Properties of Recombinant HIV and SIV antigens

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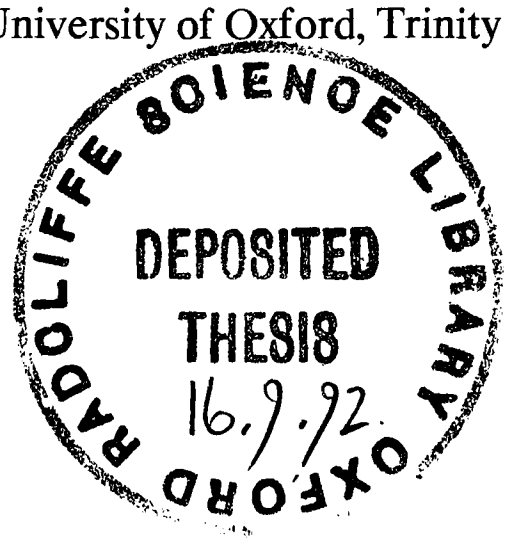

Preface

The work described within this thesis was carried out at the Natural Environment Research Council Institute of Virology and Environmental Microbiology, Oxford, between May 1989 and April, 1992. I am grateful to the Medical Research Council AIDS directed programme and the Evelyn Francis Jowett trust for financial support.

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This dissertation has not been submitted, whole or in part to any other university. This dissertation is the result of my own work and includes nothing, except where stated, which is the outcome of work done in collaboration.

Abstract

When expressed in *Spodoptera frugiperda* cells using recombinant baculoviruses, the HIV - 1 *gag* gene product, p55, self assembles to form immature HIV core like particles that are secreted into the culture medium. Using this system, the *gag* open reading frame was progressively truncated from the carboxy terminus, and each deleted Gag protein examined for its ability to produce core like particles. Deletions that removed the distal region of the Gag nucleocapsid domain, including both Cys - His motifs thought to function as RNA capture signals, did not disrupt particle formation. Analysis of the truncation mutants by north - western blot with a radiolabelled HIV - 1 RNA encapsidation signal, confirmed that only precursors with the Cys - His motif present were able to bind the probe. It was concluded that HIV - 1 Gag particle formation *per se* does not require RNA encapsidation. This finding clarifies uncertainties proposed previously regarding the role of RNA encapsidation in particle assembly. Further deletion mutants led to the delineation of the carboxy terminal boundary of a Gag assembly domain.

Properties of the SIV *env* encoded TM glycoprotein were also explored. Previous reports have found that the cytoplasmic tail of this membrane spanning protein was preferentially deleted *in vivo* when SIV was grown in a variety of cultured human cells. However, when propagated in cells of simian origin, the TM glycoprotein cytoplasmic tail was retained. Analysis of the function of this domain was addressed by expression of both the truncated and the full length proteins in insect cells using recombinant baculoviruses, coupled with a study of

their biochemical characteristics. A role for the cytoplasmic tail was identified in the post - translational modification and localization of the glycoprotein.

The use of the Gag particles in designing vaccines was investigated. Acting as non - infectious carriers of immunogenic antigens, the particles represent a safe and efficient means for presentation of epitopes for eliciting a protective immune response. Two possible approaches for loading the particles with foreign antigen were examined. In the first, the deleted portion of Gag was replaced with a sequence encoding the foreign antigen. The second method involved co - expression of Gag with another recombinant virus that expressed a foreign antigen on the surface of the cell. Both methods were found to be feasible, although limitations were identified when addition of the fusion protein promoted excess degradation of the Gag precursor.

Abbreviations

1°	Primary
2°	Secondary
3°	Tertiary
α	Alpha
ß	Beta
ϒ	Gamma
AcNPV	*Autographa californica* nuclear polyhedrosis virus
CA	capsid
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dATP	2' - deoxyadenosine 5' - triphosphate
dCTP	2' - deoxycytidine 5' - triphosphate
dGTP	2' - deoxyguanosine 5' - triphosphate
dTTP	2' - deoxythymidine 5' - triphosphate
ddATP	2' - dideoxyadenosine 5' - triphosphate
ddCTP	2' - dideoxycytidine 5' - triphosphate
ddGTP	2' - dideoxyguanosine 5' - triphosphate
ddTTP	2' - dideoxythymidine 5' - triphosphate
ER	endoplasmic reticulum
xg	Multiples of the standard acceleration of gravity (9807 mm/sec^2)
IN	integrase
kb	kilobase pairs
kDa	kilodaltons
LTR	long terminal repeat
MA	matrix
MW	molecular weight

mAb	monoclonal antibody
moi	multiplicity of infection
NC	nucleocapsid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PR	protease
RNA	ribonucleic acid
RNAase	ribonuclease
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
Sf	*Spodoptera frugiperda*
SRP	signal recognition particle
SU	surface glycoprotein
$TCID_{50}$	tissue culture infectious dose - 50% infection
TM	transmembrane glycoprotein

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General Introduction

Retroviruses

Viruses were first distinguished from bacteria as a cause of disease in 1898 by the work of Beijerinck (1898), on the tobacco mosaic virus and that of Loeffler and Frosch (1898), investigating the cause of foot and mouth disease. Thirteen years later in 1911, a retrovirus was discovered by P. Rous, who demonstrated that a type of malignancy in chickens could be transmitted by a "filtrable agent" (Rous, 1911a, b). This agent is now known as the Rous Sarcoma Virus (RSV), and is the prototype of a large family of RNA - containing viruses called the Retroviridae. The main classifying criterion of this family is the RNA - dependant - DNA - polymerase or reverse transcriptase enzyme (RT), which makes a DNA copy of the viral genomic RNA, a reversal of the normal flow of genetic information. On the basis of morphological, biological and molecular characteristics, members of the retroviridae family are classified into three sub - families, the oncovirinae, spumavirinae and lentivirinae. The oncovirinae family has been further sub - divided into types A, B, C and D according to specific morphological properties.

Members of the Retroviridae share a common genetic structure. This includes, in order from the 5' to the 3' end of the genome, the *gag* gene (group specific core antigen), the *pol* gene (encoding the polymerase), and the *env* gene which encodes the outer envelope glycoproteins. The genome is flanked by two long

terminal repeats (LTR), that include sequences for the promotion and enhancement of viral RNA transcription. In the provirus, the LTR consists of 3 regions, from 5' to 3', the U3 region, the R (repeat), region and the U5 region. Morphologically, the retroviral particle has a central core that contains the diploid RNA genome complexed with the reverse transcriptase enzyme and a specific transfer RNA molecule that primes DNA synthesis (Varmus, 1988). The particle is enveloped in a lipid membrane derived from the previous host cell, and is studded with virally encoded glycoproteins. The life cycle of the generic retrovirus begins with adsorption and fusion with the target cell plasma membrane mediated by the viral glycoproteins. Following uncoating in the cytoplasm, the reverse transcriptase synthesizes a double stranded DNA copy of the RNA genome which is integrated into the host cell genome as a provirus by the action of the virally encoded integrase (IN), on the LTRs. Transcription and translation, directed from the LTR, results in the production of viral proteins and genomic RNA. Either during of after assembly, the virus buds from the plasma membrane of the cell acquiring the lipid envelope and the viral glycoproteins therein (Levy, 1986). The intricacies of the life cycle of the immunodeficiency virus are discussed in more detail later.

Retroviruses have been linked to malignant disease in a wide variety of animals including humans (Levy, 1986). Although the mechanism of tumour induction is in general poorly understood, in some cases it has been attributed to an additional gene carried by the retroviral genome. The presence of this gene, known as an oncogene, confers upon the retrovirus the ability to transform infected host cells. The oncogene product has, in some cases, been shown to resemble normal cellular growth factors or their receptors, and their inappropriate or uncontrolled expression, *via* the viral LTR, to cause altered

growth kinetics of the cell. Not all retroviruses carry an oncogene, yet they can still cause disease, and to account for this observation other mechanisms of pathogenesis have been proposed. One of these suggests that integration of the proviral genome into the host chromosome may occur in the proximity of cellular genes that are involved in regulation of growth and / or differentiation. Enhanced transcription and translation of the cellular genes driven by the nearby viral promoter have been shown to result in altered growth characteristics.

Type C and D oncoviruses have also been associated with immunodeficiency and in some cases the host may die of opportunistic infections, resulting from impairment of the immune system, prior to the development of tumours. Members of the lentivirus sub - family have also been implicated as a cause of immunodeficiency, together with a range of other pathological conditions, that include neurological disorders, anemia, pneumonia and arthritis. Furthermore the progression of disease induction by the lentiviruses is slow, and is typically heralded by an extended incubation period of apparent clinical latency, where viral activity is either absent or very low. The lentivirinae, as do the spumavirinae, contain a number of auxiliary genes in addition to the *gag*, *pol* and *env* open reading frames and have become known as the complex retroviruses. It has been proposed that the auxiliary gene products of the lentivirinae may play a role in the maintenance and regulation of viral latency, accounting for the extended incubation period, which shall be addressed in more detail below (Levy, 1986).

Human Retroviruses

The most recent additions to the retrovirus family have been those of human origin. The first of these was the human foamy virus, a member of the spumavirinae sub - family, discovered in cultures of cells derived from human cancers in 1971. While it was observed to cause degenerative vacuolization of cultured cells *in vitro*, its link to disease, in man, has not yet been established (Achong *et al.*, 1971; Weiss, 1988). Nine years later, with the development of improved tissue culture techniques, a new member of the retrovirus family was discovered, and associated with adult T - cell leukemia in humans (Poiesz *et al.*, 1980). Named accordingly the human T - cell leukemia virus (HTLV type I), this was the first retrovirus to be identified as a cause of disease in humans. It was found primarily in populations of people from the southern islands of Japan, from the Caribbean and among the Black population in south - eastern United States of America (Poiesz *et al.*, 1980; Hinuma *et al.*, 1981). While many people were found infected, they did not invariably develop disease, and in the group that did, there was a characteristic long incubation period that persisted for up to 10 years. Genetically, the isolates, from Japan and the USA, were highly conserved by restriction fragment analysis. In tissue culture, the virus was observed to preferentially infect and replicate in human cell lines, although animal cells could also be infected, albeit less efficiently. While the virus has been observed to transform normal T - cells in culture into continuous cell lines, the mechanism of its pathogenesis in humans remains poorly understood (Hoshino *et al.*, 1983; Nagy *et al.*, 1983). A variant of this virus was recovered from a patient with hairy cell leukemia and termed the human T - cell leukemia virus type II (HTLV - II). However its link to disease in man has not yet been established (Kalyanaraman *et al.*, 1982).

Discovery of AIDS

In 1981, a report was published that documented four male homosexual patients who had presented with *Pneumocystis carinii* pneumonia, extensive mucosal candida infection and chronic viral shedding (Gottlieb *et al.*, 1981). Analysis of their peripheral blood T - cell sub - population, revealed virtual elimination of the Leu 3+ helper / inducer subset, coupled with an increased percentage of the Leu 2+ suppressor / cytotoxic subset, which amounted to an overall inversion of the ratio of these T - cell sub - populations by comparison to healthy patients. Their immunoprolific responses to soluble antigens were markedly attenuated, and the patients were both anergic and lymphopenic. This was the first report of a syndrome, that is now commonly known as the acquired immunodeficiency syndrome (AIDS), and is transmitted primarily by blood to blood contact, or through transmission of blood products. In the developed nations, promiscuous homosexual contact in populations of gay men, as well as the sharing of improperly sterilized needles in intravenous drug using populations, were identified as the main routes of transmission of the disease in the early stages of the epidemic. Heterosexual contact to a lesser extent, has also been found to be a cause of transmission, while spread *via* casual contact has been limited.

The AIDS epidemic is now widespread, with nearly half a million cases reported to the World Health Organization up to the 1st January, 1992. Of these reports, just over 200,000 are from North America, with 65% of the cases in homosexual or bisexual men, constituting the highest risk group. Approximately 30% of cases have been reported in the intravenous drug using population, with most of the remaining cases having been reported in groups of haemophiliacs, blood transfusion recipients and those that have acquired the disease through

heterosexual contact (WHO Report, 1992). The pattern of infection observed in the USA, is similar to that seen in other parts of the Western World, however in developing nations, particularly those in Africa, which has the second largest number of reported cases (130,000), the distribution is more evenly spread between males and females. The cause for this different distribution pattern, although not well understood, has been thought to result from differing levels of hygiene and patterns of sexual behaviour. While these statistics document the number of cases of AIDS disease reported, the number of people who are infected carriers, but remain healthy has been estimated to be more than 2 million Worldwide (Adler, 1991). The AIDS epidemic thus represents a serious problem to public health services, particularly in the developing countries, where resources are limited.

Discovery of HIV

Two years after the initial reports of AIDS, a new retrovirus was isolated from peripheral blood lymphocytes in tissue culture, derived from patients suffering from the immunodeficiency syndrome (Barré - Sinoussi *et al.*, 1983). After further characterization combined with the observation that antibodies raised against the new retrovirus were cross reactive to antigens derived from the HTLV family, the new retrovirus was designated the human T - cell lymphotropic virus type III (HTLV - III), to indicate its similarity to the HTLV family (Gallo *et al.*, 1984; Schupbach *et al.*, 1984). At about the same time, a group of researchers based in France, reported the isolation of a retrovirus from peripheral blood lymphocytes derived from a patient who had been suffering from lymphadenopathy, a condition that typically precedes the onset of AIDS.

This retrovirus was called the lymphadenopathy associated virus (LAV), to emphasize the type of symptoms thought typical of its pathogenesis (Barré - Sinoussi *et al.*, 1983).

During this period, samples were regularly exchanged between these two laboratories, and it wasn't until much later, when these viral isolates were cloned and sequenced, that it became apparent that they were nearly identical. Following numerous investigations, the isolates were found to be the result of contamination by one strain (now known as HIV_{LAI}), which was particularly well adapted to growth in tissue culture (Gallo, 1991; Reitz *et al.*, 1991; Wain - Hobson *et al.*, 1991).

Shortly after the first two reports of a new retrovirus, a third laboratory identified another retrovirus, in specimens taken from patients suffering from AIDS in San Francisco (Levy *et al.*, 1984). This was called the AIDS - associated retrovirus (ARV). Currently DNA sequences from 28 separate isolates of HIV have been published, derived from different individuals from a variety of geographical locations, all these isolates are distinguishable from one another (Myers *et al.*, 1991). In a series of studies, the sequencing of PCR amplified fragments of the HIV genome derived from a single patient over several years, demonstrated that the clones were all highly related but distinguishable from one another. It was thus apparent that the virus existed as a quasi - species, where no two samples, from the same individual, were identical (Meyerhans *et al.*, 1989; Delassus *et al.*, 1991; Martins *et al.*, 1991). This genetic variation was notably different from the pattern seen from isolates of the HTLV family, where the genome was generally well conserved. Morphologically, the new retroviruses could be distinguished from the HTLV group, with the use of electron

microscopy. It was found that while the HTLVs display type C oncovirus morphology (centrally located isometric core), the new retrovirus more closely resembled members of the lentivirus sub - family (cone shaped core). In tissue culture there were also differences noted, the HTLV group immortalized T - cells, while the new virus induced cytopathic changes that lead to cell fusion, syncytial formation with giant cells and balloon degeneration (Gallo *et al.*, 1984; Klatzman *et al.*, 1984; Levy *et al.*, 1984; Levy, 1986).

The Link between HIV and AIDS

The etiological link between HIV and the disease AIDS, was initially proposed by Popovic and colleagues (Popovic *et al.*, 1984), who demonstrated that the virus could be readily isolated from patients presenting with AIDS and pre - AIDS conditions. Immunological evidence was also found, where antibodies, directed against the HIV proteins, were identified in sera from AIDS and pre - AIDS patients, but not in control groups (Sarngadharan *et al.*, 1984). At first these links were tenuous, however, over the last decade they have been reinforced by an accumulation of molecular, immunological and epidemiological data, such that HIV has become accepted as the current etiological agent of AIDS. An international committee of virologists recommended that the retrovirus be called, universally, the human immunodeficiency virus (HIV), to indicate the strong association of this virus with the nature of the disease it caused in humans (Coffin *et al.*, 1986).

The Natural History of Infection

The natural history of infection with HIV usually begins with a seroconversion illness. The symptoms occur within 6 weeks of first contact with the virus and are non - specific, varying widely from almost no symptoms to a glandular fever like illness sometimes coupled with acute encephalitis and meningitis. During this period, which can extend up to 3 months, virus is typically found in the blood (viremia), and evidence for high levels of viral replication reported. After seroconversion, when antibodies first appear in the blood stream, a period of clinical latency ensues that is characterized by low levels of viral replication and a constant or slowly decreasing number of CD4 positive T - cells. Within 10 years of the initial contact with the virus, approximately 50% of male homosexuals progress to disease (Biggar, 1990). Throughout this period of latency, it is difficult to isolate virus from the blood, and viral antigens are often undetectable. In some patients, however, the period is interspersed with minor and short lived upsurges of viremia (Ratner, 1989; Nowak *et al.*, 1991). Progression to disease is heralded by bouts of persistent generalized lymphadenopathy (PGL), from which approximately two thirds of patients recover. The remainder go on to develop the AIDS related complex (ARC), which is typified by fever, weight loss, diarrhoea, night sweats and immune suppression. The final phase of pathogenesis of the disease, called full blown AIDS, constitutes an increase in severity of the ARC symptoms, together with a series of opportunistic infections, the most common of which is *Pneumocystis carinii* (Adler, 1987). Some patients may also develop malignant tumours (Smith,N and Spittle, 1991), Kaposi's sarcoma and malignant lymphomas being the most commonly seen types, while others develop severe neurological disorders (Carne, 1991). During the development of AIDS, viral isolation

becomes increasingly easy, with the proportion of infected cells in peripheral blood 2 to 3 orders of magnitude greater than in asymptomatic patients (Ho *et al.*, 1989).

