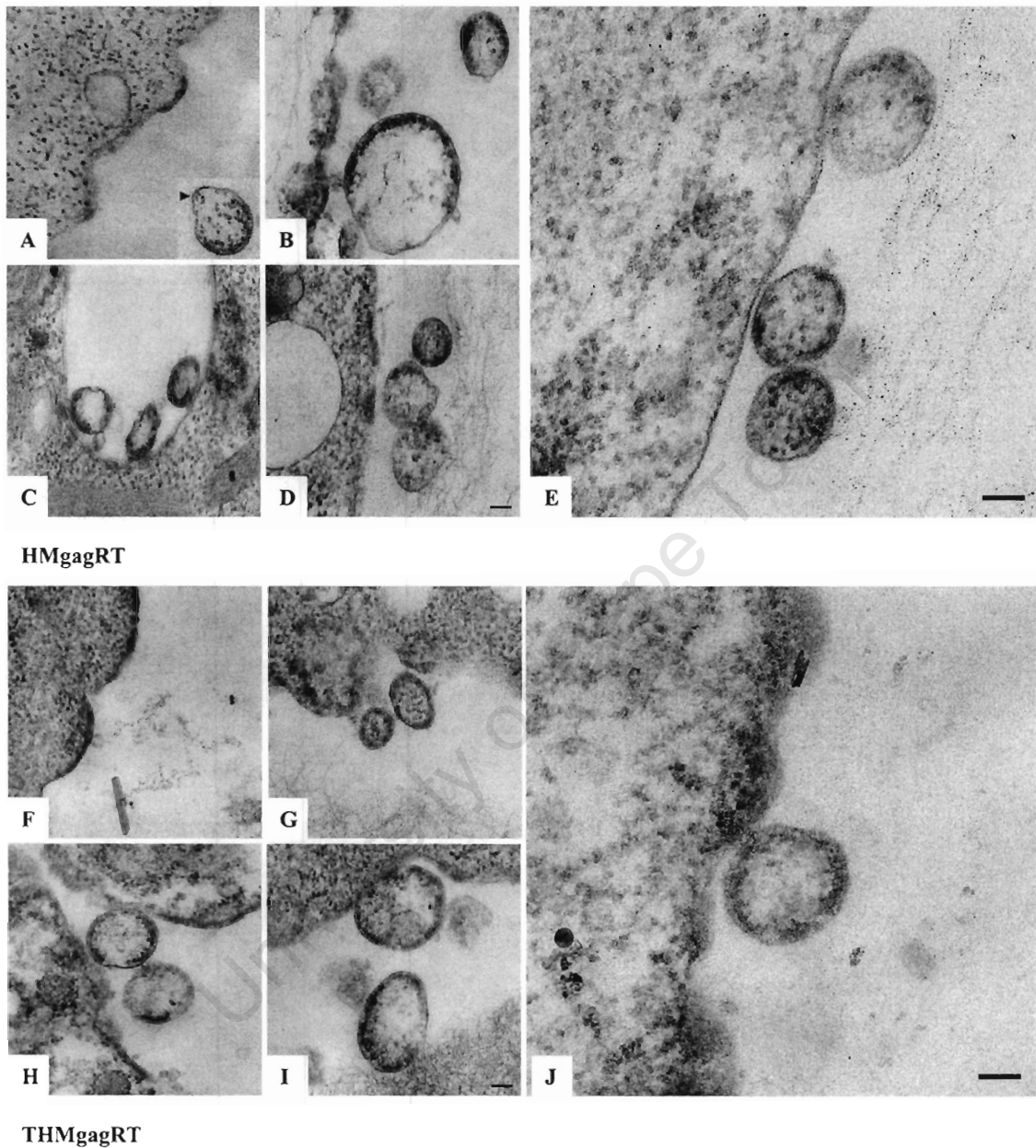


FIGURE 4.17. HMgagRT and THMgagRT VDPs.

Budding and release of extracellular VDPs from rAc-HMgagRT and rAc-THMgagRT infected cells. Inset (A): incompletely formed particle, arrowhead indicates where particle pinched off from the cell and only the cell derived membrane remains in this section of the structure. Bars = 100 nm (A-D, F-I), 100 nm (E, J).

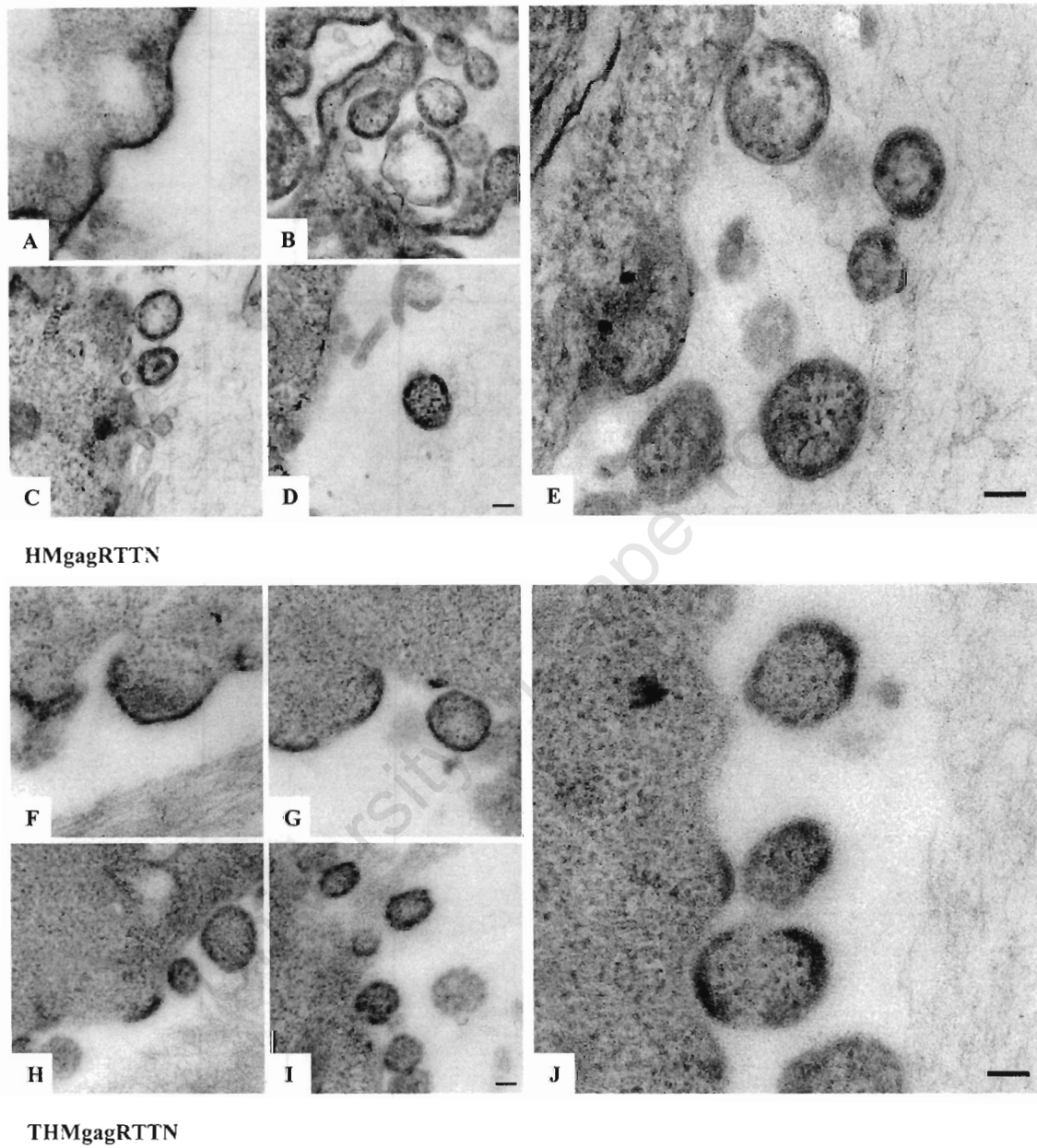


FIGURE 4.18. HMgagRTTN and THMgagRTTN VDPs.

Budding and release of extracellular VDPs from rAc-HMgagRTTN and rAc-THMgagRTTN infected cells. Bars = 100 nm (A-D, F-I), 100 nm (E, J).

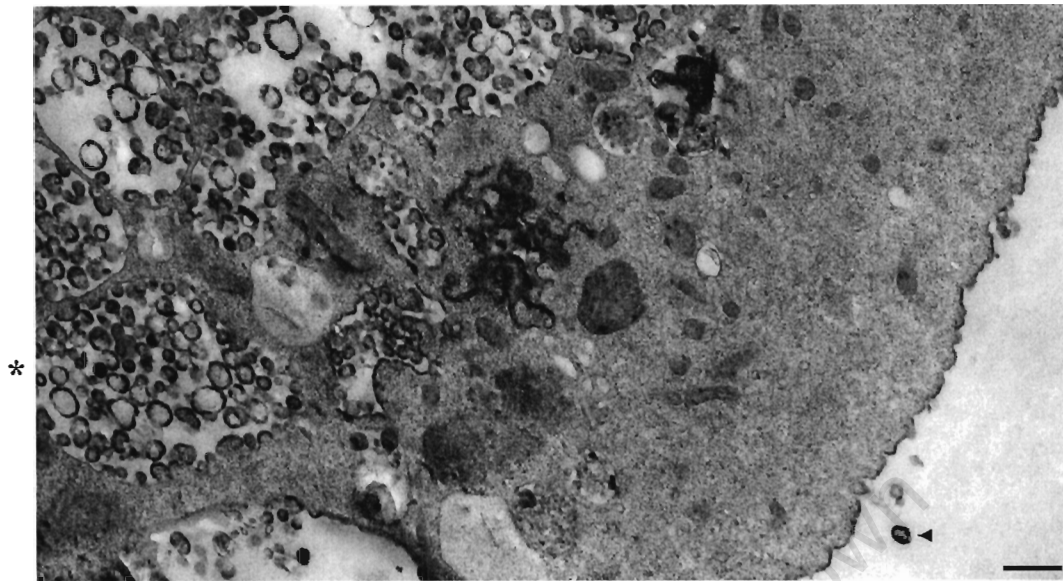


FIGURE 4.19. Example of VLP budding into intracellular compartments.

This cell, infected with rAc-THMgag3'TN, showed high concentrations of VLPs that had budded into membrane bound regions within the cytoplasm. The compartment indicated with an asterisk is filled with both complete and partly formed particles. All Myr¹ Gag proteins exhibited this phenomenon to some extent. Budding at VLP formation was also present at the cell surface, and an extracellular VLP is shown with an arrowhead. Bar = 500 nm.

The size data for VLPs and VDPs from the RTTN-derived constructs is summarized in Table 4.1 and the correlation between fusion length and particle size is compared in Figure 4.22.

4.3.3.4 Gag-PE chimaeras

Surprisingly, HMgagPE and THMgagPE, which carry the second shortest of the fusions (155 aa), did not generate VLPs or even VDPs. Cells expressing Gag-PE constructs appeared to extrude irregularly sized blebs or vesicles, that rarely budded completely (Fig. 4.20A-J). In both cases, structures 250 – 400 nm across were most common (Fig. 4.20E, J), but structures measuring up to 1 μ m were also observed. These structures could be distinguished from the polymorphic features at the cell membranes of Sf21 cells (Fig. 4.5) by their electron dense budding surface and predominantly spherical shape. In addition, cells expressing HMgagPE or THMgagPE showed unstructured electron dense cytoplasmic aggregations (Fig. 4.21). These were not seen in cells expressing other constructs or in uninfected controls. Immunogold labeling of sections probed with anti-SV5-Pk mAb could not confirm that extracellular blebs or cytoplasmic aggregates contained full-length Gag-PE proteins (data not shown).

TABLE 4.1. Diameters of chimaeric Gag VLPs and VDPs based on tsTEM measurements

| Construct ¹ | Range in diameter ² (nm) | Average diameter ³ (nm) | SD ⁴ (n = 40) |
|------------------------|-------------------------------------|------------------------------------|--------------------------|
| <i>VLP controls</i> | | | |
| WTgagC | 95 - 120 | 106 | 6.8 |
| HMgagC | 95 - 120 | 104 | 6.8 |
| THMgagC | 95 - 110 | 103 | 4.1 |
| <i>Gag chimaeras</i> | | | |
| HMgag3'RT | 105 - 135 | 122 | 7.7 |
| THMgag3'RT | 95 - 110 | 106 | 4.8 |
| HMgag3'TN | 130 - 240 | 164 | 26.8 |
| THMgag3'TN | 115 - 200 | 149 | 28.8 |
| HMgagTN | 125 - 195 | 158 | 19.3 |
| THMgagTN | 120 - 160 | 138 | 11.2 |
| HMgagRT | 155 - 290 | 224 | 41.9 |
| THMgagRT | 170 - 410 | 270 | 67.6 |
| HMgagRTTN | 155 - 290 | 233 | 46.7 |
| THMgagRTTN | 170 - 300 | 243 | 39.0 |

¹ Gag-PE constructs omitted, as these constructs did not form discrete particles.

² Each value rounded to nearest 5 nm. Only intact VLPs were measured - partly formed, disintegrated or fused particles were excluded.

³ The diameter of each particle was measured twice on perpendicular axes since particles were often not completely circular in cross section. These mean values (n = 40) were then averaged.

⁴ Standard deviation, rounded off to nearest decimal point.

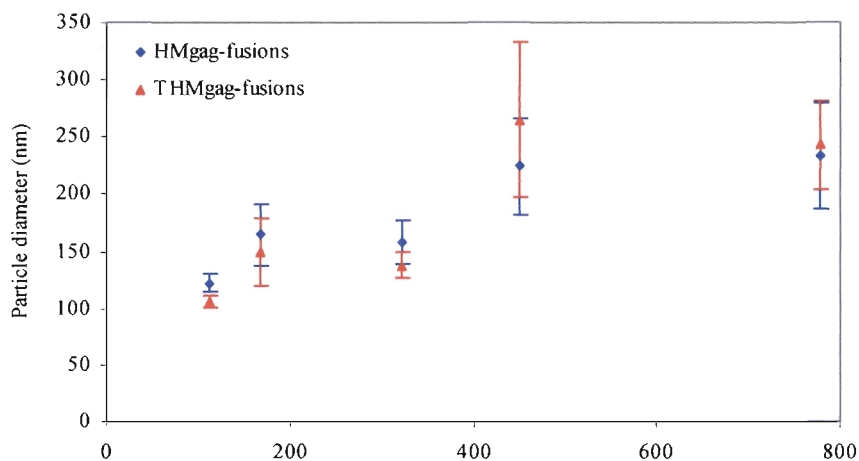


FIGURE 4.22. Size of chimaeric particles in relation to fusion sequence length. Average particle diameter and standard deviation (error bars) based on tsTEM measurements (Table 4.1).

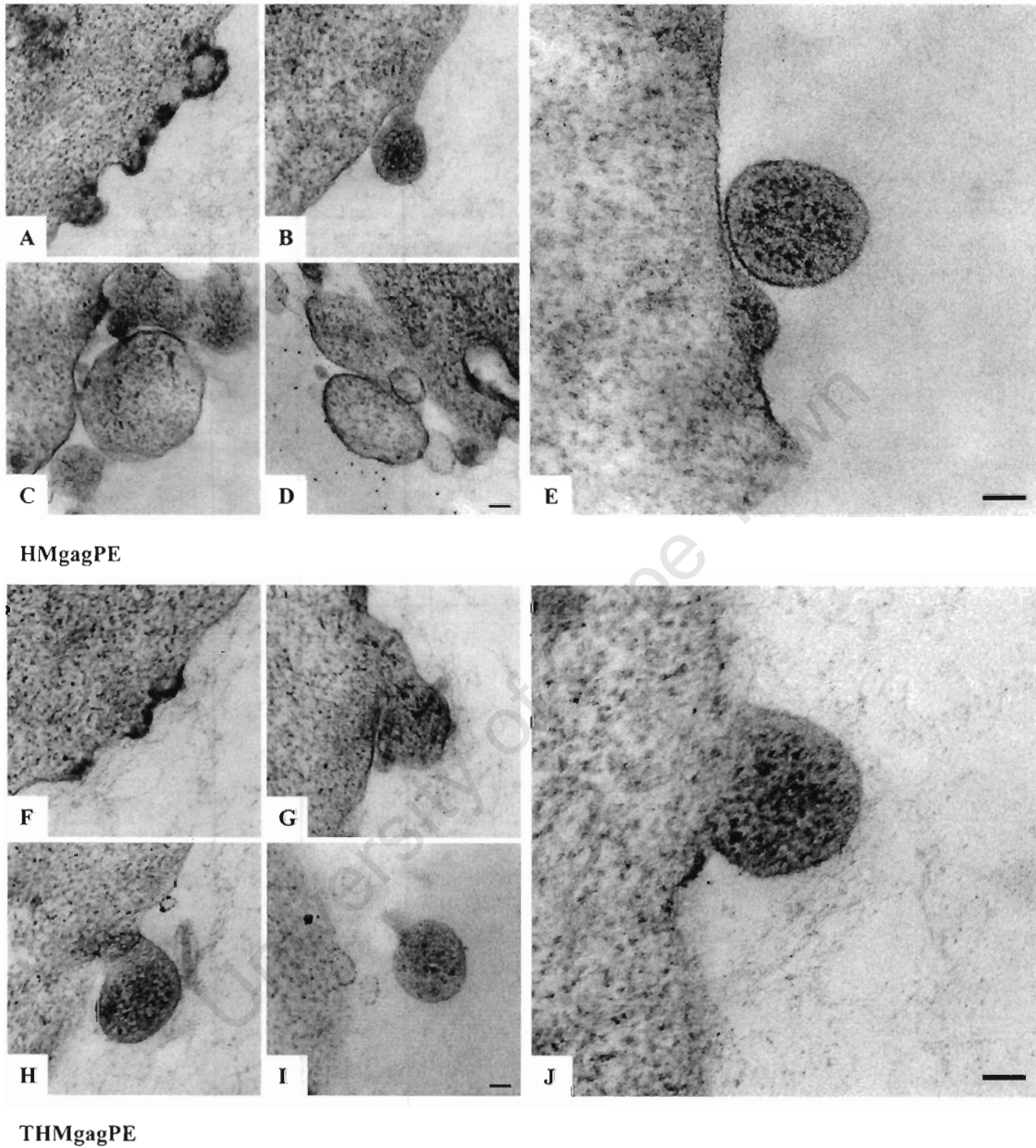


FIGURE 4.20. Budded HMgagPE and THMgagPE blebs.

Cell membrane associated blebs in cells infected with rAc-HMgagPE and rAc-THMgagPE. Bars = 100 nm (A-D, F-I), 100 nm (E, J).