Three years after the discovery of HIV, a closely related retrovirus was isolated from AIDS patients in West Africa (Clavel *et al.*, 1986). This retrovirus, although very similar to HIV in both biological and morphological properties, could be distinguished by both serological assays and genetic profile (Guyader *et al.*, 1987). To distinguish it from the earlier HIV isolates, it was called HIV type 2 (HIV - 2), with the original isolates being renamed to HIV type 1. The amino acid sequence divergence between the two viruses was found to be approximately 45% (Guyader *et al.*, 1987; Desrosiers, 1990), although this was not uniformly distributed across the genome being mainly localized to the *env* region (Chakrabarti *et al.*, 1987). Pathogenically this virus appeared less virulent in humans than the HIV - 1 strains.

Discovery of Non - Human Immunodeficiency Viruses

With the discovery of HIV in humans, a search for related retroviruses in other primates was initiated. This led to the identification in 1985, of a non - human primate retrovirus reported by Kanki and colleagues working at the New England Regional Primate Research Centre (Daniel *et al.*, 1985). Isolated from rhesus macaques (*Macaca mulatta*), the viruses were T - cell tropic with lentivirus morphology similar to HIV, and shared immunologic cross reactivity (Kanki *et al.*, 1985). Because of the degree of similarity of this retrovirus to HIV and the nature of its pathogenesis, it was originally called the simian T - cell

lymphotropic retrovirus (STLV - III), to indicate its relation to HTLV - III, the name for HIV at the time, but is now known as the simian immunodeficiency virus (SIV_{mac}). Comparison of the nucleic acid sequences of the viruses, showed 75% similarity between SIV_{mac} and HIV - 2, while both show only 40% homology to HIV - 1. This was reflected in the *pol* gene products, which are the slowest evolving proteins of the retroviruses (Chakrabarti *et al.*, 1987; Schneider and Hunsman, 1988; Doolittle *et al.*, 1990). Further isolates, identified in African green monkeys (*Cercopithecus aethiops*), termed SIV_{agm}, and Gabon mandrills (*Papio (Mandrillus) sphinx*), SIV_{mnd}, have revealed less similarity to HIV - 2 (60%), in their *pol* gene products, being the same as their similarity to HIV - 1 (Daniel *et al.*, 1988; Fukasawa *et al.*, 1988; Tsujimoto *et al.*, 1988). Another SIV isolate from a sooty mangabey (*Cercocebus atys*), SIV_{sm} has shown greater similarity (85%), to HIV - 2 (Hirsch,VM *et al.*, 1989b), while in 1990 an isolate from a wild chimpanzee (*Pan troglodytes troglodytes*), termed SIV_{cpz}, showed greater homology to HIV - 1 (85%), than to either HIV - 2 (60%), or other SIV isolates (60%) (Huet *et al.*, 1990). It has been suggested that these isolates may represent links in the chain of evolution of HIV, however the chronology of the virus crossing species barriers and the origins of the immunodeficiency viruses themselves remains unclear.

In recent years immunodeficiency viruses of other vertebrates have been reported. A bovine immunodeficiency virus (BIV), was identified in cattle (Gonda *et al.*, 1987), with 52% amino acid sequence homology in the *pol* domain to HIV and feline immunodeficiency virus (FIV), in domestic cats (Pedersen *et al.*, 1987), which was found to be more closely related to equine infectious anaemia virus (EIAV), and to visna virus, than it was to the HIVs or SIV (Olmstead *et al.*, 1989). These non - human immunodeficiency viruses are being

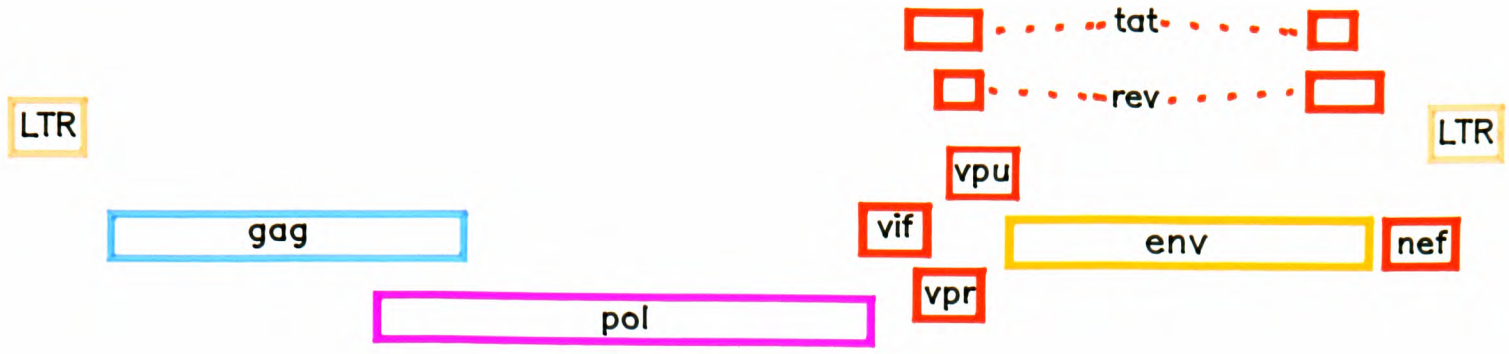
further characterized with the aim of developing an animal model for AIDS in humans in which to test drugs and potential vaccines. However, SIV infection of monkeys currently represents the most characterized of the potential animal models and several vaccine candidates and immunization regimes are presently being assessed (Gardner and Luciw, 1989; Newmark, 1989; Groopman, 1991; Hu *et al.*, 1992). Additionally, the SIV / monkey model has been used for studying the roles of individual genes in the infection process *in vivo* (Kestler *et al.*, 1991).

In pursuit of developing a treatment or vaccine for AIDS in humans there has been a strong drive toward understanding the molecular biology of the immunodeficiency viruses. The great majority of data gathered thus far has been derived from studies on HIV type 1, however many of the mechanisms involved in the replication of this virus appear to be shared by both HIV type 2 and SIV. The genomic organization of the immunodeficiency viruses is more complex than is typical for the retrovirus family (Cullen and Warner, 1990). In addition to the characteristic *gag*, *pol* and *env* open reading frames, flanked by the long terminal repeats (LTR), the genome also contains several smaller open reading frames that encode proteins which are translated from singly or multiply spliced messenger RNA (mRNA), species (figure 1). Some of these smaller proteins have regulatory functions in transcription, translation and messenger transport while the roles of others remain unclear. A brief summary of the role of each of the proteins encoded by HIV as a representative of the family of immunodeficiency viruses follows. Throughout this study, the standard nomenclature introduced for viral proteins, common to all members of the Retroviridae, has been adopted (Leis *et al.*, 1988). For genes that are found only in the immunodeficiency virus group, the standard nomenclature laid down by Gallo and colleagues has been used (Gallo *et al.*, 1988).

Figure 1.

The HIV - 1 genome

The upper section of the drawing is a diagrammatic representation of the HIV - 1 genome showing the *gag* gene (blue), *pol* gene (magenta) and *env* gene (orange). The auxiliary genes *tat*, *rev*, *vpu*, *vif*, *vpr* and *nef* are shown in red, with the genome flanked by the viral LTRs (peach). Shown in the lower half of the diagram are the products of the *gag*, *pol* and *env* open reading frames, with their fully processed subunits below. Each subunit is labelled and the MW given in parentheses (kDa).



Gag precursor

(p55)

Polymerase precursor

(p160)

Envelope precursor

(gp160)

Matrix (p17)

Capsid (p24)

(p15)

(p7) (p6)

Nucleocapsid

Surface (gp120)

Transmembrane (gp41)

Protease (p10)

Reverse Transcriptase (p64/p51)

Integrase (p32)

0 1 2 3 4 5 6 7 8 9

Kilobases

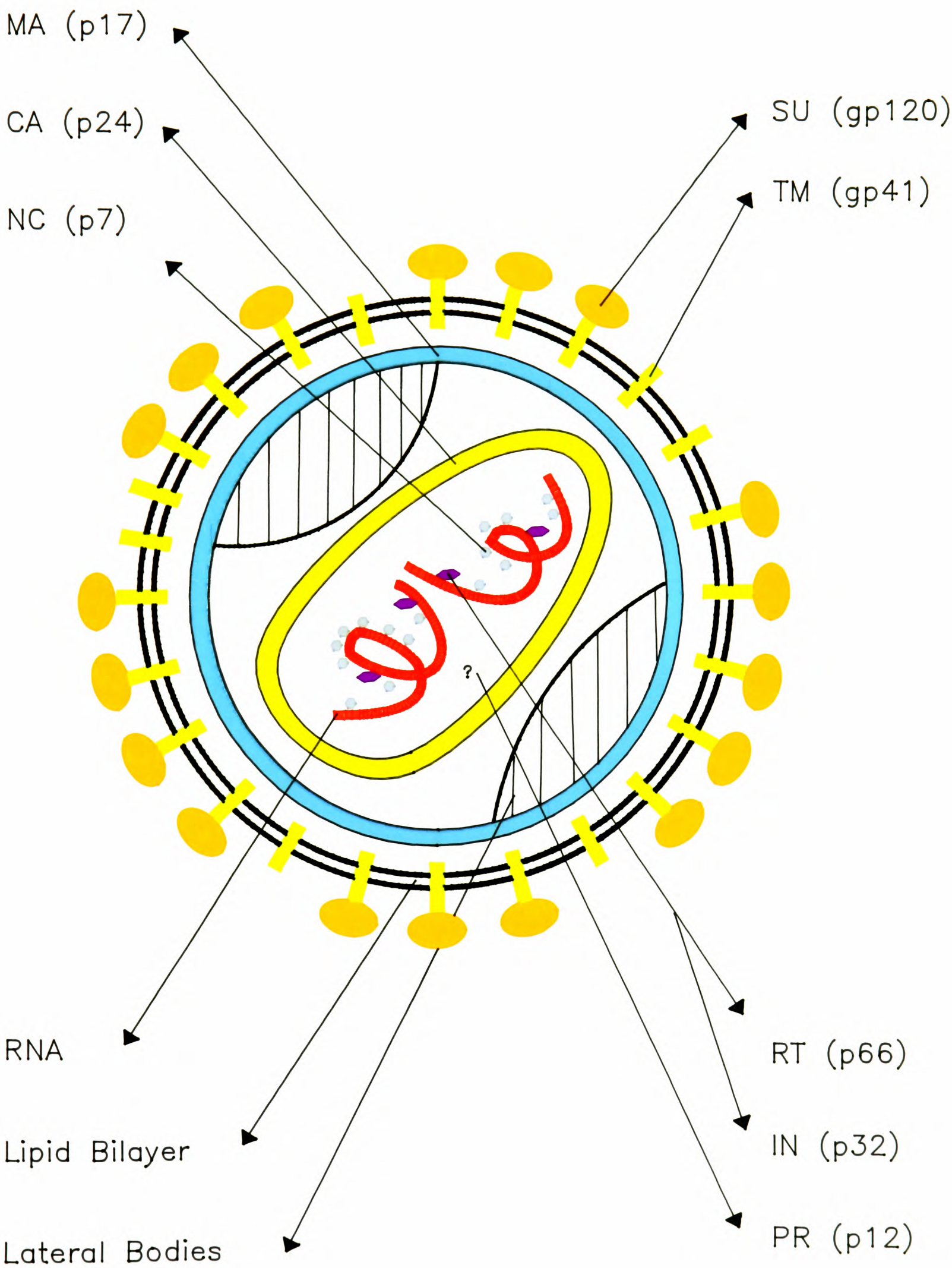
Human Immunodeficiency Virus: Structural Proteins

The *gag* or group antigen gene (figure 1), encodes a 55 kDa protein (p55), whose role is primarily structural. The p55 precursor undergoes post - translational modification where cleavage of the amino terminal methionine exposes a glycine residue which is subsequently myristoylated in the cytosol. The myristoylation is necessary and sufficient to direct the precursor to the plasma membrane where oligomerization and assembly of the virus particle takes place (Magee and Hanley, 1988; Gheysen *et al.*, 1989; Gottlinger *et al.*, 1989; Bryant and Ratner, 1990). The virus particle, as it is being formed, causes an evagination of the plasma membrane which culminates in its budding and release from the cell. Either soon after or during release from the cell, the protease begins cleavage of the Gag precursor into its four major domains, p17, p24, p7 and p6 (Tritch *et al.*, 1991). This process, referred to as particle maturation, coincides with the condensation of an outer electron dense ring to a cone shaped core (Gelderblom *et al.*, 1988). The myristoylated p17 domain or matrix protein (MA), derived from the amino terminus of the Gag precursor, covers the inner side of the viral envelope (figure 2), and may act as a scaffold during the assembly of the virus at the plasma membrane. The capsid antigen (CA), p24, which is the only phosphorylated of the Gag derived proteins, is found localized to the central conical core of the virus after maturation (Gelderblom *et al.*, 1987). p24 is also the most abundant of the proteins found in the virion. p7 and p6 are derived from the p15 intermediate precursor which is cleaved from the carboxy terminus of the Gag protein. The nucleocapsid domain (NC), p7, contains two repeats of a highly conserved Cysteine - Histidine motif (Cys - His), that is thought to play a role in the binding and encapsidation of nucleic acid, (addressed in Chapter Two). The role of p6 has recently been shown to facilitate the release of

Figure 2.

Slice view of the HIV - 1 virus particle

Diagrammatic representation of a section through a mature HIV - 1 virus particle showing the location of the viral components. The 2 letter codes used are according to standard retroviral nomenclature (Leis *et al.*, 1988), and are as follows: MA - matrix, CA - capsid, NC - nucleocapsid, SU - surface glycoprotein, TM - transmembrane glycoprotein, RT - reverse transcriptase, IN - integrase and PR - protease. The MW are indicated in parentheses (kDa). The diploid genomic RNA (red), is represented within the central core, while the lateral bodies (hatched black), of unknown function and composition are also indicated. The p6 domain, derived from the Gag precursor, not shown, is of unknown location.


MA (p17)
CA (p24)
NC (p7)
SU (gp120)
TM (gp41)
?
RNA
Lipid Bilayer
Lateral Bodies
RT (p66)
IN (p32)
PR (p12)

assembled particles from the plasma membrane (Gottlinger *et al.*, 1991), the mechanism of this however remains poorly understood. Based on electron microscopy, a core - envelope - link (CEL), has been proposed that is located between the narrow end of the core and the external matrix / envelope layer. It was speculated that the p6 protein may be a constituent of this structure by analogy to a proline rich protein of EIAV, however confirmation of this has yet to be found (Gelderblom, 1991).

The largest open reading frame, the *pol* gene, is translated as a fusion protein that arises as a result of a ribosomal frameshifting event as the full length HIV transcript is read (see Chapter One for detail). This 160 kDa precursor encodes the protease (PR), reverse transcriptase (RT), and integrase (IN), enzymes (figure 1), with the latter two found localized to the central core of the mature virus particle and associated with the diploid genomic RNA (figure 2). The protease is autocatalytic and is functional as a homodimer (Wlodawer *et al.*, 1989; Krausslich, 1991), its role, as previously referred to, is in the processing of the Gag and Pol precursors during maturation of the newly budded virus particles to their subunits. It has been demonstrated that when the protease was rendered inactive the released virions remained in an immature state, and were also found to be non - infectious (Kohl *et al.*, 1988; Peng *et al.*, 1989; Gelderblom, 1991). The RT in its active form is a heterodimer with ribonuclease H (RNAase H), activity, and is responsible for synthesizing a DNA copy of the single stranded RNA genome (Veronese *et al.*, 1986). The RNAase H activity is essential for the synthesis of a linear double stranded DNA copy of the genome. Being unique in nature, and found only in the retrovirus family, the RT enzyme has become the obvious target for antiviral therapy in attempting to disrupt the viral life cycle. The integrase enzyme is involved in the integration of the double

stranded DNA copy of the viral genome into the host cell genomic DNA *via* an interaction with the viral LTRs. A nucleoprotein complex, which included the integrase enzyme, has been isolated from the cytoplasm of infected cells and shown to have the ability to integrate linear DNA *in vitro* (Farnet and Haseltine, 1990). Further analysis revealed that the integrase was the only viral protein found in the nucleoprotein complex and it was suggested that its presence was sufficient for the integration of the retroviral DNA into its target (Farnet and Haseltine, 1991b).