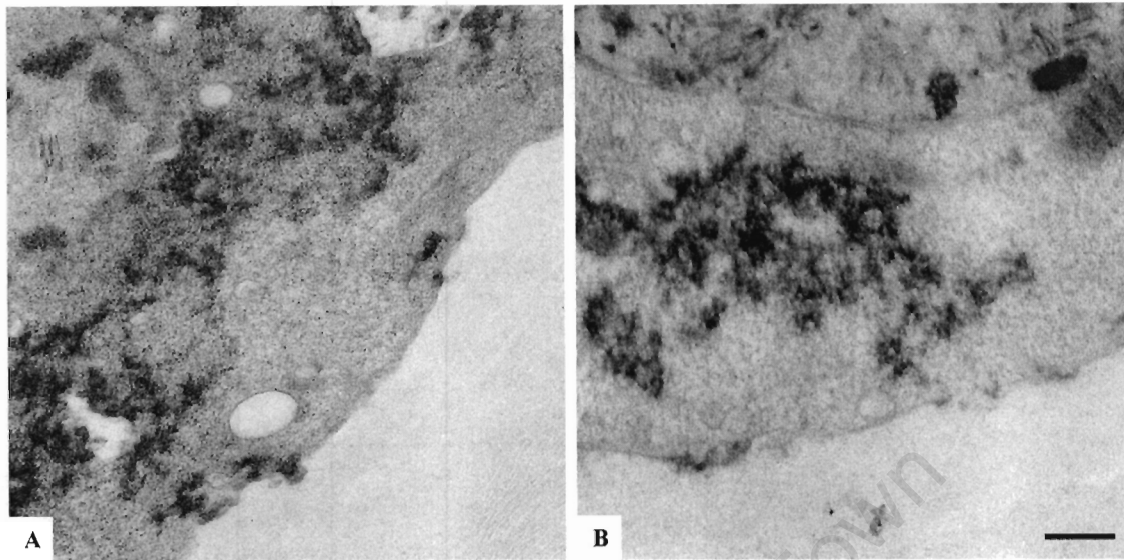


FIGURE 4.21. **Cytoplasmic HMgagPE and THMgagPE aggregations.** Accumulation of cytoplasmic aggregations (not discernibly ordered) in rAc-HMgagPE and rAc-THMgagPE infected cells. Bars = 100 nm (A-D, F-I), 100 nm (E, J).

4.3.4 SGC of Sf9 suspension culture supernatant

IEM was unsuccessful in confirming that the VLPs and VDPs seen by tsTEM contained the full-length Gag chimaeras (by detecting the fusion component). Therefore, concentrated particle purifications were required so that the protein composition could be analyzed by western blot. Using SCC and SXC, the levels of Gag-RT and Gag-RTTN were too low for this analysis, so SGC with 60 ml volumes of culture supernatant was conducted. This would also be the next step toward optimizing the production of VLP/VDP batches for immunogenicity testing, so a suspension culture system using Sf9 cells was chosen.

From the 10 RTTN-derived Gag chimaeras, the 5 fusions to HMgag (Fig. 2.10) were chosen for complete analysis of SGC samples. Evidence from western blots of culture supernatants (3.3.2.2), SCC samples (4.3.1.1), SXC samples (4.3.1.2) and tsTEM data (4.3.3.3) suggested that THMgag is not necessarily a superior carrier of larger antigens than HMgag. In this case HMgag-fusions would be preferable as potential vaccine candidates, as they contain more potential Gag epitopes.

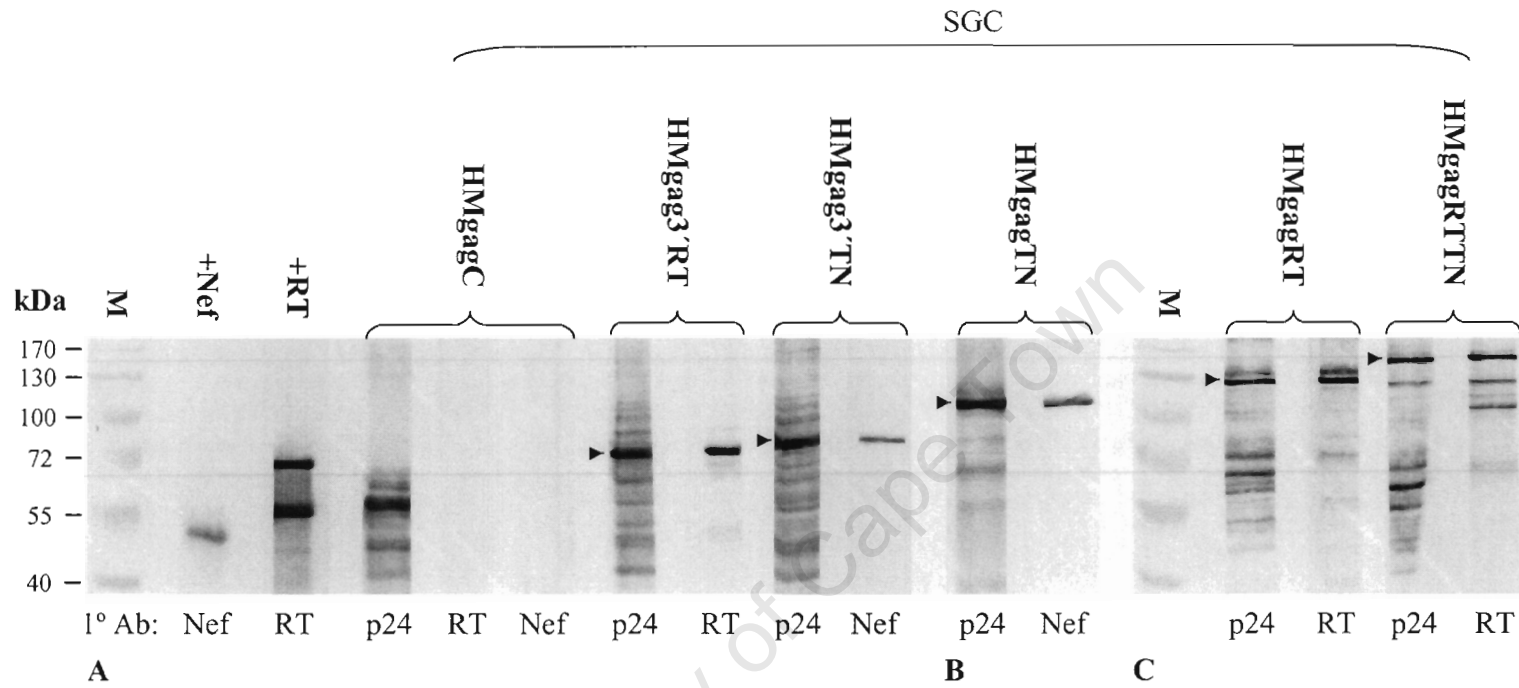


FIGURE 4.23. Purification of chimeric HMgag-fusion VLPs and VDPs from Sf9 suspension culture using SGC.

Particles were purified from Sf9 suspension cultures by centrifugation through a continuous sucrose gradient. Cells were infected with rAcNPV expressing the indicated constructs. In adjacent wells on the same gel (A-C), 30 μ l of SGC sample was loaded and membrane lanes were probed with p24-specific antiserum (p24), RT antiserum (RT) or anti-Nef mAb (Nef) as indicated. Control proteins: +RT = 100 ng, +Nef = 50 ng. M = MW marker, 1° Ab = primary antibody.

In western blots of purified preparations from the HMgag-fusion constructs (Fig. 4.23), samples were run in adjacent lanes on a single blot to confirm that different primary antisera/antibodies were indeed detecting the same band. The full-length proteins were present in all SGC samples, providing strong evidence that at least some of the particles visible by tsTEM for the corresponding constructs were composed of the complete chimaera. apTEM of the HMgag-fusion SGC samples (Fig. 4.23) confirmed the presence of particles of comparable size to those visible by tsTEM (Fig. 4.24). However, these particles were not highly concentrated in the samples used to prepare EM grids, particularly for the HMgagRT and HMgagRTTN SGC samples.

The 5 THMgag-fusions with RTTN components (Fig. 2.10) were also detected in initial SGC sample preparations (by western blot with p24-specific antiserum), but at a lower intensity than HMgag-fusions under the same conditions (data not shown). In pooled SGC preparations (from 120 ml of culture supernatant), each Gag-PE protein was just detectable with anti-SV5-Pk mAb, but not with p24-specific antiserum (data not shown).

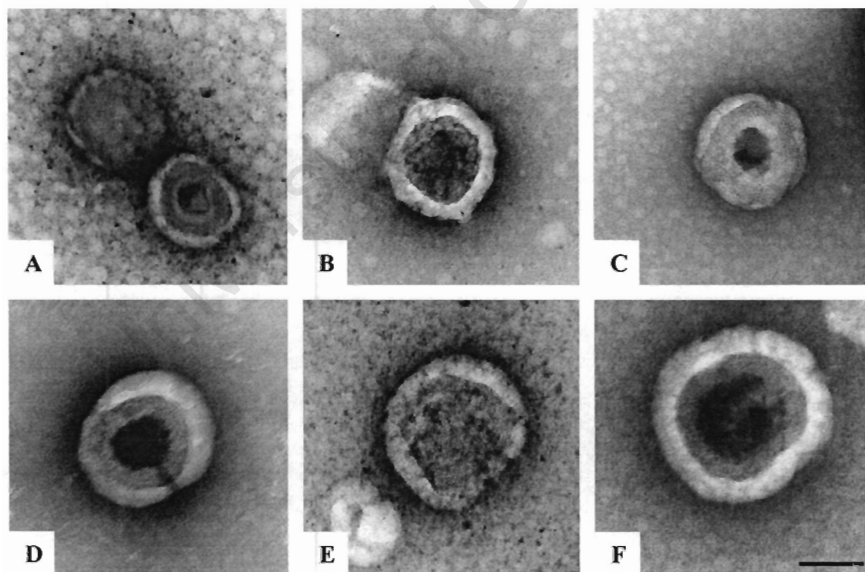


FIGURE 4.24. Structures resembling VLPs are present in Sf9 SGC samples.

The HMgag-fusion SGC samples were analyzed by apTEM: (A) HMgagC (B) HMgag3'RT (C) HMgag3'TN (D) HMgagTN (E) HMgagRT (F) HMgagRTTN. Bar = 100 nm, all micrographs at the same scale.

4.4 DISCUSSION

The expression of WTgagC, HMgagC and THMgagC led to the production of VLPs, which were indistinguishable from one another. The absence of p6 did not preclude VLP formation or visibly affect the particle morphology, consistent with previous reports using the baculovirus system (Royer *et al.*, 1991; Jowett *et al.*, 1992; Griffiths *et al.*, 1993). This observation supports the theory that p6 does not contribute to critical Gag-Gag interactions during immature HIV-1 virion assembly and release (Wang *et al.*, 1998; Spearman *et al.*, 1994). Furthermore, p6 does not appear to affect VLP size, unlike HIV virions where p6 has been shown to be a principle determinant of virion size, and the removal of p6 led to the production of very large HIV particles (Garnier *et al.*, 1998). It has been suggested that p6 is required for efficient detachment of budding virions from the cell membrane (Yu *et al.*, 1995), but the results of this study indicate that this L-domain function of p6 does not extend to Gag or chimaeric Gag VLPs produced in insect cells. Expression of the Myr⁻ Gag controls caused defined Gag microstructures to form within the cell but did not lead to membrane association of Gag, or the budding and release of extracellular VLPs. These results confirm that myristylation is required for VLP formation (Gheysen *et al.*, 1989), and support suggestions that it is involved primarily in forming stable membrane associations (Bryant & Ratner, 1990; Resh, 2004) rather than Gag multimerization (Morikawa *et al.*, 2000), which can occur in the absence of membranes (Gross *et al.*, 1998). It was noted that the expression of Myr⁺ Gag controls caused decreased adhesion of cells in monolayer culture relative to those expressing Myr⁻ Gag. Most likely, the budding and release of VLPs from the cell surface promoted cellular detachment.

The accumulation of HΔMgagC (which is synthesized in the cytoplasm) in the nucleus suggests that Pr55^{gag} contains one or more nuclear localization signals (NLSs) that are suppressed in the Myr⁺ protein. Since THMgagC accumulates in the cytoplasm in addition to the nucleus, p6 may contain its own NLS that could function synergistically with others in Gag (there are two present in MA (Scarlatà & Carter, 2003)) to cause the complete nuclear import evident in HΔMgagC expressing cells. Alternatively, the presence of p6 may increase the functionality of other NLSs in distant domains. Royer *et al.* reported similar results for analogous constructs in insect cells, but they noticed no morphological difference between nuclear and cytoplasmic structures (Royer *et al.*, 1991).

Furthermore, low levels of Myr⁻ Pr55^{gag} clusters were found in the cytoplasm (Royer *et al.*, 1991), whereas in this study these were not observed in rAc-HΔMgagC infected cells. The latter may be due to a difference in NLS sequences, as the two potential karyophilic signals that were identified in their Gag construct sequences (KKKYKLLKH and KSKKKA, (Royer *et al.*, 1991)) are not present in HΔMgagC (F.1).

The nuclear aggregations from Myr⁻ Gag constructs (Fig. 4.8B & 4.9D) showed indistinguishable structure using tsTEM, and therefore it is unlikely that p6 plays an important role in the assembly of these multilayered structures. Since THΔMgagC showed individual structures in the cytoplasm resembling VLPs, but larger aggregations in the nucleus, there may be a concentration-dependent effect controlling the type of structure formed. Alternatively, micro-environmental properties of the cytoplasm and nucleus may govern what structure will form in each region.

The budding of RTTN-derived particles at the cell surface agrees with IIF evidence for the localization of the RTTN-derived chimaeric proteins to the cell membrane (3.3.3.2). HMgag- and THMgag-based chimaeras carrying the same C-terminal sequence produced comparable particles (based on size and ultrastructure visible by tsTEM) for all RTTN-derived fusions. Therefore, for this set of chimaeras, the removal of p6 in the Gag carrier did not obviously alter particle morphology, and HMgagC could incorporate the same length C-terminal fusions as THMgagC. Griffiths *et al.* observed that ~50% of VLPs produced from in-frame V3 fusions to a p6-truncated Gag were found in the nucleus of insect cells (Griffiths *et al.*, 1993), but this was not observed for any of the Myr⁺ chimaeric Gag constructs in this study. It would appear that in the absence of p6, the V3 sequence interfered with cellular localization of the chimaeric protein, whereas in this study the fusion sequences did not have this effect.

Expression of Gag fused with 3'RT, 3'TN and TN led to the production of budded particles that exhibited a typical Pr55^{gag} VLP structure in cross section. TN was ~120 aa longer than the hypothesized limit (Deml *et al.*, 2005) for an in-frame fusion to Gag that would allow VLP formation. HMgagTN and THMgagTN carry the longest fusion within a type I in-frame VLP reported to date. The particles formed by expressing Gag-RT and Gag-RTTN constructs appear less compact, more granular and do not generally have a distinct electron dense ring of protein near the VLP periphery. A comparison of the particles formed by Gag-TN and Gag-RT fusion

proteins (Fig. 4.16 & 4.17) shows a clear transition from typical VLP morphology (Gag-TN VLPs) to budded particles with a less defined internal structure (Gag-RT VDPs). The Gag-RT and Gag-RTTN particles, termed here as VDPs, have in-frame fusions of 450 and 778 aa respectively. The Gag-RTTN VDPs carry the longest in-frame fusion to *gag* reported to date, that has still produced extracellular particles in the baculovirus system, albeit with differing ultrastructure to typical VLPs.

As the length of RTTN-derived fusions increased, the resultant chimaeric particles appeared larger (rising from ~100 nm to ~250 nm in average diameter), less compact in structure and more variable in size. However, there were exceptions to this trend. Thus, although the full TN polypeptide (322 aa) is almost double the length of the 3'TN polypeptide (169 aa), HMgag- and THMgag- fusions with TN generated similar sized VLPs to corresponding fusions with 3'TN. Likewise, the -RTTN (778 aa) fusions did not generate larger VDPs than -RT (450 aa) fusions; in fact the largest VDPs (up to 410 nm in diameter) were from THMgagRT. The VLP and VDP sizes based on average tsTEM measurements (Table 4.1) are most likely less than the actual average particle dimensions, since sections that do not pass through the center of the particle will show structures of smaller diameter. Therefore, the actual size range and heterogeneity of particles for each construct may be lower than is apparent from these measurements.

The chimaeric RTTN-derived particles can be purified from Sf9 suspension culture supernatant by SGC. The bands of lower MW observed in western blots for HMgagRT and HMgagRTTN (Fig. 4.23C), suggest that some of the particles visible by tsTEM may be composed of individual breakdown products or a combination of such proteins. This may partly account for the apparent size heterogeneity in the larger VDPs. However, the presence of the full-length protein band in SGC samples is sufficient evidence to show that chimaeric Gag particles with large in-frame C-terminal fusions (up to 778 aa) can be produced, which was not though possible until this study. This greatly expands the use of Gag-based antigen carriers as candidate HIV-1 vaccines.

Pr55^{gag} chimaeras with long C-terminal polypeptide additions have been reported previously (zur Megede *et al.*, 2003; Young *et al.*, 2004; Buonaguro *et al.*, 2001). Zur Megede *et al.* constructed vaccine plasmids encoding several variants on Gag-Pol in-frame C-terminal fusions by mutating the *gag-pol* frameshift signal (zur Megede *et al.*, 2003). However, although they

made a construct that encoded a C-terminal fusion to Pr55^{gag} of inactivated HIV-1 PR and RT, which was larger (149 kDa) than the largest construct in this study (HMgagRTTN, 144 kDa), Gag proteins from this construct were not detectable in the culture supernatant of transfected HEK293 cells by western blot. Young *et al.* made DNA vaccines expressing larger Gag-Pol chimaeras that were capable of forming VLPs in primate cell lines (Young *et al.*, 2004). However, these were generated by making use of the *gag-pol* frameshift signal, so that the added DNA sequence was in the same frame as *pol*. Consequently, only 5% or less of the Gag molecules in the resultant VLP would be expected to carry the extended protein sequence (Jacks *et al.*, 1988; Wilson *et al.*, 1988). Likewise, Buonaguro *et al.* used *gag-pol* ribosomal frameshifting to express Gag-Pol-Nef (~160 kDa) chimaeric VLPs (Buonaguro *et al.*, 2001), and Tobin *et al.* expressed chimaeric VLPs incorporating the C-terminal 273 aa of HIV-1 gp120 (Tobin *et al.*, 1997) - both groups used a baculovirus system.

In the present study, *gag* sequences were used where the *gag-pol* frameshift signal had been eliminated through prior sequence alteration to reflect optimal human codon usage (van Harmelen *et al.*, 2003). Thus, the 3'-terminal gene fusions in this study were in the same frame as the *gag* gene sequence, allowing for the expression of chimaeric VLPs incorporating a 1:1 ratio of Gag carrier protein to polypeptide insert. Thus, each VLP would in theory carry approximately 20-fold more fused polypeptide antigen than would result from the *gag-pol* frameshifting approach, so that these type I in-frame VLPs would be anticipated to show greater fusion sequence-specific immunogenicity than would type I frameshift VLPs carrying the same fusion.

The phenomenon of Pr55^{gag} VLP (Royer *et al.*, 1991; Vernon *et al.*, 1991, A. Jaffray, *pers. comm.*) and type I in-frame VLP (Brand *et al.*, 1995) budding into cytoplasmic vesicles has been reported previously, and was demonstrated for all chimaeric RTTN-derived constructs in some of the rAcNPV-infected cells. This agrees with IIF results (3.3.3.2), where defined regions of cytoplasmic IIF staining were observed in some cells in addition to cell membrane staining. Even if this occurs at a low overall level, the particle yield in purification strategies could potentially be increased by the incorporation of cell lysate, although this may complicate the protocol.

Although the relative particle yields from the RTTN-derived fusions could not be quantified (MOI not standardized among infections), the data from the western blots of culture supernatant

(Fig. 3.3) and numbers of particles budding per cell (tsTEM observations, Fig. 4.14 - 4.18) suggests a trend that particle yield decreases with chimaeric protein size. A similar trend has been noted previously for Env-derived C-terminal Gag fusions (Luo *et al.*, 1992).

Expression of the Gag-PE constructs in insect cells did not lead to VLP or VDP production. Rather the cells extruded irregular blebs and accumulated unstructured electron dense aggregations in the cytoplasm. Since the Gag-PE constructs produced similar intracellular- but lower extracellular levels of protein (3.3.2.3), and far fewer budding structures than the RTTN-derived constructs, these aggregations are hypothesized to be composed of chimaeric protein that remains in the cell. This hypothesis could not be proven with IEM, but was supported by IIF results (3.3.3.3). The low levels of Gag-PE proteins that could be detected in SGC samples (4.3.4) may have been due to the release of these aggregates into the culture medium (from limited cell lysis) in conjunction with the co-purification of the extracellular blebs (Fig. 4.20).