The *env* gene encodes a 160 kDa glycoprotein precursor that is processed by host cell derived proteases to the surface gp120 (SU), and transmembrane gp41 (TM), glycoproteins (figure 1). The precursor is synthesized from a singly spliced messenger RNA (mRNA), and carries at the amino terminal end a hydrophobic signal sequence (approximately 30 amino acids in length), that directs the protein to the cellular secretory pathway where the signal sequence is removed and extensive N - linked glycosylation of both the SU and TM proteins takes place (Kozarsky *et al.*, 1989). By comparison of DNA sequences from different isolates of HIV, it was determined that the surface glycoprotein of HIV contained six conserved regions (C1 to C6), and five hypervariable regions, V1 to V5 (Modrow *et al.*, 1987). Structural analysis applied to the pattern of the cysteine residues and possible disulphide linkages, led to the proposal of a model, that included each of the variable domains, V1 to V4, as loops within the protein (Leonard *et al.*, 1990). Crystallography data to verify this model, however, has not yet been obtained. Within the gp41 transmembrane domain, a stretch of hydrophobic amino acids has been identified, approximately at the centre of the protein, that serves to anchor the molecule to the cell membrane. Despite proteolytic cleavage, the gp41 and gp120 glycoproteins remain non -

covalently associated with each other *via* highly conserved hydrophobic amino acid stretches located at each end of the gp120 glycoprotein (Helseth *et al.*, 1991). During budding the gp120 / gp41 complex is acquired by the emerging virus particle as an integral part of the viruses envelope (figure 2).

The initial stages of infection of a cell is largely dependant upon various functional domains within the surface and transmembrane glycoproteins. A brief overview of the process follows. It has been established that HIV gains entry to a target cell *via* an interaction between its surface glycoprotein and the cellular CD4 receptor, and that the glycosylation of the protein was a requirement for this interaction (Matthews *et al.*, 1987; Montefiori *et al.*, 1988; Peterlin and Luciw, 1988; Kieny, 1990). Additionally, the surface glycoprotein has been implicated in determination of the viral tropism for different cell types. Recently, the regions responsible for this determination were delineated to a small stretch of amino acids that encompass the third variable domain, often referred to as the V3 loop (Cann *et al.*, 1992).

In further studies of the steps of viral entry, a series of experiments analyzed the required components for successful infection of a target cell by HIV. The gene that encoded the CD4 receptor (also known as T4), was cloned and introduced into a primitive leukemic human T - cell line (HSB2), that was previously resistant to HIV infection. Following expression of the CD4 protein, the authors demonstrated that the cell line had become susceptible to infection and was able to replicate and produce infectious progeny virions (Maddon *et al.*, 1986). The CD4 gene was also introduced into the murine cell line, NIH 3T3. However, in this case, it was found that the cell line remained resistant to infection. Coupled with the study published by Levy and colleagues (1986), where the same cell line

(NIH 3T3), was transfected with an infectious DNA clone of HIV, and yielded viral progeny, it was suggested that the block to infection occurred at the cell surface. These findings supported the proposal that the CD4 receptor was required, but was not the only factor necessary for successful infection by HIV.

There have been some findings reported that may lead to the identification of the additional co - factor or co - factors on the cell surface that participate in the infection process. Trypstatin, a Kunitz type protease inhibitor, was found to block syncytia formation in tissue cultured CD4 positive T - cells (Hattori,T *et al.*, 1989). The inhibitor, which acts upon the trypsin and tryptase proteases, was found to have homology to the highly conserved sequence, GPGR (in single letter amino acid code), found on the tip of the V3 loop. In support of this, further data was published on the proteolytic susceptibility of the V3 loop of the HIV surface glycoprotein to the thrombin and tryptase proteases (Clements *et al.*, 1991). Currently, efforts are underway to locate and characterize a possible cell surface expressed protease, and to assess its role in HIV infection.

Other studies have established that the co - factors, present on some human, rhesus and African green monkey cell lines, confer differential susceptibility to HIV - 1, HIV - 2 and SIV. It was also noted that the small, but highly conserved, sequences within the V3 loops (or the equivalent domains), within the surface glycoproteins of HIV - 2 and SIV, were different from one another (Clapham *et al.*, 1991). These findings together may provide an explanation where the cell tropism of a virus type (or strain), could be determined by the susceptibility of the V3 loop to cleavage by the putative cell surface expressed protease on a CD4 positive target cell.

Although not formally proven, the proposed model of viral entry includes the binding or adsorption of the surface glycoprotein to the cellular CD4 receptor, followed by the cleavage of the V3 loop by a putative cell surface expressed protease. This results in a conformational change that exposes the amino terminal fusogenic domain of the TM glycoprotein gp41 (Gallaher, 1987; Gallaher *et al.*, 1989; Kowalski *et al.*, 1991). Through mechanisms that are not well understood, the viral core is then internalized and the replication cycle ensues.

Regulatory Proteins

The *trans* - activator of transcription gene (*tat*), encodes a 14 kDa nuclear protein that self associates to form metal ion linked dimers. Translated from a doubly spliced mRNA species (figure 1), Tat is an essential protein for viral gene expression (Rosen *et al.*, 1986), and appears to increase both the rate of initiation and elongation of transcription by about two orders of magnitude (Cullen and Warner, 1990). It interacts with the Tat responsive element (TAR), localized within the LTR, to relieve a transcription block within TAR, just downstream of the transcription start site (Kao *et al.*, 1987; Braddock *et al.*, 1989; Cullen and Warner, 1990; Kieny, 1990). It was observed that Tat also acts post - transcriptionally to promote translation in a test system using Xenopus oocytes (Braddock *et al.*, 1989, 1990), however similar experiments done in primate cells, permissive for HIV replication, have shown no such effect (Chin *et al.*, 1991). Recent studies have demonstrated that a host nuclear localized factor (68 kDa protein), may also be involved in the function of the Tat - TAR regulation of gene expression (Marciniak *et al.*, 1990; Rosen, 1991).

Another essential gene product is the 19 kDa phosphorylated regulator of expression of virion proteins (Rev), protein. Like Tat, it is translated from doubly spliced mRNA (figure 1), and found in the nucleus of the infected cell. Its action induces the transition from the early to the late phase of viral gene expression in the replication cycle. This is characterized by the reduction in expression of proteins encoded by the fully spliced mRNAs and the predominant expression of the singly spliced and unspliced mRNAs giving rise to Nef, Vpr, Vpu, Vif and the structural proteins Gag, Gag - Pol and Env (Cullen and Greene, 1989; Schwartz *et al.*, 1990, 1991; Garrett *et al.*, 1991). The Rev protein has been shown to act upon a 234 nucleotide RNA target sequence, termed the Rev responsive element (RRE), that is located within the *env* open reading frame (Malim *et al.*, 1989). Rev has the ability to form multimers, however when this capability was disrupted, its function was abolished (Malim and Cullen, 1991; Zapp *et al.*, 1991). Additionally, the Rev protein appears to interact with a host derived nuclear factor termed the NF_{RRE} (Vaishnav *et al.*, 1991). The mechanism of action of the Rev protein is complex and currently remains poorly understood. However its overall effect is in the inhibition of transport of multiply spliced mRNAs coupled with the promotion of export of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm. The Rev protein does not appear to directly disrupt splicing events itself (Green and Zapp, 1989; Cullen and Warner, 1990; Rosen, 1991). Recent work on Rev has revealed an additional mode of action in the modulation of translation. It is thought to relieve a block in the assembly of polysomes onto the exported cytoplasmic mRNA (Arrigo and Chen, 1991).

Auxiliary Proteins

In contrast to Tat and Rev, the negative factor (*nef*), gene product is non - essential for viral replication *in vitro*. It is a myristoylated 27 kDa phosphoprotein that is translated from singly spliced mRNA and is found associated with the inner side of the cytoplasmic membrane within expressing cells (Franchini *et al.*, 1986). It was shown sometime ago that Nef - defective viruses were able to multiply faster in culture than those that produce the intact product (Terwilliger *et al.*, 1986). Furthermore, down regulation of LTR directed viral gene expression has been observed *via* an interaction between Nef and the negative regulatory element (NRE), located in the LTR (Ahmad and Venkatesan, 1988). More recently however, these effects on viral replication and gene expression have not been apparent in experiments done by others (Hammes *et al.*, 1989; Kim *et al.*, 1989), thus Nefs function remains controversial. The protein had been reported to possess GTP binding, GTPase and autophosphorylation activities, suggesting that it may be a member of a group of "G" proteins that play a significant role in cellular signal transduction pathways (Guy *et al.*, 1987; Kieny, 1990). These observations too have now been challenged by Kaminchik and colleagues, who found no G protein enzymic activities could be attributed to the purified Nef protein (Kaminchik *et al.*, 1990).

Recently, an additional role for the Nef protein was proposed, in down regulation of expression of the CD4 receptor molecule at the cell surface. Nef expression in human CD4 positive cell lines, *via* retroviral vectors, demonstrated a reduction in surface expressed CD4, but had no effect on steady state levels of CD4 RNA or CD4 protein within the cell (Garcia,JV and Miller, 1991). This may have the double function of preventing superinfection of the cell and

causing disruption to the normal pathway of T - cell activation. Furthermore, experiments done *in vivo*, using SIV infection of rhesus monkeys, have demonstrated that there was a strong requirement for intact Nef protein (Kestler *et al.*, 1990), and that the deletion of the *nef* gene resulted in much lower virus loads and decreased pathogenic potential of the virus (Kestler *et al.*, 1991). This was in contrast to experiments *in vitro*, where deletion of Nef had only minor effects. This served to emphasize the limitation of tissue culture in determining the individual functions of some HIV encoded proteins. More information is required to assess the role and significance of this protein in the replication cycle of the immunodeficiency viruses.

The viral infectivity factor (*vif*), gene, encodes an unusually basic protein of 23 kDa molecular weight. Experiments *in vitro* have shown that Vif negative virions were severely impaired (1000 - fold decrease), in their ability to infect target cells, despite an otherwise normal appearance under the electron microscope (Fisher *et al.*, 1987; Strebel *et al.*, 1987). Vif protein however, has not been detected in the virus particle and the precise mechanism by which it exerts its effect on the infectivity of the cell - free virion is unclear. Nevertheless, there are several possibilities, one of these prescribes Vif a role in the processes of virion maturation and / or morphogenesis (Cullen and Warner, 1990; Kieny, 1990). The most recent of these, identifies a role for the Vif protein as a thiol protease, which acts upon the HIV Env encoded glycoproteins during processing (Guy *et al.*, 1991). It was consequently proposed that Vif negative virions had impaired infectivity due to improper processing of the *env* encoded glycoproteins which are involved in mediating viral attachment, fusion and entry into the target cell.

General Introduction

The viral protein R (Vpr), is not essential for HIV replication *in vitro*. It is a 15 kDa protein, that has been observed to increase the rate of replication and cytopathic effects of HIV - 1 in CD4 positive T - cells (Ogawa *et al.*, 1989; Cohen *et al.*, 1990b). Vpr can also act in *trans* to increase expression of heterologous genes from the HIV - 1 LTR as well as other promoters, the mechanism involved is unclear, but may include interactions with host derived cellular factors (Yuan *et al.*, 1990). Interestingly, it was the first and, so far, only regulatory gene product of HIV - 1 to be detected within the virus particle, suggesting a role in facilitating the early steps of infection prior to *de novo* viral protein synthesis (Cohen *et al.*, 1990a). Similar findings have been reported for the SIV *vpr* gene product (Yu *et al.*, 1990), while in HIV - 2, the protein was also implicated in the modulation of cell tropism of the virus (Hattori,N *et al.*, 1990).

Unique to HIV - 1, is the viral protein U (*vpu*), gene. It is intriguing that the closely related viruses HIV - 2 and SIV lack this gene (with the exception of SIV_{cpz} which is more closely related to HIV - 1 viral isolates in genetic structure than other SIV and HIV - 2 isolates), and possess a different open reading frame unique to themselves, the viral protein X (*vpx*), gene, which will be discussed below. *vpu* encodes a hydrophobic 16 kDa protein that is not essential to HIV replication *in vitro* and is found associated with cytoplasmic membranes (Strebel *et al.*, 1989). Disruption of the function of Vpu resulted in a 5 to 10 fold decrease in the production of progeny virions (Strebel *et al.*, 1988). As the biosynthesis of proteins remained unaltered it was thought likely that the effect was elicited at the point of particle release, which resulted in the accumulation of cell associated HIV - 1 virions (Terwilliger *et al.*, 1989; Klimkait *et al.*, 1990). A recent publication has shown that Vpu can enhance the intracellular processing of the gp160 precursor to its gp120 and gp41 subunits in CD4 expressing HeLa

cells, while this effect on gp160 was not seen in the absence of the CD4 protein. On the basis of these findings the authors proposed a role for Vpu in destabilization of an intracellular sequestering event where viral gp160 and CD4 form complexes (Willey *et al.*, 1992). A possible mechanism for this was suggested where Vpu may exert its effect as a membrane sited pH or ion modulator of the endoplasmic reticulum (ER). This hypothesis was based on the observation that Vpu is both biochemically and structurally similar to the Influenza A viral protein M2, which is known to act as a modulator of pH in the ER lumen and thereby prevent premature conformational changes within the haemaglutinin protein (Lamb, 1991. Keystone Symposia on the Molecular Biology of Human Pathogenic Viruses. Lake Tahoe, CA, USA). However, further experimental evidence is still required to confirm or contest this hypothesis.

The Vpx protein is 14 kDa in size and produced in large quantities by cells infected with either HIV - 2 or SIV (Henderson *et al.*, 1988). It is present within the virus particle making up nearly 10% of its total protein content and may be localized to the lateral bodies (figure 2), although this has not been confirmed (Gelderblom, 1991). The function of the protein is unclear, however some data has suggested that Vpx may participate in the cell tropism of the virus (Yu *et al.*, 1991). Initial studies have revealed that Vpx mutants were unable to establish infection in primary blood lymphocytes, however they would propagate normally in established cell lines (Guyader *et al.*, 1989). More recently, experiments using immortalized T - cell lines showed that the mutants replicated to low titres with progeny virions being reduced in infectivity by three orders of magnitude relative to wild type (Kappes *et al.*, 1991). While Vpx defective virions have been distinguishable from wild type in tissue culture, the effects commonly seen

were subtle and appeared to be dependant on cell type thus raising the question of cellular factor involvement (Marcon *et al.*, 1991). Further work has been ongoing in this area to establish the role of the gene product *in vivo*.

Finally, a 28 kDa protein has been reported that is made up of segments of Tat, Rev and Env protein *via* multiple RNA splicing events. The protein termed Tev, was localized almost exclusively to the nucleoli of the cell and was weakly phosphorylated. The function of the protein remains unclear, but has been implicated in regulatory events during the initial phase of virus expression (Benko *et al.*, 1990).

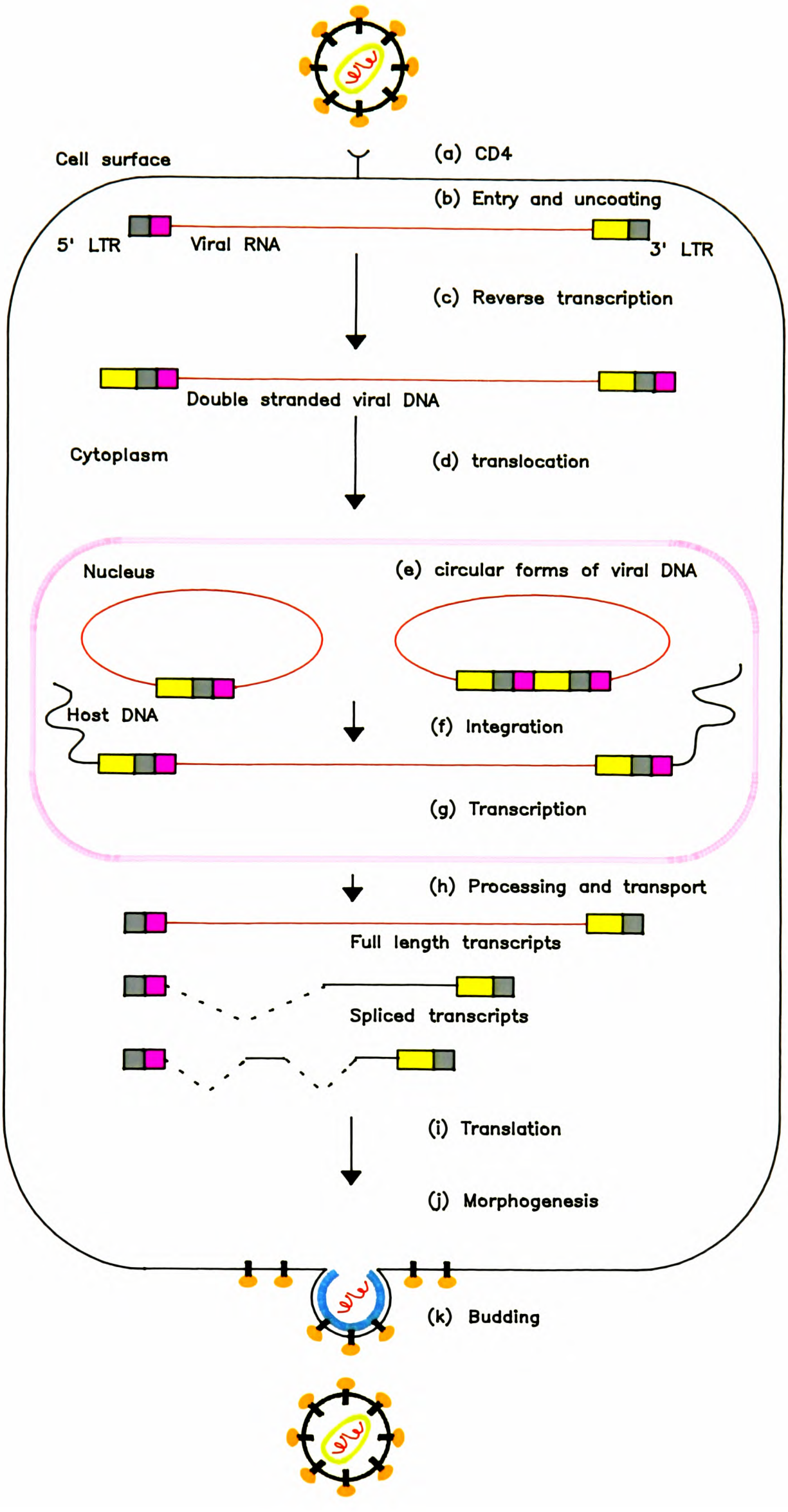
Life Cycle

The replication cycle of HIV is like that of most other retroviruses (figure 3). The virion adsorbs to the host cell *via* a specific high affinity interaction between the viral gp120 glycoprotein and the cell surface associated CD4 receptor. Following adsorption, pH - independent fusion occurs between the lipid bilayer of the virus and the plasma membrane of the target cell, mediated by the hydrophobic amino terminus of the gp41 (Gallaher, 1987; Gallaher *et al.*, 1989; Kowalski *et al.*, 1991). Uncoating and reverse transcription by the virally encoded enzyme first produces hybrid RNA / DNA molecules, which are converted to double stranded linear DNA that contain two copies of the viral LTR. The linear DNA is then translocated to the nucleus whereupon covalently closed circular forms are generated *via* the action of cellular factors with either one or two copies of the LTR (Farnet and Haseltine, 1991a). The HIV DNA is then integrated into the host cell genome by the virally encoded integrase (Farnet and Haseltine, 1990), however which form or forms (circular or linear),

Figure 3.