The fact that neither Gag-PE construct formed particles, whereas all RTTN-derived constructs formed either VLPs or VDPs, suggests that sequence composition, in addition to sequence length, plays a critical role in chimaeric Gag particle formation. PE is 155 aa long, and although shorter C-terminal Gag-fusions have not produced VLPs (Table 1.2), -3'TN (169 aa) and -TN (322 aa) fusions in this study did produce VLPs, and the fusion of RTTN (~5x longer than PE) to Gag carriers produced VDPs. Therefore, sequence composition may have more influence than sequence length *per se* on tolerable C-terminal fusions that can produce chimaeric Gag particles.

Particle formation is probably dependent on permissible protein interactions between the Gag carrier molecule and the fusion protein or peptide, along with the folding of the fusion component into an overall structure that does not sterically hinder particle assembly. It is possible that the artificial PE fusion adopted conformations that inhibited particle assembly in this manner without preventing membrane binding. Furthermore, it is possible that the presence or absence of the p6 region may indeed affect VLP formation for other C-terminal fusion sequences, even though this was not the case any of the fusion sequences used in this study. Ultimately, the length and sequence limitations of fusions relative to a specific Gag carrier that can be packaged into VLPs (as either C-terminal or internal Gag fusions) need to be empirically determined, as there is no indication from this study, or in the literature, that they can be accurately predicted.

CHAPTER 5

Conclusions

The development of an effective HIV vaccine is one of the greatest challenges facing mankind in the 21st century. In 2005, an estimated 7 400 people were newly infected with HIV each day in sub-Saharan Africa alone (UNAIDS, 2006). In a geographical region with over 12 million AIDS orphans in 2005 (UNAIDS, 2006), where the vast majority of AIDS patients do not have access to antiretroviral therapy, the need for a preventative HIV vaccine cannot be over-emphasized. Since traditional vaccine approaches have failed to elicit NAb responses to HIV (Letvin, 2005), novel vaccine approaches have been increasingly pursued. Chimaeric Gag VLPs are one such novel vaccine strategy that has shown encouraging results for stimulating cellular immune responses (Deml *et al.*, 2005; Wagner *et al.*, 1996b). Furthermore, since HIV-1C Pr55^{gag} VLPs successfully boosted murine immune responses to a pTHgagC DNA vaccine prime (Jaffray *et al.*, 2004), chimaeric Gag VLPs may boost the immune responses to DNA vaccines such as pTHr.HIVA and pTHr.grttnC.

In this study, 12 chimaeric Gag proteins were constructed and tested for their ability to form VLPs in insect cells using the baculovirus system. During this process a number of reported functions of the myristylation signal and p6 within Pr55^{gag} were reaffirmed. Myristylation was required for extracellular VLP formation but not Gag multimerization, and may contribute to Gag membrane targeting by suppressing NLSs in Gag. The absence of p6 did not affect the formation, release, size or ultrastructure of Gag-only VLPs, and p6 may contain a NLS or enhance the function of NLSs elsewhere in Gag.

This study demonstrated that Pr55^{gag} and Pr50^{gag} could tolerate far longer C-terminal polypeptide fusions additions than previously estimated, while still forming regular budded particles. It was also demonstrated, for RTTN-derived fusions at least, that the presence or absence of the p6 region of Gag does not have an impact on the length of polypeptide that can be carried C-terminally by Gag, or on the overall appearance and size of the particle formed by a specific chimaeric Gag protein. However, particle yield in Sf9 cells was apparently higher for constructs with a Pr55^{gag} carrier, but this observation would need to be confirmed with quantitative data.

A 322 aa fusion (TN) to Gag formed typical VLPs, while the full RTTN polyantigen (778 aa), which is almost twice the length of Pr50^{gag} itself, can be incorporated into an exact VDP analogue (THMgagRTTN) of the pTHr.grttnC DNA vaccine. In practice however, shorter chimaeras that produce higher yields of uniform VLPs, as opposed to lower yields of VDPs from longer chimaeras may be preferable for vaccine preparation purposes. Therefore, long protein additions (e.g. RT) could be carried in sections by several shorter chimaeras (e.g. HMgag3'RT) in a heterogeneous VLP preparation. However, the larger VDP chimeras (e.g. HMgagRT) are potentially more immunogenic and may elicit strong immune responses at lower doses.

Unlike any of the RTTN-derived Gag fusion proteins, the Gag-PE chimaeras did not form VLPs or VDPs. Therefore, a particle-based analogue to pTHr.HIVA could not be reliably produced. This was a surprising result given that PE (155 aa) was within the hypothesized fusion length limit of ~200 aa. Moreover, Gag-PE chimaeras have a fusion sequence that is ~5x shorter than the Gag-RTTN chimaeras, which formed VDPs. Therefore, it appears that the sequence composition of the fusion may have a greater bearing than sequence length *per se* on chimaeric Gag particle formation, at least for C-terminal Gag fusions.

The fusion length limit of ~200 aa for type I in-frame VLPs proposed by Deml *et al.* (Deml *et al.*, 2005) was based almost exclusively on VLPs with V3- and CD4BD-derived fusions (Table 1.1). The apparent importance of fusion sequence composition in type I in-frame VLP formation may reconcile the difference in tolerable fusion length observed in this study compared to the previously proposed limit. Although artificial polypeptides allow for the concentration of immunological epitopes in a small sequence space, our contrasting results obtained with Gag-PE and Gag-TN (shuffled epitope fusion sequence) suggest that artificial polypeptides must be tested empirically for VLP formation. The results with Gag-RT and Gag-RTTN chimaeras suggest that predominantly native peptide sequences may be preferable for generating chimaeric Gag VLPs with long fusions, and should be considered in future chimaeric Gag VLP design.

Recent work by Masemola *et al.* indicated that the majority of T-cell responses against HIV-1C proteins in humans were directed against Nef, Gag and Pol components, in this hierarchical order (Masemola *et al.*, 2004). Based on these data, the chimaeric Gag particles produced in this study could potentially be highly immunogenic; however this hypothesis can only be verified by

immunogenicity testing of selected constructs, probably starting in a mouse model. To this end, the titre of rAcNPV stocks would need to be determined, so that the optimal MOI for particle production from selected constructs in Sf9 cells could be obtained. The visualization of the particle band in the sucrose density gradient by white light *trans*-illumination is a convenient method for particle purification, but the same density range is not reliably selected in replicate purifications. Complete sucrose sedimentation analysis of selected constructs should allow for the concentration, purity and reproducibility of particle preparations to be improved by defining a constant fraction for purifying particles. Although apTEM was not suitable in this study for determining which chimaeras formed particles, this technique has application in assessing the quality of concentrated particle preparations to be used in immunogenicity testing.

Although it was possible to make a direct VDP analogue to pTHr.grttnC (THMgagRTTN), apparently only relatively low culture supernatant protein levels and VDP yields from either THMgagRTTN or HMgagRTTN were observed. HMgagTN is probably the most promising individual candidate HIV-1 vaccine produced in this study based on particle composition, structure and apparent yield. Therefore, a combination of HMgagTN and HMgagRT particles offers an attractive boost antigen combination to pTHr.grttnC. Evaluation of the CTL responses to HMgagTN and HMgagRT in mice should confirm whether this proposed prime-boost strategy with pTHr.grttnC is worth pursuing further.

In conclusion, this study has provided evidence that depending on the sequence composition, type I in-frame VLPs can tolerate much longer foreign gene sequences than previously thought possible. This greatly expands on the application of chimaeric Gag particles as candidate HIV-1 vaccine candidates. Herein, 10 HIV-1C VLP and VDP vaccine candidates have been produced, and should immunogenicity testing of selected particles in mice show favourable immune responses, these selected particles will be used in a prime-boost vaccination trial with pTHr.grttnC in primates. Such a vaccination regime, with HIV-1C based antigens, is applicable for sub-Saharan Africa where HIV-1C predominates and the need for an HIV vaccine is the greatest.

APPENDICES

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Appendix A – Reagents and standard methods

A.1 Bacterial strains and growth conditions

E. coli DH5 α TM and DH10BacTM (Invitrogen) were cultivated at 37°C in Luria-Bertani (LB) broth with agitation, while LB agar plates contained 1.5% Bacto Agar. Ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), gentamycin (7 μ g/ml), tetracycline (10 μ g/ml) isopropyl- β -D-thiogalactopyranoside (IPTG, 40 μ g/ml) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, 100 μ g/ml) were added to the medium where necessary.

A.2 Reagents and buffers

Reagents

All restriction and DNA modification enzymes were purchased from Roche, antibiotics were obtained from Sigma and all other chemicals were reagent grade or better.

Buffers

PBS (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl, 2.5 mM KCl; pH 7.4)

SDS-PAGE

5x sample loading buffer (315 mM Tris-HCl, 10% (w/v) SDS, 40% glycerol, 40% 2-mercaptoethanol, 0.025% bromophenol blue; pH6.8)

Western blotting

Transfer buffer (50 mM Tris base, 40 mM glycine, 20% (v/v) methanol; pH 9.2)

Blocking buffer (3% (w/v) BSA, 0.1% (v/v) Tween 20 in PBS)

Washing buffer (0.1% (v/v) Tween 20 in PBS)

IIF

PBS-IIF (10 mM Na₂HPO₄, 2.5 mM NaH₂PO₄, 140 mM NaCl; pH 7.4)

Blocking buffer (1% BSA (w/v) in PBS-IF)

A.3 DNA preparation and manipulation

All bacterial plasmids were maintained in *E. coli* DH5 α TM (Invitrogen). Plasmid preparations were obtained by using the Plasmid Mini Prep Kit (QIAGEN[®]) following manufacturer's instructions, and DNA concentration was determined by measuring absorbance at 260 nm with a NanoDrop ND-1000 Spectrophotometer. Restriction endonucleases, T4 ligase, shrimp alkaline phosphatase, mung bean nuclease, Klenow enzyme and appropriate enzyme buffers were used in cloning strategies as specified by the manufacturer (Roche). Restriction enzyme digest fragments and PCR products were purified from 0.8% agarose gels (Sambrook *et al.*, 2001) using the High Pure PCR Product purification kit (Roche).