Life cycle of HIV - 1

Diagrammatic view of the stages in the life cycle of HIV - 1. Beginning with (a) the adsorption of the HIV particle onto the surface of the target cell *via* an interaction between the viral SU glycoprotein and the cellular CD4 receptor, followed by (b) entry and uncoating of the viral particle, (c) reverse transcription of the single stranded RNA to double stranded DNA flanked by the viral LTRs in the cytoplasm, and (d) translocation to the nucleus of the cell (pink). (e) circular forms of the viral genome are created containing one or two copies of the LTR sequence, followed by (f) integration of the viral DNA into the host cell chromosome. Activation of the integrated provirus gives rise to (g) transcription, (h) processing and transport of spliced and unspliced forms of RNA to the cytoplasm, (i) translation and subsequent modification of the viral proteins, and (j) transport of the products to the plasma membrane where viral particle morphogenesis occurs. (k) budding and release from the plasma membrane is followed by maturation of the viral particle mediated by the active viral protease. In this diagram, the LTRs are depicted with the three regions colour coded, U5 (magenta), R (grey) and U3 (yellow).


Cell surface
(a) CD4
(b) Entry and uncoating
5' LTR
Viral RNA
3' LTR
(c) Reverse transcription
Double stranded viral DNA
Cytoplasm
(d) translocation
Nucleus
(e) circular forms of viral DNA
Host DNA
(f) Integration
(g) Transcription
(h) Processing and transport
Full length transcripts
Spliced transcripts
(i) Translation
(j) Morphogenesis
(k) Budding

is preferentially integrated is not known. At this stage of the life cycle, the provirus may remain latent for an extended period of time (Peterlin and Luciw, 1988).

After activation of the cell harbouring the retrovirus (discussed below), host cell derived transcription factors together with virally encoded *trans* - activators lead to HIV replication and gene expression (figure 3). The Gag precursor drives assembly of the viral core structure at the plasma membrane and also incorporates the virally encoded enzymes together with two copies of the genomic RNA. Upon budding from the cell, the viral particle acquires a lipid bilayer together with the surface and transmembrane viral glycoproteins. This step, interestingly, appears to exclude most other cell surface expressed proteins. Either during or shortly after release of the virion, maturation ensues where the protease is activated and cleaves the Gag and Pol precursors to their subunits resulting in infectious virus particles (Peterlin and Luciw, 1988).

Pathogenesis

The intricacies of HIV replication within the body and its interaction with the immune system is highly complex. Additionally the pathogenesis and progression of the disease include a wide range of factors that are the subject of much clinical research. This remains a very significant part of understanding the mechanisms of disease induction by HIV and will be essential to developing treatment strategies, thus a brief overview of the main features is presented.

Pathogenically the manifestation of disease in humans can be broadly categorized into four groups. 1) immune deficiency with the consequent

opportunistic infections, 2) infection of the brain with the resulting central nervous system abnormalities, 3) cell proliferation where particular stimulation of some types of cells leads to hyperplasia and neoplasia as in Kaposi's sarcoma and B - cell lymphomas, 4) miscellaneous associated diseases due to independent infection because of lifestyle and / or immune impairment. HIV can only replicate in activated cells, a state which is essentially transient in nature giving the impression that HIV can remain clinically latent. This is of advantage to the virus in avoiding surveillance and eradication by the immune system. Molecularly, it has been suggested that this is not *bona fide* latency, but a low level of expression of HIV protein is probably occurring all the time (Gallo, 1990). The integrity of replication of the HIV genome is relatively poor with the estimated rate of error being 10^{-4} per base, or one misincorporation per genome per replication cycle (Dougherty and Temin, 1988; Nowak, 1990). Given this high rate of mutation in the HIV genome and the large numbers of progeny produced, eventually escape mutants will arise that allow the virus a small burst of replication and spread until the new variant is checked by the immune system.

One possible explanation for the transition from apparent latency to active viral replication is as follows. Over a period of time there is an accumulation of events that lead to the activation of cells harbouring integrated provirus (discussed in more detail below), giving rise to viral gene expression. This is coupled with the gradual impairment of the immune system leading to increasing inability of the system to monitor and hold in check virus spread, consequently, more cells become infected and are available to express virus when triggered. The impairment to the immune system may be linked to the cytopathic effects of the virus observed *in vitro*, where cells that harbour HIV provirus, express viral

glycoproteins on their surface. These interact with the CD4 receptors of neighbouring uninfected cells and result in cell to cell fusion with syncytia formation and the appearance of giant multi - nucleated cells, which subsequently leads to cell death. This mechanism results in the destruction of both infected and uninfected T - cells, and would thus help to explain the pathogenesis of the virus. The main CD4 expressing cells in the immune system are a subset of the T - lymphocyte lineage, otherwise known as T4 cells. These are known to be critical in the orchestration of events in the immune response, thus their disruption may represent a large part in the cause of immune dysfunction (Gallo, 1990).

Recently a mathematical model was proposed that describes the gradual degeneration of the immune system over time. Coupled with this are intermittent bursts of viral replication, and a gradual increase in diversity of strains of HIV within the body. This diversity is encouraged by natural selection pressures as a result of immune surveillance. The authors proposed that, at a certain time point, a threshold of diversity is exceeded, that results in the inability of the immune system to further control viral replication. This leads to chronic viremia, and the uncontrolled spread of the virus throughout the body, with the fastest replicating strains becoming the dominant species. The model is supported by clinical and molecular data, however, as noted by the authors, further testing is required for its confirmation or falsification (Nowak *et al.*, 1991).

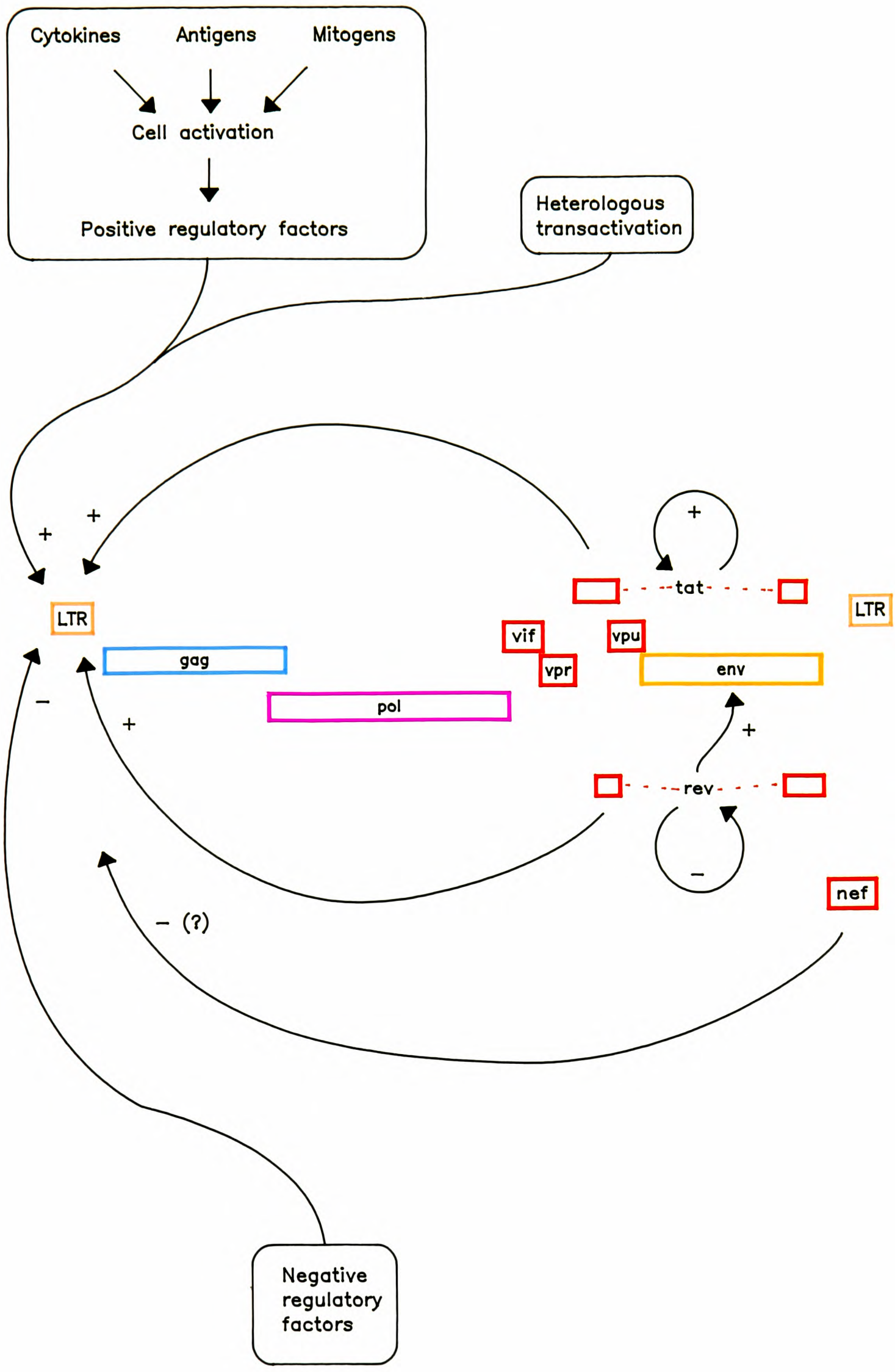
Molecularly, much research has been directed at understanding the complex regulatory mechanisms of the immunodeficiency viruses mediated primarily through the LTR, particularly the *tat* and *rev* gene products that are essential to

virus replication (summarized in figure 4). This in part has been aimed at shedding light on the mechanism of viral latency, where the virus is either dormant or replicates at a very low level. Many cellular factors derived from the host cell exert their influence on the replication cycle of HIV, which complicates the picture further. Some of these have a positive or up - regulatory function, such as the inducible transcription factor NF - *k*B (Nabel and Baltimore, 1987), and are produced during cellular activation which can be triggered by a number of mechanisms including cytokines, antigens and mitogens (figure 4). Other transcriptional factors that up - regulate HIV gene expression are constitutive, such as Sp1 (Jones,KA *et al.*, 1986), and TFIIID (Garcia,JA *et al.*, 1987). Conversely, while in a state of quiescence, negative regulatory proteins (e.g. RPT - 1), are present in the T - cell that may down regulate expression of the proviral genome (Patarca *et al.*, 1988). In this way, the HIV provirus can respond appropriately to the intracellular milieu of its host cell (Cullen, 1991; McCune, 1991). Additionally, heterologous activation by other viruses either directly, by *trans* - activation of the HIV LTR, or indirectly, *via* stimulation of positive regulatory cellular factors (figure 4), has been demonstrated (Steffy and Wong - Staal, 1991). By gaining a better understanding of the complicated balance between the cellular factors and the virally encoded *trans* activators that maintain latency or trigger active viral replication, it may be possible to develop means of stalling or preventing active viral replication and the progression of disease.

Figure 4.

Regulation of HIV - 1 gene expression

Flanked by LTRs (peach), the HIV - 1 genome is shown with the *gag* gene (blue), *pol* gene (magenta), *env* gene (orange) and the auxiliary genes (red). A number of signals act upon the 5' LTR to positively (+), induce transcription of the proviral DNA. These include the viral *tat* and *rev* gene products in addition to positive regulatory factors derived from the host cell produced in response to cytokines, antigens and mitogens. Heterologous activation by other viruses can also up - regulate viral gene expression. Down - regulation (-), is thought to be mediated by the viral *nef* gene product, in addition to some cellular derived factors. The Tat protein also increases expression of its self, while the Rev protein down - regulates self expression and boosts expression of singly and unspliced mRNAs by acting on the Rev responsive element in the *env* open reading frame.


Cytokines
Antigens
Mitogens
Cell activation
Positive regulatory factors
Heterologous transactivation
+
+
+
LTR
gag
pol
vif
vpr
vpu
env
tat
rev
LTR
+
+
−
−
−
(?)
nef
Negative regulatory factors

The Project Outline

In this thesis two functions of the HIV Gag precursor come under investigation. The first involves the characterization of domains that are required in the protein - protein interactions that promote the oligomerization of the p55 precursor to form the Gag particle. The second aspect of Gag precursor function that is addressed concerns the capability of the Gag precursor protein to bind nucleic acid. As a way of introduction to these specific topics, the general molecular aspects of both protein - protein and protein - nucleic acid interactions are briefly discussed below. Within retroviruses some of these interactions have yet to be understood in detail, consequently examples have been drawn from well characterized systems and their relevance to the retroviral scheme highlighted where appropriate.

In addition to studies on the Gag antigen p55, chapter three of this thesis investigates some aspects of the post - translational modification of the SIV transmembrane glycoprotein. Accordingly, an introduction is given below of the mechanisms involved in protein post - translational processing including targeting to the cellular secretory pathway, folding, glycosylation and oligomerization.

Finally, an investigation into a possible practical use of the Gag particle as a carrier of foreign antigen has been described in chapter four. A brief introduction of other protein carrier systems already in use such as the yeast Ty element virus - like particle (Ty - VLP), is also given below.

General Introduction

As the baculovirus protein expression system has been used extensively for the production of recombinant proteins throughout this work, an explanation of the key principles involved is given.

Protein - Protein Interactions

The protein - protein interaction makes up a fundamental part of many biological processes including gene regulation and expression, protein synthesis, protein folding with subsequent modification and general intracellular trafficking and organization. Among some of the better characterized protein - protein interactions are those for which the three dimensional structures of the participating proteins have been determined by X - ray crystallography. These include a number of proteases and their interaction with either a substrate or inhibitor as well as the protein binding capacity of some antibodies to their respective antigens. Janin and Chothia (1990), conducted a survey of 15 protease - substrate / inhibitor and 4 antigen - antibody pairs with the aim of establishing a pattern or preference for certain amino acids involved in the interaction. They reported that most of the recognition sites involved in the contact region had a similar chemical composition to the accessible surface of the average protein; 55% non - polar, 25% polar and 20% charged. It was thus concluded that generally there was no particular amino acid composition found for the recognition sites studied, however it was noted that nearly half of the contact residues in the antibody - antigen interaction sites were aromatic, a preference that had been previously reported (Padlan, 1990). Both types of interaction involved similar numbers of hydrogen bonds ranging from 8 - 13, with an average of 10. However, whilst two thirds of the hydrogen bonds in the

protease - substrate / inhibitor interaction were between main chain atoms, most of the bonds within the antigen - antibody complex were between side chains of the polypeptide. In addition to hydrogen bonds, between 6 and 12 polar atom interactions, through a bridging water molecule, were found to be typical of well defined structures. The calculated size of the contact area for these complexes was approximately 1600 Å^2 (Janin and Chothia, 1990).

These interfaces were also compared to the contact areas between the subunits of known oligomeric proteins. Generally it was noted that the area of the oligomeric protein interaction was larger than that involving the complexes stated above and covered up to 5000 Å^2. The larger interface was thought to stabilize both the association of the subunits and the individual tertiary structure of the monomer. Interactions between oligomeric subunits were noted as being more hydrophobic in nature and generally involved fewer hydrogen bonds than those involved in functional recognition (Janin *et al.*, 1988).

The study of viral capsid assembly mechanisms is a large field which makes up a part of the discipline of protein - protein interactions. Structurally viruses are broadly categorized into enveloped and non enveloped viruses depending on whether or not they have acquired a lipid bilayer from their previous host cell. This is determined by the mechanism of exit from the cell that the virus employs. The viral structure is either rod shaped or spherical, a characteristic affected by the nature of the viral capsid subunit protein - protein interactions.

A wealth of information is available on the various types of viral structures and their mechanisms of assembly. For the purposes of this introduction however, particular examples have been selected to illustrate certain points. For example

a rod shaped virus, the well characterized tobacco mosaic virus (TMV), has been compared and contrasted below with the structure and assembly of the spherical or icosahedral viruses to which group the Retroviridae members belong. Similarly, the icosahedral group of viruses has been represented by an overview of Sindbis virus, a togavirus which is one of the structurally least complex of the spherical enveloped group of viruses.

The oligomerization of protein subunits has been studied extensively in the self assembly mechanism of the Tobacco Mosaic Virus (TMV), a plant RNA containing virus of the Tobamo group. This was the first example of *in vitro* assembly of purified protein subunits together with RNA to yield infectious virus (Fraenkel - Conrat and Williams, 1955). The three dimensional structure of the subunit had been determined by X - ray crystallography and the various interactive domains defined. The oligomerization was found to be driven largely by hydrophobic interactions between the monomers. The resulting structure of the multimer was polymorphic, the characteristics of which were found to be influenced largely by the pH and ionic strength of the environment in which the subunits were placed. In contrast to purified protein *in vitro*, the *in vivo* formed infectious virus is made up of a rod of protein constituting a right handed helix of subunits, 16 2/3 subunits per turn, complexed with RNA. This type of structure was only formed *in vitro* under conditions of low pH in the absence of RNA, while at higher pH values the predominant form was a disk like structure together with smaller coat protein aggregates termed A - protein. It was later established that these alternative forms played a catalytic role in nucleation of subunit oligomerization and subsequent extension of the multimer. Additionally the presence of RNA was found to enhance nucleation as well as favour the formation of the helical rod structure (Butler, 1984). This example illustrates

well that viral assembly and RNA encapsidation can be separated but nonetheless shows an important link between these two processes.

In contrast to the helical rod viral structure, a description of the mechanism of assembly of a spherical or icosahedral virus is presented below using the Sindbis virus as an example. This virus is a member of the alphavirus genus, the main genus of the Togaviridae, a family of small enveloped vertebrate viruses (Fraenkel - Conrat *et al.*, 1988). The three dimensional structure of the capsid monomer has recently been determined for this virus by X - ray cystallographic techniques and the mechanism of capsid assembly extrapolated from the subunits characteristics. The core protein subunit is approximately 29 kDa in molecular weight and self assembles to form an icosahedral capsid with T = 4 symmetry. The T or triangulation number of the surface lattice of icosahedral structures refers to the number of multiples of 60 subunits that are required to make up the structure. Hence with T = 4, the number of subunits required is 240. The structural unit was found to be a dimer of the core protein, with the contact points of the monomer localized to a narrow neck of 6 amino acids that made up a ß strand. Each subunit consisted of 264 amino acids with a remarkably high proportion of basic residues as well as prolines. While the monomer was roughly spherical the dimer was found to have a more oblong structure. Interestingly a "Greek key" ß - barrel fold within the monomer was found to bear striking similarity to the structure of mammalian serine proteases of the chymotrypsin family. This family of viruses are the only family found thus far for which the protease of the capsid protein has been established to make up an essential part of the viral core structure (Choi *et al.*, 1991). The RSV is the only well characterized retrovirus found to date with the protease domain fused to the Gag assembly precursor. However it has been shown that its deletion does

not affect the particle assembly proficiency of the Gag protein (Voynow and Coffin, 1985). Thus it was concluded that the protease is not an essential part of the structure of the retroviral capsid.

The polyomaviruses, SV40 and polyoma are both double stranded DNA viruses with icosahedral symmetry that are members of the Papovaviridae. The structure of the capsids in these viruses represents a step up in complexity from the Sindbis virus. SV40 and polyoma contain 360 copies of the major protein VP1 assembled as 72 pentamers, 12 of which lie on five fold axes while the remaining 60 fill the intervening surface in a close packed array. Each of these intervening pentamers is surrounded by six others. The overall structure is equivalent to a T = 7 symmetry, however because some pentamers are surrounded by 5 other pentamers, and others are flanked by 6, the geometry is more complex than that of the basic icosahedral structure (Rayment *et al.*, 1982). The *in vitro* assembly of purified *E. coli* expressed VP1 subunits into pentamers and capsid like assemblies has been observed. The pentamers that associate with others were found to form readily, while hexameric association of the pentamers was found to be the result of spontaneous bond switching specificity during assembly (Salunke *et al.*, 1986; Garcea *et al.*, 1987).

The study of the positive strand RNA containing viruses has yielded one of the most interesting and unexpected discoveries to come out of research into the structure of viral capsids. All of these small viruses studied so far appear to be variations on a common theme. The theme concerns the subunit structure found in all the assembly units, and is based on the ß sheet organization and referred to as either a "jelly - roll ß barrel", a "Swiss - roll ß barrel", or an "eight - strand, anti - parallel ß barrel". The barrel is based on a framework of two apposed ß sheets,

each of which is made up of 4 anti - parallel strands. It has been noted that the particular size, shape and disposition of the loops within this structure are typical of the RNA virus capsid assembly units (Harrison, 1991). The members of the picornavirus family that belong to this group and have had their structures determined are the human rhinovirus 14 (Rossman,JG *et al.*, 1985), mengovirus (Luo,M *et al.*, 1987), the poliovirus (Hogle *et al.*, 1985) and the foot and mouth disease virus (Acharya *et al.*, 1989). The general architecture is the same for all, with a T = 3 symmetry and an outer diameter of approximately 30 nm. The structural assembly unit is made up of three smaller subunits VP1, VP2 and VP3 each of which contain an eight - strand, anti - parallel ß sheet.

This finding is perhaps particularly significant from the perspectives of this project because a possible homology between the HIV p24 core protein, centrally located within the Gag precursor, and the above eight - strand anti - parallel ß sheet has been reported (Rossman,MG, 1988; Argos, 1989). Thus, the assembly of the p55 Gag core particle may be related to the known positive strand RNA virus structures detailed above. However, direct evidence for this so far is lacking.

At a molecular level the detailed capsid structure of any member of the retrovirus family has yet to be determined. To date there has been no successful crystallization of the retroviral Gag precursor that has lead to the solving of its molecular structure by X - ray crystallographic techniques although the structure of a sub domain of p55, p24 is currently in progress. It may be envisaged that there are unidentified domains within the Gag precursor that impede the crystallization process. A better understanding of the domains within the Gag precursor that drive assembly may ultimately lead to the construction of "cut

down", assembly - proficient Gag precursors deleted for the regions that inhibit crystal formation. The work in this thesis is aimed at better defining the assembly domains within the HIV Gag precursor protein, which may eventually lead to the generation of an assembly proficient cystallizable Gag derived protein.

Protein - RNA Interactions

The interaction of nucleic acid with protein is a feature of many metabolic processes in eukaryotic and prokaryotic systems. The molecular basis of such interactions has been the subject of a large number of studies. A great variety of roles for nucleic acid binding proteins have been identified that include RNA / DNA replication, transcription, chromatin folding and unfolding, recombination, strand scission and in encapsidating and maintaining the integrity of nucleic acid in virion particles. The great majority of data gathered thus far on nucleic acid binding proteins have been based on protein - DNA interactions, hence the following discussion shall begin with this group.

A number of DNA binding protein structural motifs have been identified and can be classified into 4 main groups; the helix - turn - helix, two kinds of zinc finger and the leucine zipper (Struhl, 1989). A brief description of each follows.

The helix - turn - helix motif has been the best characterized of this group and is made up of 2 α helices separated by a β turn. The motif is widespread in the prokaryotic world being found in transcriptional activator and repressor proteins (Pabo and Sauer, 1984), while in eukaryotes the motif was first identified in a

family of *Drosophila* proteins involved in control of early development and cellular differentiation. In these proteins a highly conserved region containing a helix - turn - helix has been termed the homeodomain, examples of which have now been identified in a variety of eukaryotic organisms ranging from the yeast to the human (Levine and Hoey, 1988).

The leucine zipper motif contains four or five leucine residues that are spaced exactly seven residues apart and hence could be viewed as being repeated every two turns of an α helix. It is thought that leucine residues positioned along one side of an α helix associate with the leucine residues of another parallel α helix, resulting in dimerization perhaps as a coiled coil structure (Landschulz *et al.*, 1988). This dimerization of two subunits has been found to make up a DNA binding domain such as that found in the Fos and Jun family of transcription factors (Freemont *et al.*, 1991). The proposed function of protein dimerization in preparation for nucleic acid binding could also be viewed as a protein - protein interaction. Interestingly, Delwart and Mosialos (1990), have identified a "leucine zipper" - like motif within the extracellular domain of a number of retroviral transmembrane glycoproteins, and noted that this may contribute to the regions promotion of oligomerization and stabilization of the retroviral glycoprotein structure (Doms *et al.*, 1990; Thomas *et al.*, 1991).

The two varieties of zinc fingers are differentiated by the sequence of conserved cysteine and histidine residues within the motif either C_2H_2 or C_X (Evans,RM and Hollenberg, 1988). The motif is made up of pairs of cyteines and histidines, separated by a variable number of amino acid residues, that form a tetrahedral arrangement around a zinc atom, with the intervening loop forming a finger like structure. An alternative model based on the structures of known

metalloproteins presents the zinc finger as an anti - parallel ß sheet and α helix between the zinc coordination sites (Berg, 1988; Evans,RM and Hollenberg, 1988). The motif has been found in many DNA binding proteins in a varying number of juxtaposed copies.

The interactions of proteins with RNA is more difficult to analyze and has been less extensively studied. RNA often has a particular three dimensional structure unlike DNA, and therefore protein - RNA interactions tend to show a greater complexity of structure than occurs with protein - DNA interactions. However, certain common features of a number of RNA - binding proteins have been found such as a stretch of 80 - 90 amino acid residues, denoted the RNA binding domain. The domain has been identified in the primary amino acid sequence of a number of proteins associated with mammalian nuclear RNAs and the nucleolin family of RNA binding proteins. Within this domain, a more highly conserved sequence of 8 amino acids was also found, and termed the "ribonuclearprotein (RNP) consensus sequence". The general formula for this sequence was; basic - G - aromatic - A / G - F - aliphatic - polar - F in single letter amino acid code (Adam *et al.*, 1986; Mattaj, 1989; Query *et al.*, 1989).

RNA binding proteins are a feature of many viral life cycles ensuring that the assembling virion includes a copy of its genome. In this instance the retroviruses are no exception. A putative RNA binding motif that is precisely conserved has been found in all retroviruses sequenced to date and has been denoted the Cys - His motif, where X is any amino acid residue. The formula of this motif bears striking similarity to the above mentioned zinc finger motifs, Cys - X_2 - Cys - X_4 - His - X_4 - Cys, and has a demonstrated ability to bind zinc atoms (South *et al.*,

1990). One or two copies of the motif have been localized to the carboxy terminal region of the retroviral Gag protein.

Deletion of this region in HIV has been found to abolish the ability of the Gag precursor to form particles (Gheysen *et al.*, 1989). Furthermore, the deletion of the specific *cis* - acting RNA encapsidation signal from the genomic RNA of HIV was observed to result in the assembly of virions with reduced RNA packaging and aberrant particle morphology (Clavel and Orenstein, 1990). This raised the possibility that the encapsidation of RNA has a catalytic function in the assembly of the virion as has been discussed above for TMV, where RNA plays a catalytic role in the formation of the correct viral structure. However, a study in which point mutations were introduced into the conserved Cys and His residues of the HIV nucleocapsid domain resulted in reduced RNA encapsidation but otherwise normal particle morphology (Gorelick *et al.*, 1990). As the alterations in the Cys - His motifs were relatively minor it remains possible that the region was still able to contribute to the Gag assembly mechanism, and as RNA was still present within the particles, that RNA encapsidation *via* the Cys - His motif could play a role in particle morphogenesis. The role of the Cys - His motif in particle assembly and RNA binding has been addressed in chapters one and two of this project.

Post Translational Modification of Viral Glycoproteins

All proteins are initially translated from mRNA by ribosomes in the cytoplasm of the cell, however a subset of these proteins that are destined for the secretory pathway contain signals that direct them to the endoplasmic reticulum (ER),

where they may undergo a variety of post translational modifications such as glycosylation, cleavage and oligomerization prior to being transported to their destination. The specific ER targeting signal, known as the signal sequence is made up of a hydrophobic of stretch 15 to 30 amino acids. The signal sequence functions by causing the ribosome to pause after translating approximately 70 amino acids. The peptide sequence outlying the ribosome - mRNA complex, (30 - 40 amino acids in length), is recognized by and associates with the signal recognition particle (SRP), which subsequently docks with the SRP receptor on the ER membrane (Darnell *et al.*, 1990; Rapoport, 1990). Translation then continues and the protein is extruded into the lumen of the ER. Within integral membrane proteins there is a second signal, termed the stop - transfer signal, which arrests the transfer of the polypeptide into the ER lumen and anchors the protein within the membrane. This leaves the remaining portion of the translated molecule free in the cytoplasm. Conversely in secreted proteins the stop - transfer signal is absent and the entire protein is translocated through to the ER lumen (Blobel, 1980; Sabatini *et al.*, 1982; Adams,GA and Rose, 1985).

The signal sequence and stop - transfer sequence are good examples of topogenic sequences that occur within proteins and direct the protein through the appropriate pathways to its final destination. There are a variety of characterized topogenic sequences that have a number of roles including directing proteins to organelles such as mitochondria and to intracellular lysosomes (Horwhich *et al.*, 1991; Pfeffer, 1991; Hopkins, 1992).

Amongst the group of integral membrane proteins the signal sequence is cleaved off in the majority of instances, following translocation through to the ER lumen, by the signal peptidase resulting in a class I topology where the carboxy terminus

remains in the cytoplasm and the amino terminus in the lumen. A class II orientation of membrane spanning proteins occurs when the signal sequence is not cleaved but remains fused to the protein. The final orientation of this protein is opposite to that of the class I type, amino terminal in the cytoplasm and carboxy terminus in the lumen. There have been 2 further classes defined, class III where the protein is anchored in the same orientation as class I, but by a single internal signal sequence (no amino terminal signal sequence), and the class IV proteins which span the membrane several times and have been denoted polytopic proteins. The orientation of the membrane spanning segments within this latter class has been proposed to be influenced by the charge distribution around the first hydrophobic domain (Einfeld and Hunter, 1991)

The portion of the protein within the ER lumen is available for extensive glycosylation reactions of which four main types have been identified in eukaryotic cells. The first is N - linked glycosylation which involves the addition of a complex oligosaccharide to the amino group of asparagine residues; second, glucose residues are often transiently added to high mannose type glycoproteins; third, N - acetylglucosamine residues are linked to the protein *via* serine or threonine residues (O - linked glycosylation); and four, glucosamine and mannose residues are incorporated into the glycosylphosphatidylinositol anchor of some membrane proteins (Abeijon and Hirschberg, 1992).

The folding of the protein and formation of disulphide bonds also occurs in the ER. Disulphide bonds form between cysteine residues and have a stabilizing effect on the tertiary structure of the protein. Additionally if the protein is a subunit of a larger structure, oligomerization typically occurs in the ER. Interestingly if a protein fails to fold or oligomerize correctly it is often retained

in the ER, unable to progress to the golgi complex for example, such retention has been demonstrated for the vesicular stomatitis virus (VSV) glycoprotein G (Kreis and Lodish, 1986) and the influenza hemagglutinin (Gething *et al.*, 1986). This is thought to be mediated by a class of binding proteins (BiP), that bind to improperly folded proteins and promote their retention in the ER. Glycosylated proteins then typically proceed through the golgi complex where further modification of its oligosaccharide side chains occurs together with specific proteolytic cleavage events prior to being sorted for its final destination. The role of glycosylation in glycoprotein function has yet to be clearly defined, however the addition of oligosaccharide moieties appears to play a role in protein transport, solubility, conformation and interaction with other proteins (Darnell *et al.*, 1990).

The glycoproteins of all retroviruses are directed through the secretory pathway where glycosylation, folding and oligomerization take place prior to transport to the site of viral assembly (Hunter and Swanstrom, 1990). One of the most studied glycoproteins is that encoded by the HIV *env* gene.