A.4 DNA sequencing

Sequencing reactions were performed with the DYEnamicTM ET Dye Terminator Cycle Sequencing Kit for MegaBACETM (Amersham Pharmacia Biotech) according to manufacturer's instructions. Cycle sequencing products were synthesized using primers FBDS1, HMgagS1 (Table 2.2) or pUC/M13 forward and reverse sequencing primers (Promega, Cat.# Q5601/Q5421 respectively), Thermo SequenaseTM II DNA polymerase and the thermocycler cited above. These products were analyzed on a MegaBACE 500TM sequencer (Amersham Pharmacia Biotech) and the sequence data was examined with the corresponding Sequence Analyzer version 2.4 software.

A.5 Bac-to-Bac[®] Baculovirus Expression System

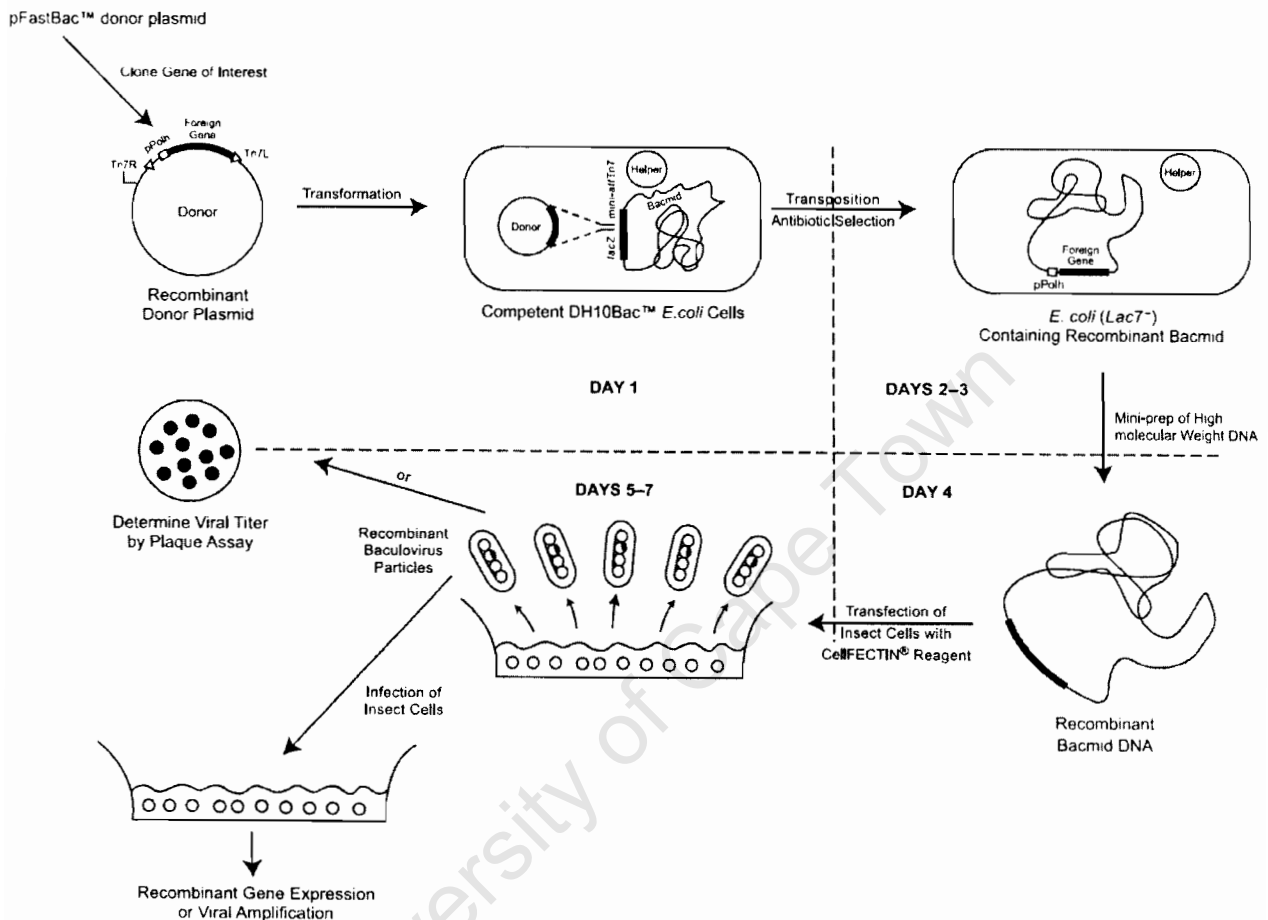


FIGURE A.1. Generation of recombinant baculovirus using the Bac-to-Bac[®] Baculovirus Expression System. The gene of interest is cloned into a pFastBac[™] donor plasmid (such as pFastBac[™]Dual) as a mini-Tn7 element under either the p10 or Polh promoter. The recombinant donor plasmid is transformed into competent *E. coli* DH10Bac[™] cells containing a resident bacmid (with a mini-attTn7 target site) and a helper plasmid. The bacmid *lacZ* α gene complements a *lacZ* deletion on the bacterial chromosome to produce blue colonies in the presence of a chromogenic substrate (e.g. X-gal) and the inducer IPTG. Transposition proteins provided *in trans* by the helper plasmid allow for the mini-Tn7 element in the donor vector to be transposed into the bacmid mini-attTn7 target site. Successful transposition disrupts the *lacZ* α gene and white colonies containing recombinant bacmids can be selected. Bacmid DNA is transfected into insect cells to generate recombinant baculovirus expressing the gene of interest, and plaque assays are the recommended method to determine the viral titre. *Source:* Bac-to-Bac[®] Baculovirus Expression Systems (Invitrogen), Instruction Manual, CAT. NO. 10359-016/10608-016.

Appendix B – Polymerase chain reaction (PCR) parameters

The PCR parameters are listed in PCR-1, with only the changes from this protocol given for subsequent PCR reactions. Plasmids and primers are detailed in section 2.2.1.

B.1 PCR-1

Amplification of the 5' region of *gag* (with abrogated myristylation signal) in pTHgagC was done using a Mastercycler[®] Gradient PCR machine (Eppendorf[®]) with the primers HMgagF1 and HMgagR1. Site directed mutagenesis (C₅→G₅) within *gag* restores the myristylation signal. Approximately 5 ng of pTHgagC was used as template in a final reaction volume of 50 μ l containing: 100 μ M dNTPs, 1.5 mM MgCl₂, 2.5U Super-Therm[®] DNA polymerase (Southern Cross Biotechnology), 1 \times Super-Therm[®] reaction buffer and 10 ρ M of each required primer. An initial denaturation step at 94°C (2 min) was followed by 30 cycles of denaturation at 94°C (30 s), annealing at 63°C (30 s), and elongation at 72°C (30 s). A final extension was performed at 72°C (1 min).

B.2 PCR-2

A *Bam*HI site was introduced at the 5' end of *gag* using primers HMgagF2 and HMgagR1, with approximately 1 ng of PCR-1 product as template DNA. The annealing temperatures during the 30 cycles of amplification were as follows: 3 cycles at 63°C, 3 cycles 54°C and 24 cycles 63°C.

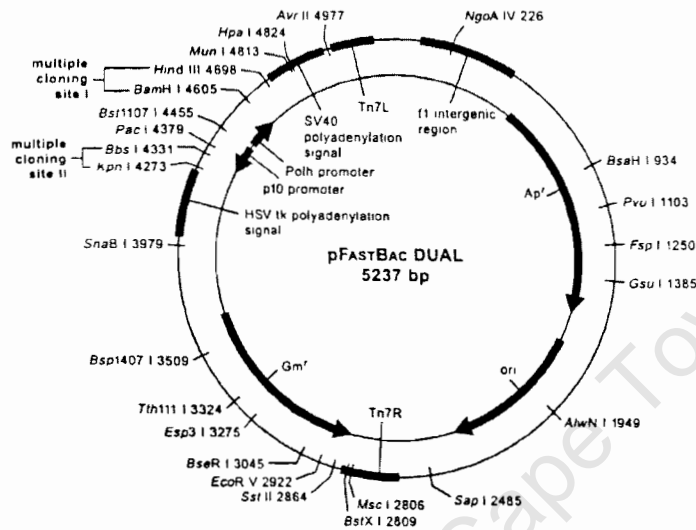
B.3 PCR-3

To remove the Gag stop codon (TAA), the 3' end of *gag* in pTHgagC was amplified with primers HMgagF3 and HMgagR2. In this case the annealing temperature was 56°C and elongation occurred for 1 min.

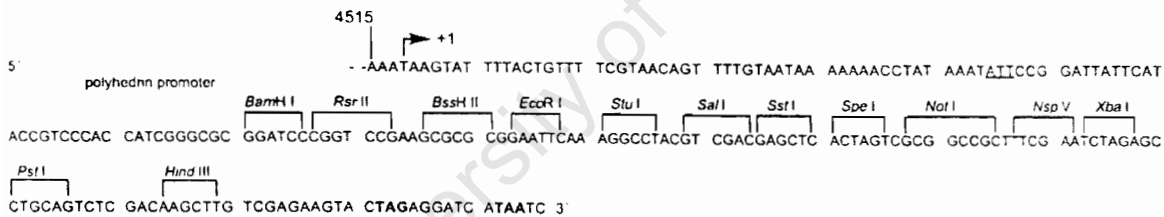
B.4 PCR-4

A stop codon (TAA) was inserted immediately downstream of the *Bgl*I site in *gag* (upstream of p6). Primers HMgagF3 and HMgagR3 were used to amplifying the *Nar*I-*Bgl*I region of *gag* in pTHgagC. Again the annealing temperature was 56°C and elongation occurred for 1 min.