Earl *et al.* (1991a), studied the synthesis, folding, dimerization, cleavage and shedding of the HIV *env* encoded glycoprotein gp160 using an assay based on CD4 binding efficiency as a marker for correct assembly of the glycoprotein. Folding can be measured indirectly by determining when a protein acquires a certain degree of functional competence which in this case is the ability to bind CD4 (Hurtley and Helenius, 1989). It was determined that newly synthesized polypeptide bound to GRP78 - BiP, a class of BiP specific for this protein, with a half time ($t_{\frac{1}{2}}$) of 15 minutes. At the end of this time period disulphide bond formation and folding had been completed in greater than 90% of the total

protein. Additionally within this same time period, greater than 90% of the Env protein was found to have acquired CD4 binding competence. Assembly was also highly efficient with approximately 90% of all molecules forming dimers with a $t_{\frac{1}{2}}$ of 30 min. Thomas *et al.* (1991), have recently established that the gp41 transmembrane glycoprotein is responsible for the interaction that stabilizes the full length gp160 precursor dimer. This observation is in agreement with earlier cross - linking studies of the molecule, that suggested gp41 could form oligomers in the absence of gp120 (Schwaller *et al.*, 1989).

There was evidence to suggest that correct folding and oligomerization were pre - requisites of transfer between the ER and golgi complex as has been defined for influenza hemagglutinin and the VSV G protein. As previously mentioned the gp160 molecule is subject to extensive N - linked glycosylation. Interestingly Fennie *et al.* (1989), established that glycosylation contributed significantly to the ability of the gp120 molecule to bind CD4. It would thus appear that glycosylation, folding, CD4 binding competence and oligomerization are all closely linked events in the biosynthesis of the Env glycoprotein.

Cleavage of the gp160 precursor to the gp120 surface and gp41 transmembrane glycoproteins has been shown to be an important step for the infectivity of the progeny virion (Guo *et al.*, 1990). The event has been localized to the golgi complex although in which compartment the cleavage occurs remains controversial. Additionally the enzyme responsible for the proteolytic cleavage has yet to be determined, although it is known to be of cellular origin. The $t_{\frac{1}{2}}$ of cleavage was 80 min, and was established as being a post ER event. While there was found to be a marked difference in cleavage efficiency between various cell types, the efficiency of shedding of the glycoprotein exhibited much less

variability. A point of interest was that $CD4^+$ cells were slightly less efficient at shedding, raising the possibility of an intracellular sequestering event inhibiting transport. Sequestering of this nature has since been shown directly by Buonocore and Rose (1990), and Crise *et al.* (1990).

It was determined that about 50% of the gp120 generated by the endoproteolytic event was released into the culture medium. Interestingly, there was a peak of gp120 shedding observed at 2 - 4 hr after synthesis, after which shedding ceased or proceeded at a very low rate. However, the explanation for this bi - phasic shedding has yet to be determined (Earl *et al.*, 1991b).

While much of the available data on post - translational modification of retroviral glycoproteins given above has been drawn from studies on HIV - 1, the general pattern of biosynthesis appears to be shared by other members of the retrovirus family (Hunter and Swanstrom, 1990). On this premise, the studies presented on the SIV *env* encoded glycoprotein in chapter three have been placed in context.

Particulate Carrier Systems

In the cause of developing a vaccine against viral infection, one of the favoured approaches has been to use live attenuated virus. However when reliable attenuation is difficult to achieve, this method becomes unacceptably hazardous. As an alternative, one approach being developed involves the use of non - infectious particulate carriers to present relevant epitopes to the immune system.

An example of this is the exploitation of the yeast retrotransposon Ty elements ability to make virus - like particles. The haploid genome of the laboratory yeast strain contains between 30 and 35 copies of the Ty element at various chromosomal locations (Klein and Petes, 1984). The retrotransposon can move from one location to another *via* an RNA intermediate that is packaged into virus - like particles (Mellor *et al.*, 1985). It is one of the subunit proteins of the particle, encoded by the *TYA* gene, that can have foreign domains fused onto its carboxy terminus without disrupting assembly (Kingsman and Kingsman, 1988).

A hybrid HIV - Ty VLP was produced by Adams *et al.* (1987), where approximately 200 bp of the *env* gene of HIV - 1 was cloned in frame with the *TYA* coding sequence. The resulting hybrid Ty - VLP was found to be easily purifiable by density gradient centrifugation and able to generate an immune response in rabbits (Adams,SE *et al.*, 1987).

In a similar experiment p24, a subunit of the HIV - 1 Gag protein, was expressed on Ty - VLPs. However, a protease cleavage site was engineered at the junction of the yeast protein and HIV p24. The presence of the site, specific for the factor Xa protease, allowed removal of the p24 antigen from the Ty - VLP following purification, yielding significant quantities of a highly purified preparation of p24 antigen (Gilmour *et al.*, 1989). This type of antigen delivery system demonstrates a further practical application of particulate carriers in facilitating the biosynthesis and delivery of foreign antigens in a highly purified state.

In addition to the yeast Ty VLPs, other carrier systems have been developed for the presentation of antigen. One of these involves the use of the hepatitis B core

particle in a similar manner as described above for the yeast Ty element. A foreign domain is fused to the amino terminus of the *C* gene product, and is expressed on the resulting particle. Using a 19 amino acid peptide derived from the foot and mouth disease virus VP1 protein, Clarke *et al.* (1987), were able to demonstrate production of particles using a vaccinia virus expression system. It was determined that the hybrid particles were 500 times more immunogenic than the free peptide, and were almost as immunogenic as the whole inactivated virus particle. The same system was later refined for the production of hybrid particles in bacteria, allowing greater yields of particles (Clarke *et al.*, 1990). Similar findings have also been reported for the presentation of antigenic epitopes on hepatitis B virus core particles from other viruses (Francis *et al.*, 1990).

In a slightly different system to those presented above, antigen presentation on particles has also been demonstrated using the poliovirus as a carrier. In this system, the foreign epitope replaces a loop at an known immunodominant site on the exterior of the particle, the structure of which has been determined as mentioned above (Hogle *et al.*, 1985; Burke *et al.*, 1988). The system has been used to express 18 amino acids from the Env glycoprotein of HIV - 1 and found to elicit broadly reactive neutralizing antibodies (Evans,DJ *et al.*, 1989). The authors also noted an advantage of this system over others, such as the hepatitis particle carrier described above, in that the chimeric particles were able to induce a secretory as well as a systemic humoral immune response. The presence of secretory antibodies in mucous fluids may potentially reduce the efficiency of transmission of HIV.

The above systems describe the practical uses of particulate carriers in the generation of immune responses. Various benefits within the different systems were also illustrated such as increased immunogenicity of a fusion domain or its ease of purification. The use of retroviral Gag precursors to facilitate the purification of fused proteins has been described by Weldon *et al.* (1990). The authors adapted the RSV Gag protein to be efficiently synthesized and released in mammalian cells and demonstrated that the fusion protein, *iso* - 1 - cytochrome *c* derived from the yeast *Saccharomyces cerevisiae*, could be isolated by recovery of the extracellular Gag particles by centrifugation.

Exploiting the budding mechanism of retroviral Gag particles to acquire surface expressed glycoproteins would add a further dimension to the construction of chimeric virus - like particles for use as potential vaccines. As has been discussed above, glycoprotein production in the higher eukaryotes allow addition of oligosaccharide moieties that may be required for correct conformation or function.

In chapter four of this thesis, the effects on particle formation of the fusion of additional antigens onto the carboxy terminus of the HIV - 1 Gag protein is investigated. Additionally, the feasibility of loading the Gag particle with integral membrane glycoproteins, as it buds from the plasma membrane of the eukaryotic insect cell, is addressed.

The Baculovirus Expression System

Baculoviruses are one of the most widely studied groups of insect viruses. They are double stranded DNA containing viruses whose restricted host range has lead to their development as biological pesticides. They have been developed as vectors for foreign gene expression and the resulting helper independent baculovirus expression system (Smith,GE *et al.*, 1983), has since been used to express a wide variety of genes (Luckow and Summers, 1988a; Bishop and Possee, 1990). It has been particularly valuable in expression of proteins that require a eukaryotic environment and which could not be produced in active form in lower eukaryotic or prokaryotic expression systems. In addition the virus itself is non pathogenic and allows abundant expression of recombinant proteins that are often antigenically, immunologically and functionally similar to their authentic counterparts.

Autographa californica nuclear polyhedrosis virus (AcNPV), isolated from the lepidopteran noctuid *Autographica californica* is the prototype virus of the baculovirus family. AcNPV replicates abundantly in several cell lines including the *Spodoptera frugiperda* (Sf) cell line Sf9. The AcNPV has a supercoiled circular genome of 130 kb. During infection of Sf cells, two types of viral progeny are produced, extracellular virus (ECV), released from the cells at 12 hr post infection and occluded virus (OV), after 4 - 5 days, the latter of which is enclosed in occlusions called polyhedra (King and Possee, 1992).

The polyhedra are composed of the 29 kDa polyhedrin protein, which constitutes approximately 30 % of the total cellular protein. The occluded virus is responsible for horizontal transmission in the natural life cycle of the virus where

the polyhedra plays a protective role against adverse environmental conditions, only breaking down to release the enclosed virions in an alkaline environment such as is found in the mid - gut of larvae. The OV gain entry to the insect gut cell *via* fusion or endocytosis, and the nucleocapsid is directed to the nucleus where replication takes place. As soon as 12 hr post infection, ECV are released by budding from the plasma membrane to generate secondary infections. The polyhedrin protein is expressed late in infection from 12 hr, with polyhedrin occlusions becoming visible within the cell under the light microscope from about 18 hr post infection. The level of ECV production, having peaked at about 24 - 36 hr post infection, gradually decreases and the cells lyse after 4 - 5 days releasing the polyhedra.

The baculovirus expression system is based on the exploitation of the late polyhedrin gene promoter. The gene itself is not essential for replication or synthesis of ECV and its removal or replacement results in the production of occlusion negative but viable ECV. These viruses form plaques on an Sf cell monolayer and can be distinguished from wild type by their lack of polyhedra. This method of differentiation was the original means for selection of recombinant baculoviruses.

Foreign genes are introduced into the AcNPV genome in place of the polyhedrin gene and come under control of the late strong polyhedrin promoter. The size of insert can up to 10 kb as the nucleocapsid that encases the genomic DNA has the ability to expand.

The method of introduction of the foreign gene into the AcNPV genome is *via* the use of a transfer vector which has as its fundamental feature a cloning site

flanked by stretches of polyhedrin coding sequence including the promoter and terminator regions (figure 5). The purified transfer vector DNA is co - transfected with infectious wild type AcNPV DNA into Sf cells, whereupon cell mediated homologous recombination occurs driven by the matching sequences of the polyhedrin gene. This results in the replacement of the polyhedrin coding sequence with the foreign gene and the stable integration of the latter into the AcNPV genome. Using conventional methods a recombination frequency of 0.1 to 0.5% can be achieved.

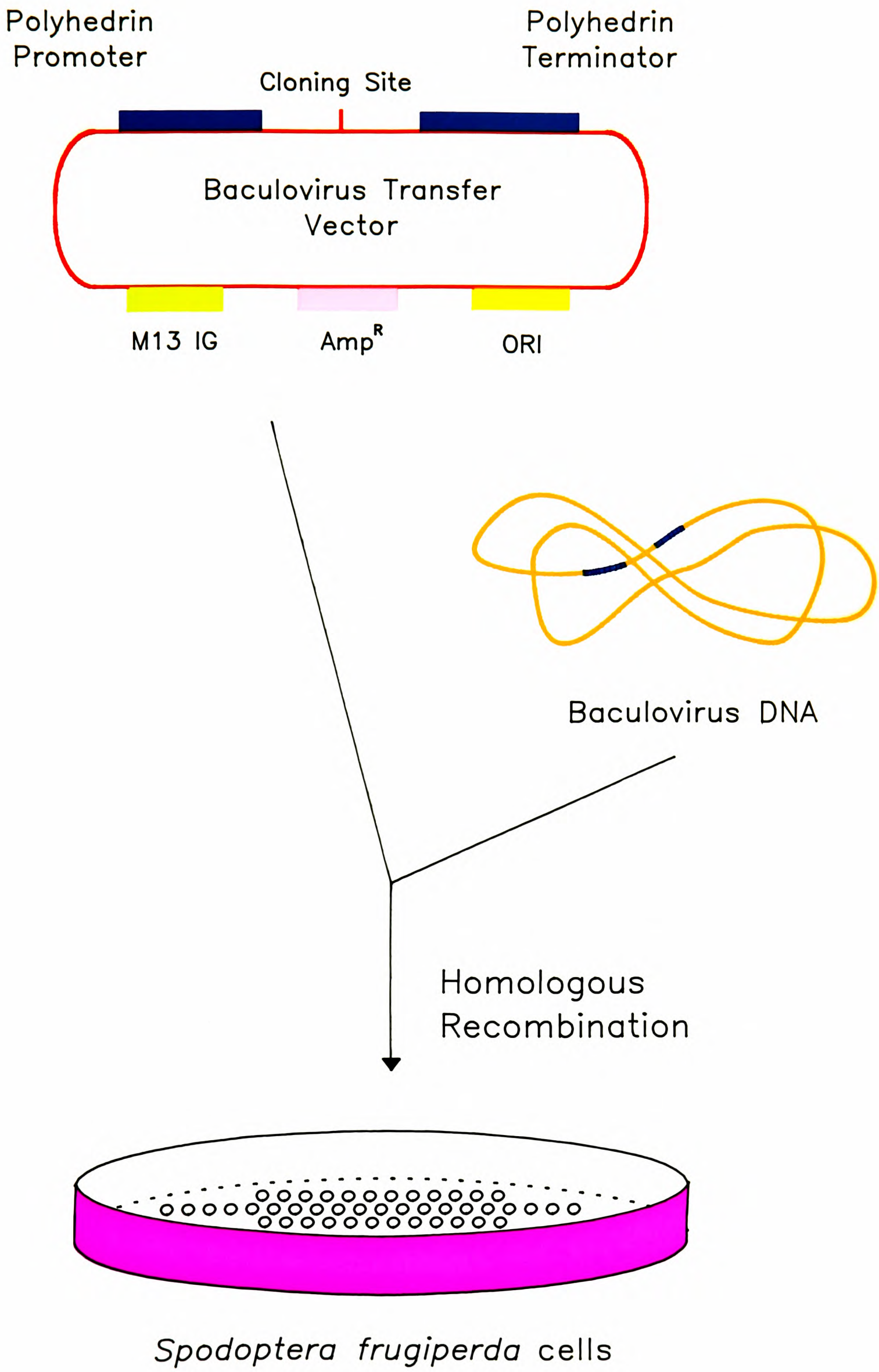
A major disadvantage of this system is that the method of selection of recombinant viruses is both labour intensive and requires a high level of skill. To improve this selection procedure a derivative of AcNPV was developed that contained the *lac Z* gene encoding the enzyme ß - galactosidase in place of the polyhedrin gene. This has the advantage of facilitating detection of recombinant viruses following co - transfection. Wild type or non - recombinants express the *lac Z* gene and plaques stained with a substrate for ß - galactosidase, X - gal (see plaque assay in materials and methods), turn blue. However, in the event of recombination the *lac Z* gene is replaced by the foreign gene and the resulting plaque remaining white can be easily distinguished from non - recombinants and picked for further testing (King and Possee, 1992)

Additionally, the introduction of a unique restriction site *Bsu* 36I within the *lac Z* gene allowed linearization of the viral DNA. As linearized DNA is less efficient at transfecting cells than circular DNA, and recombination restores the genome to being circular, there is an overall higher proportion of recombinants in the total number of progeny viruses produced. Typical recombination frequencies can be as high as 30%. However, the total number of progeny virus

Figure 5.

The Baculovirus Expression System

The upper half of the diagram shows a diagrammatic representation of a typical baculovirus transfer vector (red). The polyhedrin gene promoter and terminator regions have been included (turquoise), together with the *E. coli* origin of replication ORI (yellow), the ampicillin gene resistance marker for selection in *E. coli* (pink), and the M13 bacteriophage intergenic region IG (green) for production of single stranded DNA. The purified plasmid DNA is mixed with infectious baculovirus DNA (orange) and co - transfected into *Spodoptera frugiperda* cells indicated in the lower half of the diagram. Homologous recombination occurs with the exchange of genetic material and recombinant baculoviruses are selected.


Polyhedrin
Promoter
Polyhedrin
Terminator
Cloning Site
Baculovirus Transfer
Vector
M13 IG
Amp^R
ORI
Baculovirus DNA
Homologous
Recombination
Spodoptera frugiperda cells

is reduced although this can be off set by allowing the transfected culture to grow for an extended period of up to 5 days (Kitts *et al.*, 1991).

Once isolated, a recombinant plaque is grown to generate a viral stock of typically 2 x 10^7 pfu / ml and used for expression studies. Stocks are stored at 4 °C where the virus remains viable for many years.

Materials and Methods

Buffers and Solutions

All buffers and solutions were made up according to Sambrook *et al.* (1989), with the exception of the following:

Mung Bean Nuclease Buffer:

50 mM	CH_3COONa (NaAc) pH 5.0
30 mM	NaCl
1 mM	$ZnSO_{4\odot}$

Western Blot Blocking Buffer:

5%	Dried skimmed milk powder (Marvel)
0.05%	Polyoxyethylenesorbitan monolaureate (Tween 20) (Sigma Cat. No. P - 1379)

in PBS solution.

TYM Medium:

20 g	Bacto - Tryptone
5 g	Yeast Extract
1 g	Glucose
2 g	$MgCl_2$

in 1 litre dH_2O, autoclave. (pH 6.8)

TYM Agarose:

To 1 litre of TYM media, add 1.5% Bacto agar, autoclave.

TC - 100 Medium:

To 900 ml of dH_2O add TC - 100 powdered medium (Gibco Cat. No. 074 - 03000). Adjust pH to 5.0 with HCl, add 0.35 g/l $NaHCO_3$, re - adjust pH to 6.2 with 10 M KOH. Sterilize by filtration.

North Western Buffer:

30 mM	Hepes [N - 2 - hydroxyethylpiperazine - N' - 2 - ethanesulfonic acid] - KOH pH 7.4 (ICN Cat. No. 101926)
200 mM	KCl
10 μM	$ZnCl_2$
2 mM	DL - Dithiothreitol (Sigma Cat. No. D - 0632)
20 units/ml	Heparin (Sigma Cat. No. H - 3125)

Cloning Techniques

Stocks of plasmid DNA were digested to completion using the selected restriction enzymes at the manufacturers recommended temperatures and conditions. The fragments were separated according to size by electrophoresis through a 1% agarose gel (Gibco - BRL Ultrapure agarose, Cat. No. 540 - 5510) in 1 x TBE buffer. The selected fragment of DNA was purified from the agarose either by the filter paper / dialysis membrane method (Girvitz *et al.*, 1980) or by dissolution of the agarose matrix. This latter technique was done in one of 2 ways, the first by using low melting temperature agarose (FMC Cat. No. 50102),

and heating the excised block of agarose containing the fragment of DNA in TE buffer at 65°C for 10 min or until it had melted. The mixture was subjected to phenol / chloroform extraction and the DNA recovered by standard ethanol precipitation with 0.1 volume 3 M NaAc (pH 5.2), and 2 - 2.5 volumes 100% ethanol (0°C, 30 min), the DNA pelleted by centrifugation in an Eppendorf microcentrifuge (12,000*xg*, 10 min), washed with 70% ethanol, repelleted, dried, and resuspended in 10 μl of TE buffer.

The alternative method was using potassium iodide to dissolve the agarose gel matrix and purification of the DNA by adsorption to glass beads (QIAGEN QIAEX DNA Agarose gel extraction kit. Cat. No. 20020). The gel dissolution and recovery with glass beads method was found to be more efficient than the filter paper / dialysis membrane method for fragments greater than 1000 base pairs, however for fragments smaller than this, the former dialysis membrane / filter paper technique was significantly superior in terms of yield. Regardless of the size of the fragment, the low melting point agarose technique was found to give lower yields.

Transformation and Screening

The fragments for cloning were ligated (20°C, 16 hr) in ligation buffer with 1 unit of ligase enzyme (B - M Cat. No. 716 359), and then used to transform *Escherichia coli* (*E. coli*) strain XL1 - Blue cells (Bullock *et al.*, 1987), or MC 1061 strain (Meissner *et al.*, 1987), using either heat shock or electroporation. The heat shock technique is described in detail by Sambrook *et al.* (1989). The electroporation method (Dower *et al.*, 1988), involved addition of 10% of the

ligation mix to 50 μl of prepared cells in a electroporation cuvette. This mixture was subjected to 2500 Volts with the capacitance and resistance set at 25 μF and 200Ω respectively, immediately diluted to 1 ml with S.O.C medium and incubated (37°C, 1 hour), before plating out onto TYM agarose plates supplemented with 100 μg/ml ampicillin (Sigma Cat. No. A - 9518)). The plates were grown (37°C, 16 hr) and the resulting colonies used to seed small 2 ml cultures in TYM medium with ampicillin (200 μg/ml) which were grown to stationary phase (37°C, 16 hr). DNA was extracted from these cultures using the alkaline phosphatase DNA extraction technique described in Sambrook *et al.* (1989). The DNA preparations were screened for the presence and orientation where necessary, of the cloned fragment by cutting with restriction enzymes and analysis of the fragments by agarose gel electrophoresis. In cases where it was difficult to obtain successful clones from a ligation experiment, the Grunstein and Hogness method of screening by DNA hybridization was used (Sambrook *et al.*, 1989). Stocks of the correctly cloned plasmids were prepared as described below.

Plasmid DNA Stocks

Stocks of plasmid DNA were prepared by the midi - prep method, a scaled up version of the mini - prep method previously described (Sambrook *et al.*, 1989), and purified by centrifugation through caesium chloride gradients. However in most instances satisfactory purification was achieved by a simpler and more convenient 3 step method described below.

Step 1; The DNA sample was treated with DNAase free RNAase (20 μg/ml; Sigma Cat. No. R - 5000), in TE buffer pH 7.4 (37°C, 30 min), extracted once with phenol / chloroform, once with chloroform, precipitated as above and resuspend in 450 μl TE buffer.

Step 2; Add LiCl to a final concentration of 2.5 M (0°C, 30 min), centrifuge (12,000*xg*, 10 min), transfer supernatant to a fresh tube, and precipitate as above. Air dry the tube and resuspend in 400 μl TE buffer.

Step 3; The DNA was further purified by $CsCl_2$ / ethidium bromide precipitation as described (Saunders and Burke, 1990), the pellet of DNA taken up in 400 μl TE buffer and fractions analyzed by electrophoresis through a 1.0% agarose gel (90 mA, 30 min) in 1 x TBE running buffer. Typical yields were 1-2 μg/μl DNA, which was sufficiently pure for use in restriction enzyme digestion and transfection of insect cells.

Site Directed Mutagenesis

The site directed mutagenesis methods used here were developed and modified by Kunkel and colleagues (Kunkel, 1985; Kunkel *et al.*, 1987), and have been described in detail in Sambrook *et al.* (1989). A brief description of the technique follows. The *E. coli* strain XL - 1 blue, containing the plasmid construct was infected with M13 KO7 bacteriophage (Vieira and Messing, 1987). Progeny phage were recovered and used to transduce the mutant strain of *E. coli* RZ 1032, which has the genotype *dut*$^-$ *ung*$^-$. This strain is deficient in the enzyme dUTPase (*dut*$^-$), and thus contains elevated concentrations of dUTP, which

compete with dTTP for incorporation into the DNA chain. The *ung*$^-$ mutation results in a lack of the uracil - *N* - glycosylase enzyme, which functions to remove uracil from DNA, thus plasmid DNA produced in this strain of *E. coli* contains a small number of uracil residues. Following infection of this strain with M13 KO7, phage particles were harvested and purified by polyethylene glycol (PEG) precipitation. Single stranded DNA (containing uracil residues) was isolated and used as template for the primer elongation reaction.

Template single stranded DNA was mixed with the oligonucleotide primer, allowed to anneal, and a primer extension reaction and ligation carried out *in vitro*. This stock was used to transform *E. coli* XL - 1 blue strain competent cells by heat shock treatment. This strain degrades the strand containing the uracil residues, while the newly synthesized strand including the oligonucleotide primer is replicated. Initial screening involved the use of a slot blot apparatus (Gibco Hybri - Slot manifold Cat. No. 1052 MM), for detection of mutants by hybridization to a radiolabelled DNA probe. *E. coli* transformants were infected with M13 KO7 phage and the culture supernatant containing the phage applied to a 0.45 μm filter membrane (Hybond C - extra, Amersham Cat. No. RPN 203 E) using the slot blot apparatus. The filter membrane was baked (80°C, 2 hr), and pre - hybridized in pre - hybridization buffer (67°C, 1 hour). The appropriate oligonucleotide (50 ng), was radioactively end - labelled by incubation with 20 μCi [γ - ^{32}P] ATP (Amersham Cat. No. PB 10168), 2 units of T4 polynucleotide kinase (NBL Cat. No. 020903) in kinase buffer (37°C, 1 hour). The mixture was passed through a 0.45 μm filter (Nalge Cat. No. 190-2045), and added to 5 ml of pre - hybridization buffer containing the membrane filter. After incubation (22°C, 1 hour), the filter was washed 3 times in 6 x SSC buffer.

The temperature of dissociation or duplex melting temperature (T_m), for the oligonucleotide was calculated using the Wallace rule:

T_m = 4 x (number of G-C pairs) + 2 x (number of A-T pairs)

Progressive washing steps were done starting at 5 °C below the calculated T_m in 6 x SSC buffer, and increasing the wash temperature progressively by 2 °C steps, with exposure of the membrane filter to X - ray film (Kodak X-Omat S Film 100 Cat. No. 501 0145), following each successive wash. The radiolabelled oligonucleotide probe will dissociate from wild type DNA at a lower temperature than successful mutant DNA due to the destabilizing effects of the mismatched base pair. *E. coli* transformants were identified that contain the mutant plasmids, and the appropriate changes confirmed by DNA sequencing experiments.

Polymerase chain reaction amplification.

The theory of *in vitro* DNA amplification by the polymerase chain reaction (PCR) technique is described in detail by Sambrook *et al.* (1989). Each amplification experiment was carried out in 0.5 ml microfuge tubes in a Hybaid Thermo Reactor (Hybaid Ltd., Middlesex, U.K.) where the PCR reaction mixture included the following:

1 x	buffer - supplied by manufacturer of polymerase
10 ng/μl	forward primer
10 ng/μl	reverse primer
2.5 μm	each dATP, dCTP, dGTP and dTTP (Pharmacia Cat. No. 27 - 2035 - 01)
10 - 100 ng	template DNA or RNA
1 unit	*Taq* thermostable DNA dependant DNA polymerase

The annealing temperature, dissociation temperature, polymerization temperature and number of cycles are detailed for each experiment in the text. Over the course of this work a variety of manufacturer's thermostable DNA polymerases were used with their accompanying buffers.

DNA Sequencing

Sequencing was done by the dideoxy strand termination technique using double stranded DNA (Zhang *et al.*, 1988), however when this provided unsatisfactory results single stranded DNA was prepared and used (Sambrook *et al.*, 1989). Additionally, variations on the technique were occasionally used to yield clearer results, these included de - ionization of the polyacrylamide gel matrix prior to its polymerization. This required the addition of "Amberlite" MB - 1 mixed bed resin (2 g / 50 ml; BDH Cat. No. 55007 4 E), to adsorb out positive and negative ions. The resin was removed by filtration (Whatman 541 filter paper, Cat. No. 1541 125). Addition of dimethyl sulphoxide (DMSO), to the sequencing reactions was also found to improve results in some cases (Winship, 1989).

Viral DNA Preparation

Viral DNA was prepared from the AcRP6 SC strain, technique described elsewhere (Summers and Smith, 1987), which has been modified from *Autographa californica* nuclear polyhedrosis virus (AcNPV), such that it contains the *lac Z* gene encoding the enzyme ß - galactosidase as described in the general introduction.

Tissue Culture and Protein Expression

The AcNPV protein expression system has been used for the expression of protein in *Spodoptera frugiperda* (Sf) insect cell cultures. This expression system is capable of producing high concentrations of recombinant antigen (up to 1 mg/ml per 1 - 2 x 10^6 infected cells), that are often antigenically, immunogenically and functionally similar to their authentic counterparts (Luckow and Summers, 1988b).

Spodoptera frugiperda cells (clone 9) were propagated at 28°C in TC-100 medium containing 5 - 10% foetal calf serum (Gibco Cat. No. 011 - 06290 M), and supplemented with penicillin (50 iunits/ml) and streptomycin (50 µg/ml; Gibco Cat. No. 043 - 05070 H), kanamycin (100 µg/ml; Gibco Cat. No. 043 - 05160 H), and fungizone (2.5 µg/ml; Gibco Cat. No. 043 - 05290 F). AcNPV and recombinant viruses were grown and assayed in either confluent monolayers of Sf cells or in suspension in spinner culture flasks. The techniques and protocols used have been described in Summers and Smith (1987). Viable cell counts were done by mixing an equal volume of suspension culture with 0.1% Trypan Blue stain (Flow Cat. No. 16 - 910 - 49), loading onto a haemocytometer and counting

under an inverted microscope. All cells used for either production of proteins or for the propagation of recombinant virus were infected during the logarithmic phase of growth, density between 5 x 10^5 and 1 x 10^6 cells/ml.

The following tissue culture products were obtained from Gibco - BRL and used regularly:

32 mm dish (area 8 cm^2)	Nunclon 1 - 50318
6 x 35 mm well plate (area 9.6 cm^2/well)	Nunclon 1 - 52795
80 cm^2 flask	Nunclon 1 - 53732
175 cm^2 flask	Nunclon 1 - 56502

Co - transfection and Recombinant Virus Production

Sf cells were transfected by mixing 1 μg of linearized AcRP6 SC DNA with 5-10 μg of plasmid DNA in distilled water, adding 1 volume of Lipofectin™ (BRL, Cat. No. 8292 SA), followed by addition to TC-100 serum free medium on washed Sf cells in a monolayer of 1 x 10^6 cells in a 35 mm tissue culture dish according to manufacturers protocol. Cultures were harvested at 4 - 6 days post infection and plaque assayed. Clear plaques were selected and amplified by infecting a monolayer 5 x 10^5 Sf cells in 35 mm tissue culture wells. Cell extracts from these infections were harvested at 3 - 5 days post infection and screened for recombinant protein production by western blot probed with mAb to HIV - 1 p17 and p24. Once identified, recombinant viruses were purified by 2 - 3 rounds of plaque assay.

Plaque assay

Monolayers of Sf cells (1.6 x 10^6 cells/well), were prepared on 6 x 35 mm tissue culture plates, and infected with serial dilutions of the virus stock in TC-100 medium. After incubation (20°C, 1 hour), the innoculum was aspirated and replaced with 2 ml of a 1:1 mixture of sterile molten 3.2% low melting temperature agarose (FMC Cat. No. 50102), cooled to 39°C and TC-100 growth medium preheated to 37°C. This was allowed to set (20°C, 1 hour), and overlaid with 1 ml TC-100 medium and incubated for 3 - 4 days (28°C) in a humidified box. To identify plaques, the overlay medium was replaced with a 0.02% neutral red stain (ICN Cat. No. 16 - 911 - 49) in PBS, incubated (28°C, 1 hour) and destained for 4 hr with PBS. If the plaques were to be screened for successful recombination, a further staining step with 2 mg/ml 5 - bromo - 4 - chloro - 3 - indolyl - ß - D - galactopyranoside (X - gal; NBL Cat. No. 070306) at 28°C for 16 hr was carried out.

Detection of Recombinant Viruses

Clear plaques were picked using a sterile Pasteur pipette and ejected into 1.5 ml of TC-100. This was used to infect a 35 mm well of Sf cells (5 x 10^5 cells) and grown at 28°C for 4 - 6 days. The culture supernatant was harvested and stored at 4°C pending the results of the assay. The cells were washed from the dish, pelleted (185*xg*, 10 min), washed in 1 ml PBS, repelleted and lysed in 50 μl of SDS gel loading buffer. The samples were denatured (100°C, 10 min) and analyzed by SDS/PAGE and western blot for production of recombinant antigen.

SDS/PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), was routinely used to analyze protein fractions from cellular extracts and other sources. The methods for preparation are described in detail in Sambrook *et al.* (1989). Unless otherwise stated 10% polyacrylamide gels were used with 4% acrylamide stacking gels and electrophoresed at 180 Volts for 75 min in tris - glycine acrylamide gel electrophoresis buffer.

Total Protein Stain

Staining for total protein content of the SDS/PAGE gel was done with coomassie blue stain using the method described (Sambrook *et al.*, 1989).

Western Blot

Nitrocellulose membrane filters, 0.45 μm (Schleicher and Schuell Cat. No. 401196) were thoroughly soaked in western blot transfer buffer and placed on 3 layers of 3MM filter paper (Whatman Cat. No. 3030 - 917), also soaked in the transfer buffer, on the western blotting apparatus (Atto Model. No. AE 6670). The SDS/PAGE gel was positioned on top of the membrane, and 3 additional sheets of buffer soaked 3MM placed over the gel. The apparatus was assembled and protein transfer was done by electrophoresis at either 150 mA (90 min) or 230 mA (60 min). The apparatus was disassembled, the membrane rinsed thoroughly in PBS and blocked in blocking buffer for 1 - 6 hr on a shaking table

(20°C). The filter was probed with the 1° antibody (20°C, 16 hr shaking), washed twice with 50 ml blocking buffer followed by the 2° antibody (20°C, 2 hr), washed again as above and where applicable the 3° antibody was applied (20°C, 2 hr). All antibodies were used at a 0.1% dilution in blocking buffer unless otherwise stated. After probing with the final antibody, invariably an alkaline phosphatase conjugate, the filter was washed twice in blocking buffer and finally rinsed in PBS. The blot was developed by incubation in one of the following development solutions containing substrate for alkaline phosphatase.