Appendix C – pFastBac™ Dual vector map



pFastBac DUAL polyhedrin promoter region and MCS I:



+1 corresponds to the transcriptional start for the polyhedrin promoter.
ATT corresponds to the original translational start codon. The ATG was mutated to an ATT.
 An in-frame ATG codon must be provided by the cloned gene to initiate translation.
 Stop codons are shown in bold.

FIGURE C.1. pFastBac™ Dual: vector map and pPolh MCS.

Protein encoding sequences to be expressed by recombinant baculovirus in insect cell culture are cloned into the MCS downstream of the Polh promoter.

Source: Bac-to-Bac® Baculovirus Expression Systems, Instruction Manual, CAT. NO. 10359-016/10608-016.

Appendix D – Restriction enzyme digests of new plasmids.

TABLE D.1. Conformation of new plasmids by restriction enzyme digest patterns

| Plasmid ¹ | Size (bp) | Restriction enzyme(s) ² | Digestion products ³ (bp) |
|---|-----------|---|---|
| <i>Sub-cloning plasmids</i> | | | |
| pGEM-5'HMgag | 3611 | <i>EcoRI</i> <i>Alw44I</i> | 614, 2997 927, 1246, 1438 |
| pGEM-3'HgagNS | 3984 | <i>EcoRI</i> | 18, 969, 2997 |
| pGEM-3'THgag | 3837 | <i>EcoRI</i> | 18, 822, 2997 |
| pFBD-5'HMgag | 5817 | <i>BamHI, EcoRI</i> <i>HindIII</i> <i>BglI</i> | 601, 5216 666, 5151 1268, 2041, 2508 |
| pFBD-THMgagNS | 6560 | <i>Alw44I</i> <i>BamHI, EcoRI</i> <i>BglI</i> <i>BglII</i> <i>HindIII</i> | 225, 1223, 1246, 1768, 2098 1344, 5216 705, 1268, 2079, 2508 470, 6090 1409, 5151 |
| pFBD-HMgagNS | 6710 | <i>EcoRI, NarI</i> <i>HindIII</i> <i>SacI</i> | 953, 5757 1559, 5151 504, 6206 |
| <i>Plasmids expressing Gag control proteins</i> | | | |
| pFBD-WTgagC | 6765 | <i>EcoRI, SalI</i> <i>Alw44I</i> <i>PstI</i> | 1543, 5222 1246, 1768, 3751 963, 5802 |
| pFBD-HMgagC | 6713 | <i>EcoRI, NarI</i> <i>BamHI, EcoRI</i> <i>BglI</i> | 956, 5757 1497, 5216 858, 1268, 2079, 2508 |
| pFBD-THMgagC | 6563 | <i>EcoRI, NarI</i> <i>HindIII</i> <i>SacI</i> | 806, 5757 1412, 5151 357, 6206 |
| pFBD-HAMgagC ⁴ | 6707 | <i>HindII</i> <i>NcoI</i> | 181, 2471, 4061 317, 6397 |
| pFBD-THAMgagC ⁴ | 6557 | <i>HindII</i> | 181, 2321, 4061 |
| <i>Plasmids expressing chimaeric Gag proteins</i> | | | |
| pFBD-HMgag3'RT | 6997 | <i>Alw44I, EcoRI</i> <i>BamHI</i> <i>EcoRI, NotI</i> <i>PvuII</i> | 225, 834, 1223, 1246, 1701, 1768 1789, 5208 323, 6674 1972, 5025 |
| pFBD-THMgag3'RT | 6847 | <i>Alw44I, EcoRI</i> <i>BamHI</i> <i>EcoRI, NotI</i> <i>PvuII</i> | 225, 684, 1223, 1246, 1701, 1768 1639, 5208 323, 6524 1822, 5025 |
| pFBD-HMgagPE | 7147 | <i>Alw44I, EcoRI</i> <i>PvuII</i> <i>XhoI</i> | 225, 294, 834, 1223, 1246, 1557, 1768 1968, 5178 2264, 4883 |
| pFBD-THMgagPE | 6997 | <i>Alw44I, EcoRI</i> <i>PvuII</i> <i>XhoI</i> | 225, 294, 684, 1223, 1246, 1557, 1768 1819, 5178 2114, 4883 |

| Plasmid ¹ | Size (bp) | Restriction enzyme(s) ² | Digestion products ³ (bp) |
|------------------------------|-----------|--|---|
| pFBD-HMgag3'TN | 7176 | <i>Alw44I</i> , <i>EcoRI</i> <i>EcoRI</i> <i>NarI</i> , <i>XbaI</i> <i>PstI</i> | 225, 510, 834, 1223, 1246, 1370, 1768 510, 6666 506, 963, 5707 464, 6712 |
| pFBD-THMgag3'TN | 7026 | <i>Alw44I</i> , <i>EcoRI</i> <i>EcoRI</i> <i>NarI</i> , <i>XbaI</i> | 225, 510, 684, 1223, 1246, 1370, 1768 510, 6516 506, 813, 5707 |
| pFBD-HMgagTN | 7635 | <i>Alw44I</i> , <i>EcoRI</i> <i>BglI</i> <i>EcoRI</i> <i>NarI</i> | 225, 834, 969, 1223, 1246, 1370, 1768 219, 763, 798, 1268, 2079, 2508 969, 6666 1422, 6213 |
| pFBD-THMgagTN | 7485 | <i>Alw44I</i> , <i>EcoRI</i> <i>BglI</i> <i>EcoRI</i> <i>NarI</i> | 225, 684, 969, 1223, 1246, 1370, 1768 219, 648, 763, 1268, 2079, 2508 969, 6516 1272, 6213 |
| pFBD-HMgagRT | 8035 | <i>Alw44I</i> , <i>EcoRI</i> <i>BglI</i> <i>EcoRI</i> , <i>NotI</i> | 225, 834, 1044, 1223, 1246, 1695, 1768 1268, 2079, 2180, 2508 1361, 6674 |
| pFBD-THMgagRT | 7885 | <i>Alw44I</i> , <i>EcoRI</i> <i>BglI</i> <i>EcoRI</i> , <i>NotI</i> | 225, 684, 1044, 1223, 1246, 1695, 1768 1268, 2030, 2079, 2508 1361, 6524 |
| pFBD-HMgagRTTN ⁴ | 9047 | <i>BglI</i> <i>EcoRI</i> <i>HindIII</i> | 219, 807, 1268, 2079, 2166, 2508 2337, 6710 1040, 2856, 5151 |
| pFBD-THMgagRTTN ⁴ | 8897 | <i>EcoRI</i> , <i>HindIII</i> <i>NarI</i> | 2337, 6560 1040, 2706, 5151 2640, 6257 |

¹ Plasmid backbone abbreviations: pGEM = pGEM[®] – T Easy, pFBD = pFastBac[™]Dual. Encoded Gag proteins in pFBD plasmids are detailed in Fig. 2.10.

² Restriction enzymes were chosen based on important cloning sites within the plasmid backbone and insert. Digestion with *Alw44I* and *EcoRI* differentiates between HMgag- and THMgag-based constructs (834bp and 684bp fragments respectively).

³ Expected digestion products were calculated using DNAMAN[®] version 4.13 (Lynnon Biosoft) and the banding patterns were confirmed by agarose gel electrophoresis of digested plasmids.

⁴ Where cloning was non-directional, the expected fragments for an insert in the incorrect orientation are as follows: pFBD-HMgagRTTN (*HindIII*: 1439, 2457, 5151), pFBD-THMgagRTTN (*HindIII*: 1439, 2307, 5151), pFBD-HΔMgagC (*HindII*: 1015, 1509, 4061; *NcoI*: 1859, 4854) and pFBD-THΔMgagC (*HindII*: 1015, 1487, 4061). In each case these are easily distinguished from the correct orientation with the respective restriction enzymes.

Appendix E – Antisera and antibodies tested for use in IIF and IEM.

E.1 IIF

The antisera and antibodies ARP319, ARP432 (Table 3.2, 3.2.5); ARP313, ARP431 and D7320 (Table E.1) were used in IIF staining of rAcNPV infected Sf21 cells expressing HMgagC, THMgagC, HΔMgagC and THΔMgagC as well as an AcNPV negative control. No IIF staining could be detected using ARP319 and ARP313 with FITC conjugated anti-mouse IgG (1:500, Sigma). Probing with D7320 and secondary anti-sheep FITC conjugate (1:500, Sigma) also showed no IIF staining. When cells were probed with ARP431 and ARP432, the Gag expressing cells showed IIF staining that could not be readily differentiated from that of AcNPV infected cells (detection Alexa Flour® goat anti-rabbit, Molecular Probes)

TABLE E.1. **Additional primary antisera and antibodies used in IIF**

| Antigen | Host | Dilution | Details | Designation | Source |
|---------|--------|----------|--|-------------|------------------------------------|
| p24 | Mouse | 1:500 | Monoclonal antibody to HIV-1 p24/p55. | ARP313 | R. Fern and R. Tedder ¹ |
| p17 | Rabbit | 1:500 | Polyclonal antiserum to recombinant HIV-1 _{HXB2} p17 GST (<i>E.coli</i>). | ARP431 | G. Reid ¹ |
| p24 | sheep | 1:500 | Affinity purified polyclonal antiserum to HIV-1 p24. | D7320 | Aalto Bio Reagents ² |
| RT | Mouse | 1:500 | Monoclonal antibody to HIV-1 RT. Mapped to C-terminus (aa 431-531) | ARP384 | R. Fern and R. Tedder ¹ |

¹ National Institute for Biological Standards and Control (NIBSC), Centralised Facility for AIDS Reagents, Medical Research Council (MRC), United Kingdom (UK).

² Aalto Bio Reagents Ltd, Dublin, Ireland.

E.2 IEM

The antisera and antibodies ARP444, EVA3067.4, EVA 3068.2 (Table E.2); ARP428, 01-003, EVA3066 (Table 3.2, 3.2.5); EVA3018 (Table 3.4, 3.3.6) and ARP384 (Table E.1) were used in an IEM protocol (4.2.4.3) in an attempt to detect the RTTN-derived fusion components within chimaeric Gag particles. Results are summarized in Table E.3.

TABLE E.2. Additional primary antisera and antibodies tested in IEM

| Antigen | Host | Specificity | Designation | Source |
|---------|-------|---|-------------|------------------------|
| Nef | Sheep | Polyclonal antiserum to HIV-1 GST Nef | ARP444 | M. Harris ¹ |
| Nef | Mouse | Monoclonal antibody to HIV-1 BRU Nef, mapped to aa 161-180 | EVA3067.4 | K. Krohn ¹ |
| Nef | Mouse | Monoclonal antibody to HIV-1 JR-CSF Nef, mapped to aa 193-206 | EVA3068.2 | K. Krohn ¹ |

¹ National Institute for Biological Standards and Control (NIBSC), Centralised Facility for AIDS Reagents, Medical Research Council (MRC), United Kingdom (UK).

TABLE E.3. IEM with antisera/antibodies to the fusion components of Gag chimaeras

| Antigen | Antibody ¹ | Dilutions | Resin | Samples ² | Result ³ |
|---------|-----------------------|---------------------------|-----------------------|-----------------------|---------------------|
| SV5-Pk | EVA3066 | 1:50, 1:100, 1:200, 1:400 | Spurr's and LR White™ | HMgagPE and THMgagPE | NS |
| RT | ARP428 | 1:50, 1:100, 1:200, 1:400 | Spurr's and LR White™ | HMgagRT and HMgag3'RT | NS |
| RT | ARP384 | 1:50, 1:100 | Spurr's | HMgagRT and HMgag3'RT | ILA |
| RT | EVA3018 | 1:50, 1:100 | Spurr's | HMgagRT and HMgag3'RT | ILA |
| Nef | 01-003 | 1:50, 1:100, 1:200, 1:400 | Spurr's and LR White™ | HMgagTN and HMgag3'TN | NS |
| Nef | ARP444 | 1:50, 1:100 | Spurr's | HMgagTN and HMgag3'TN | NS |
| Nef | EVA3068.2 | 1:50, 1:100 | Spurr's | HMgagTN and HMgag3'TN | ILA |
| Nef | EVA3067.4 | 1:50, 1:100 | Spurr's | HMgagTN and HMgag3'TN | ILA |

¹ Detected with anti-sheep and anti-mouse 10 nm colloidal gold conjugate as appropriate (1:50, Sigma).

² In each case HMgagC was included as a negative control.

³ Same overall result was observed in each instance.

NS = Non-specific immunogold labeling, could not be differentiated from the negative control.

ILA = immunogold labeling absent.

Appendix F – Amino acid sequences

F.1 Amino acid sequences of Gag control proteins

Note: All constructs correspond to protein sequence from the start codon of Gag (ATG) until the stop codon (TAG, TAA or TGA) of the protein-encoding sequence.

HMgagC (492 aa)

```

1      MGARASILRG EKLDKWEKIR LRPGGKKHYM LKHIVWASRE LERFALNPGL LETSEGCKQI
61     MKQLQPALQT GTEELKSLYN TVATLYCVHE KIEVRDTKEA LDKIEEEQNK CQOKTQQAKA
121    ADGKVSQNYF IVQNLOQGMV HQAISPRTLN AWVKVIEEKA FSPEVIMFT ALSEGATPQD
181    LNTMLNTVGG HQAAMQMLKD TINEEAAEWD RLHPVHAGPI APGQMREPRG SDIAGTTSTL
241    QEQIAWMTSN PPIPVGDIYK RWIILGLNKI VRMYSVPSIL DIRQGPKEPF RDYVDRFFKT
301    LRAEQATQEV KNWMTDTLLV QNANPDCKTI LRALGPGATL EEMMTACQGV GGPGHKARVL
361    AEAMSQTNSG NIMMQRSNFK GPRRIVKCFN CGKEGHIARN CRAPRKKGCW KCGKEGHQMK
421    DCTERQANFL GKIWPSHKGR PGNFLQNRPE PTAPPAESFR FEETTPAPKQ EPIEREPLTS
481    LKSLFGSDPL SQ*

```

THMgagC (442 aa)

Identical to HMgagC, but without the sequence shown in bold (corresponding to a 50 aa C-terminal truncation).

HΔMgagC (492 aa) and THΔMgagC (442 aa)

Identical to HMgagC and THMgagC respectively, but with ala2 replacing gly2.

F.2 Amino acid sequences of C-terminal fusion sequences

Note: The six C-terminal fusion sequences are shown immediately following GKIWPSHKGR PG (THMgag-fusions) or LKSLFGSDPL SQ (HMgag-fusions).

3'RT (113 aa)

```

..EFKGGVYY DPSKDLIAEI QKQGDDQWTY QIYQEPFKNL KTGKYAKRRT THTNDVKQLT
EAVQKISLES IVTWGKTPKF RLPIQKETWE IWWTDYWQAT WIPEWEFVNS GRFRI*

```

PE (115 aa)

```

..EFPPIPVG EIYKRWIIFR DYVDRFYKTL RAIFQSSMTK ITLWQRPLVE RYLKDQQLLT
VYGVVWVWR PQVPLRPMTY KAVDLSHFLK EKGGLILKEP VHGVYHPDIV IYQYMDLTP
GPGVRYPLAC TPYDINQMLR GPGRAVVTIP NPLLGLD*

```

3'TN (169 aa)

```

..EFKALTS SNTAHNNPDC AWLQAQEEEE DVGFPVRPQV PLRPMTYKAA FDLSSFLLKEK
GGLEGLIHSK RRQDILDLDLV YHTQGYFPDW QNYTPGPGVR YPLTFGWCFK LVPVDPREVE
EANKGENNCL LHPMSQHGM E DADREVLRWV FDSSLARRHL AREKHPEYYK D*

```

TN (322 aa)

```

..EFKGMVGI SYGRKKRRQR RSTPPSSEDH QNPISKQPLP QTRGDPTGSE ESKKKVESKT
KTDPFDCKYC SYHCLVCFQT KGLGISYGRK KRMEPIDPNL EPWNHPGSQP NTPCNKCYCK
YCSYHCLVVG WPAVRERIRR TEPAAEGVGP ASQDLDKHGA LTSSNTAHNN PDCAWLQAQE
EEEDVGFPPVR PQVPLRPMTY KAAFDSLFFL KEKGGLEGLI HSKRRQDILD LWVYHTQGYF
PDWQNYTPGP GVRYPPLTFGW CFKLVDPDPR EVEEANKGEN NCLLHPMSQH GMEDADREVL
RWVFDSSLAR RHLAREKHPE YYKD*
    
```

RT (450 aa)

```

..EFCGKKAI GTVLVGPTPV NIIGRNMLTQ LGCTLNFPIS PIETVPVKLK PGMDGPKVKQ
WPLTEVKIKA LTAICEEMEK EGKITKIGPE NPYNTPIFAI KKEDSTKWRK LVDFRELNKR
TQDFWEVQLG IHPAGLKKK KSVTVLDVGD AYFSVPLDEG FRKYTAFTIP SINNETPGIR
YQYNVLPQGW KGSPAIFQAS MTKILEPFRA KNPEIVIQY MAALYVGS DL EIQHRAKIE
ELREHLLKWG FTTPDKKHQK EPPFLWMGYE LHPDKWTVQP IQLPEKDSWT VNDIQKLVGK
LNWTSQIYPG IKVRQLCKLL RGTKALTDIV PLTEEALEL AENREILKEP VHGVYYDPSK
DLIAEIQKQG DDQWTYQIQ EPFKNLKTGK YAKRRTHTN DVKQLTEAVQ KISLESIVTW
GKTPKFRPLPI QKETWEIWWT DYQATWIPE WEFVNSGRFR I*
    
```

RTTN (778 aa)

```

..EFCGKKAI GTVLVGPTPV NIIGRNMLTQ LGCTLNFPIS PIETVPVKLK PGMDGPKVKQ
WPLTEVKIKA LTAICEEMEK EGKITKIGPE NPYNTPIFAI KKEDSTKWRK LVDFRELNKR
TQDFWEVQLG IHPAGLKKK KSVTVLDVGD AYFSVPLDEG FRKYTAFTIP SINNETPGIR
YQYNVLPQGW KGSPAIFQAS MTKILEPFRA KNPEIVIQY MAALYVGS DL EIQHRAKIE
ELREHLLKWG FTTPDKKHQK EPPFLWMGYE LHPDKWTVQP IQLPEKDSWT VNDIQKLVGK
LNWTSQIYPG IKVRQLCKLL RGTKALTDIV PLTEEALEL AENREILKEP VHGVYYDPSK
DLIAEIQKQG DDQWTYQIQ EPFKNLKTGK YAKRRTHTN DVKQLTEAVQ KISLESIVTW
GKTPKFRPLPI QKETWEIWWT DYQATWIPE WEFVNSGRKL ATMVG
                                ISYGR KRRRQRRSTP
PSEDHQNP I SKQPLPQTRG DPTGSEESK KVESKTKTDP FDCKYCSYHC LVCFQTKGLG
ISYGRKKRME PIDPNLEPWN HPGSQPNTPC NKCYCKYCSY HCLV
                                VGWPAV RERIRRTEPA
AEGVGPASQD LDKHGALTSS NTAHNNPDCA WLQAQEEEEED VGFPVRPQVP LRPMTYKAAF
DLSFFLKEKG GLEGLIHSKR RQDILDWVY HTQGYFPDWQ NYTPGPGVRY PLTFGWCFKL
VPVDPREVEE ANKGENNCLL HPMSQHGMED ADREVLRWVF DSSLARRHLA REKHPEYYKD
*
    
```

RT

**Shuffled
Tat**

Nef

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