1. **ß - Napthyl phosphate method:**

   The development solution was prepared fresh for each blot and consisted of:

   25 mg ß - Napthyl Phosphate (Sigma Cat. No. N - 1132)

   25 mg Fast blue BB Salt (Sigma Cat. No. F - 3378)

   50 mg $MgSO_4$

   in 50 ml of alkaline phosphatase buffer:

   3.7 g Boric Acid

   1.8 g NaOH

   in 100 ml of dH_2O

2. **BCIP / NBT method:**

   Stock solutions of 15 mg/ml 5 - bromo - 4 - chloro - 3 - indolyl phosphate (BCIP; Biorad Cat. No. 170 - 6539) in dimethyl formamide (DMF) and 30 mg/ml nitro blue tetrazolium (NBT; Biorad Cat. No. 170 - 6532) in 70% DMF in dH_2O, were prepared and stored at 4°C protected from light. Immediately prior to use the stock solutions were diluted to 1% in carbonate buffer pH 9.8:

0.1 M $NaHCO_3$

1 mM $MgCl_2$

The latter development solution was found to be consistently more sensitive with less background than the former method.

Thin section electron microscopy

Infected cells (moi = 10), harvested at 2 days post infection, were washed in PBS and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 24 hr. Fixed cells were embedded in 1% low melting point agarose blocks, the agarose fixed in 2.5% glutaraldehyde as above and washed in 0.1 M cacodylate buffer. This was followed by fixation in 1% osmium tetroxide in 0.1 M cacodylate buffer (2 hr) and 0.5% uranyl acetate (3 hr). After dehydration in ethanol, cells were embedded *via* propylene oxide in Araldite. Sections were cut with a diamond knife on a Reichert - Jung Ultracut E ultramicrotome, post - stained with uranyl acetate and lead citrate, and examined with a Philips CM 12 electron microscope at 80 kV.

All of the photographs presented in this thesis were taken by Dr. David Hockley and Dr. Milland Nermut of the NIBSC, London.

Chapter One

Gag Particle Morphogenesis

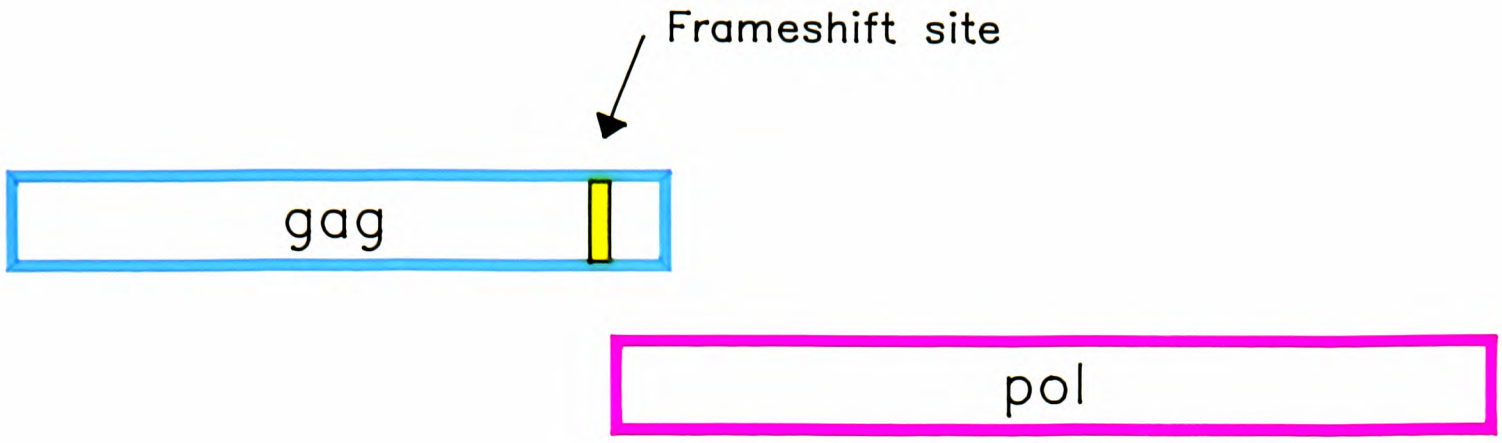
Introduction

During assembly of the virion core, HIV incorporates not only the structural proteins derived from the Gag precursor, but also the enzymes encoded by the *pol* gene. These are the protease, reverse transcriptase with associated RNAase H activity and the integrase enzymes, as described in the general introduction. From structural studies, it is apparent that the quantities of these enzymes required for replication are less than the structural Gag proteins, so how does the virus regulate the ratio of structural to enzymic protein and ensure that appropriate amounts are packaged into the virion? HIV has evolved a clever mechanism employing a ribosomal frameshift sequence encoded in the amino terminus of the *gag* open reading frame (figure 6). This ensures that the majority of structural proteins are derived from the *gag* open reading frame, but also that a small percentage of the translated proteins include enzymic activities encoded by the *pol* gene. The *gag* and *pol* genes are in different reading frames and when the ribosomal complex reads through the *gag* mRNA it encounters a sequence of six uridine residues, known as the frameshift site, where, at a frequency of approximately 5%, the ribosomal complex shifts into the *pol* open reading frame and continues on to encode a Gag - Pol fusion precursor of 160 kDa molecular

Figure 6.

HIV - 1 *gag* and *pol* gene expression

The HIV - 1 *gag* gene (blue), and *pol* gene (magenta), are shown in the upper section of the diagram. The location of the ribosomal frameshift site is delineated by a yellow box. Displayed in the lower section, are the products of transcription and translation with the Gag only precursor (blue) and the Gag - Pol fusion precursor (blue and magenta) indicated.



Transcription and Translation

Gag precursor (19/20 transcripts)

Gag – Pol fusion precursor (1/20 transcripts)

weight (Jacks *et al.*, 1988; Wilson *et al.*, 1988). This process results in production of a ratio of about nineteen Gag precursors to a single Gag - Pol fusion precursor (figure 6). Both precursors are directed to the plasma membrane by the myristoylated glycine residue at the amino terminal of Gag, where the Gag - Pol fusion precursor is incorporated into the forming virion. This system of frameshifting is also commonly found in other retroviruses (reviewed in Jacks, 1990), however in some cases ie the Moloney murine leukemia virus (MoMuLV), an alternative mechanism is used, where the *gag* and *pol* genes are in the same reading frame separated by an amber stop codon (UAG). The ratios in this case are then determined by the ability of the translational mechanism to suppress this nonsense codon (Odawara *et al.*, 1991).

The HIV - 1 Gag precursor has the ability to form particles when expressed in the absence of all other viral proteins (Gheysen *et al.*, 1989; Hu *et al.*, 1990). Being one of the proteins common to all retroviruses, similar observations supporting this function of Gag have been made in RSV (Wills *et al.*, 1989), MoMuLV (Hansen *et al.*, 1990), SIV (Delchambre *et al.*, 1989), BIV (Rasmussen *et al.*, 1990), FIV (Morikawa,S *et al.*, 1991) and HIV - 2 (Luo,L *et al.*, 1990), thus the gene has been dubbed the "particle making machine" (Wills and Craven, 1991). However when expressed in the absence of the *pol* gene products, no processing of the HIV - 1 Gag precursor was observed and the particles retained a morphology similar to that of immature HIV virions (Gheysen *et al.*, 1989; Hu *et al.*, 1990; Hoshikawa *et al.*, 1991). Experiments to locate assembly domains within the HIV Gag precursor revealed that deletion of the nucleocapsid protein p7 and the p6 regions, both downstream of the p24 capsid domain, abolished the ability of the Gag precursor to self assemble (Gheysen *et al.*, 1989; Kojima *et al.*, 1990; Hoshikawa *et al.*, 1991)

Shioda and colleagues (1990), demonstrated that expression of both the *gag* and *pol* open reading frames of HIV in the human colon carcinoma cell line SW 480, using recombinant vaccinia viruses (RVV), resulted in the production of particles containing processed Gag antigen and active RT enzyme that were similar in size and density to HIV virions. The authors also expressed a mutant where the frameshift site in *gag* had been altered so that the *gag* and *pol* genes were in the same reading frame. Although Gag and Pol antigens were detected within the cell, this construct failed to produce particles in detectable quantities (Shioda and Shibuta, 1990). In the Gag - Pol fusion precursor, the p6 domain of HIV Gag was absent due to the position of the frameshift site (figure 7).

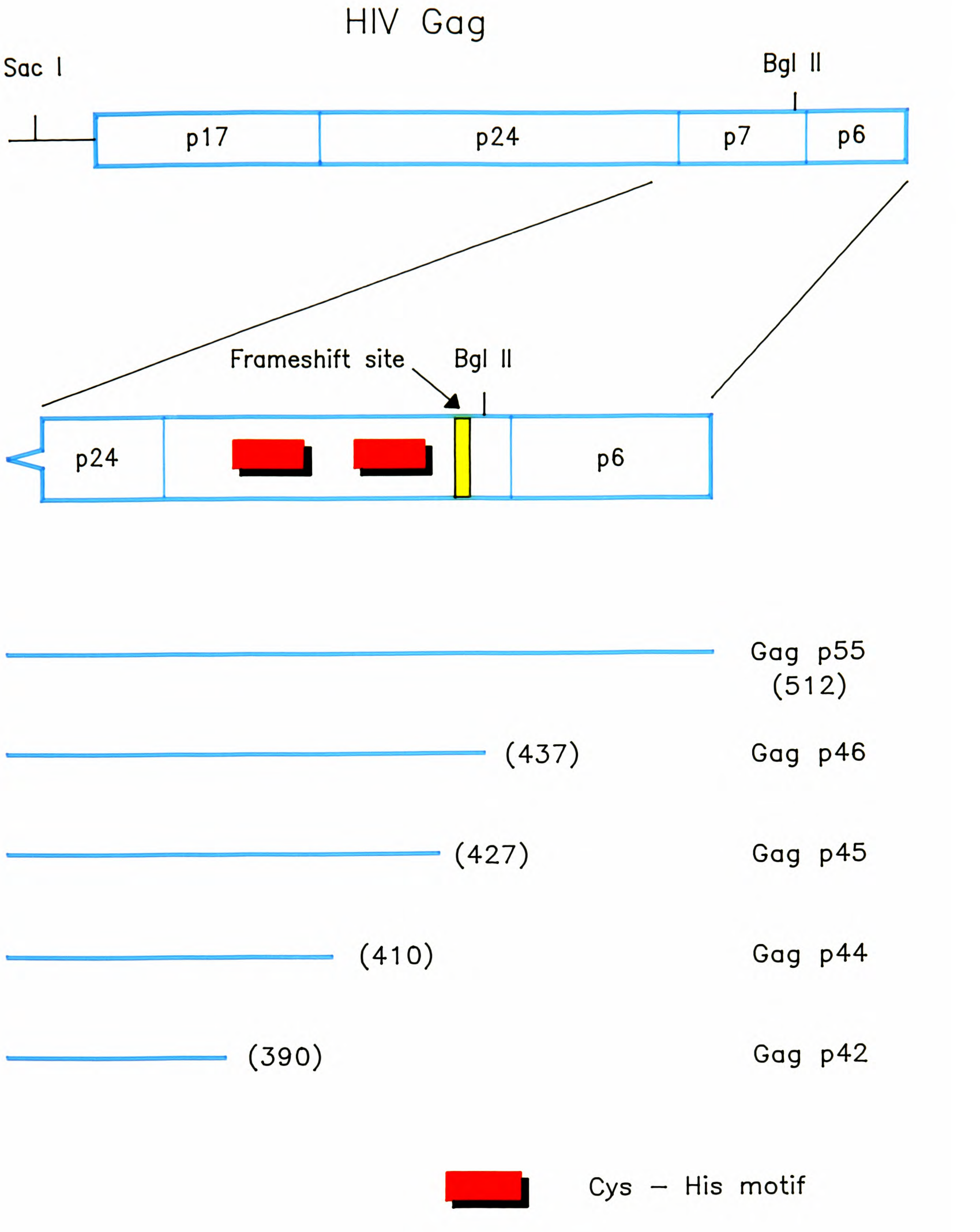
To address the question of whether the removal of the p6 domain alone was responsible for the abolition of particle assembly, a truncated HIV *gag* gene lacking the p6 region was expressed in insect cells using recombinant baculoviruses. This protein expression system was chosen for this analysis because not only has it been shown to achieve high levels of recombinant protein expression, but also that efficient Gag particle production has been demonstrated in insect cells by expression of the full length *gag* gene in recombinant baculoviruses (Gheysen *et al.*, 1989).

In addition, three more truncation mutants were prepared to assess the role of the p7 protein in particle assembly. This domain harbours two copies of a Cysteine - Histidine (Cys - His), motif which has the formula: Cys - X - X - Cys - X - X - X - X - His - X - X - X - X Cys, where X represents any amino acid. It is noteworthy that this motif is both widespread and highly conserved across the Retroviridae, with all sequenced members of the family known to contain one or

Figure 7.

The HIV - 1 *gag* gene

On the top level is a view of the HIV - 1 *gag* gene with the p17, p24, p7 and p6 domains shown. On the next level down, an exploded view of the carboxy terminus is given, where the main features of note are the Cys - His motifs (red), and the ribosomal frameshift sequence (yellow). On the lower section of the diagram can be seen a representation of the carboxy terminal boundaries of the full length (Gag p55), and four truncated Gag precursors Gag p46 (removal of p6 domain), Gag p45 (two Cys - His motifs), Gag p44 (one Cys - His motif), and Gag p42 (both Cys - His motifs deleted). The calculated number of amino acids in each precursor is given in parentheses.


HIV Gag
Sac I
Bgl II
p17
p24
p7
p6
Frameshift site
Bgl II
p24
p6
Gag p55
(512)
(437)
Gag p46
(427)
Gag p45
(410)
Gag p44
(390)
Gag p42
Cys – His motif

two copies within the Gag precursor (Wills and Craven, 1991). Its proposed function, the encapsidation of viral genomic RNA, has also been investigated in detail in the following chapter.

The Cys - His motifs, together with bound genomic RNA, have also been proposed to act as morphopoietic factors in viral assembly (Gelderblom, 1991). In support of this, a mutant was made where the *cis* - acting genomic RNA encapsidation sequence, lying between the splice / donor site and the *gag* initiation codon, was deleted. The authors observed aberrant morphology of the progeny virions produced by this mutant, coupled with a significant reduction in their infectivity (Clavel and Orenstein, 1990). Furthermore, in support of the motifs role in protein - protein interactions in addition to RNA binding, a mutant of simian virus type 40 (SV40), where an equivalent domain was deleted from the large T antigen, abolished the ability of the antigen to oligomerize into stable hexamers that are required for replication (Loeber *et al.*, 1991). In summary, it was thought that a role for the Cys - His motif in particle morphogenesis was not unlikely, and accordingly the construction of the three additional mutants was aimed at evaluation of such a role. The first was truncated so that the sequences immediately downstream of the distal Cys - His motif were deleted, the second had all of the distal motif deleted, while the third truncation had both the proximal and distal motifs removed entirely (figure 7).

During the course of this investigation, Royer *et al.* (1991), published a study asserting that deletion of the p7 domain did not disrupt particle formation of the HIV Gag precursors, in contrast to earlier work (Gheysen *et al.*, 1989; Kojima *et al.*, 1990; Hoshikawa *et al.*, 1991). The authors accounted for this discrepancy by suggesting that higher levels of expression were attained in their experiments

than in the previous reports. To clarify this issue, two additional truncation mutants were prepared under control of the same baculovirus promoter sequences as used in the other mutants in this study. The first had all sequences downstream of the p24 / p7 junction deleted while the other had half of the sequences between the p24 / p7 junction and the amino terminus of the proximal Cys - His motif removed.

In summary, the objectives of this chapter were as follows: first to determine whether or not the p6 domain is required for particle morphogenesis, second to assess the role, if any, that the Cys - His motifs play in Gag particle formation and finally to delineate the carboxy terminal boundary of an HIV - 1 Gag assembly domain.

Results

Construction of recombinant Gag expressing viruses

To generate the truncation mutant that has the p6 domain deleted, a *Sac* I - *Bgl* II fragment of HIV - 1_{LAI} strain (R3 plasmid; a gift of Simon Wain - Hobson; sequence described in Wain - Hobson *et al.*, 1985), was cloned into the baculovirus expression vector pAc CL29.1 (Livingstone and Jones, 1989), which has single stranded DNA production capability and is based on the pAc YM1 vector described elsewhere (Matsuura *et al.*, 1987). The *Sac* I site was upstream of the 5' end of the *gag* open reading frame while the *Bgl* II site was located between the frameshift sequence and the p7 / p6 junction (amino acid position 437), thus providing a convenient fragment for cloning (figure 7). An in - frame nonsense codon was positioned in the vector downstream from the cloning site, providing the translational termination signal.

This construct, termed pAc CL29.1 HIV$^{gag\ p46}$, was used for the generation of the other truncation mutants, where amber (TAG) stop codons were introduced at amino acid positions 427, 410 and 390 by altering single base pairs using site directed mutagenesis described in detail in the materials and methods section. Briefly, single stranded template DNA containing a small number of uracil residues was prepared from an *E. coli* strain (RZ 1032: genotype *dut*$^-$ *ung*$^-$).

Mutagenesis was carried out with the following oligonucleotides:

Gag p45 5' - GCCTGTCTCTAAGTACAATCT - 3'

Gag p44 5' - CAACAGCCCTATTTCCTAGGG - 3'

Gag p42 5' - TTGAAACACTAAACCATCTTT - 3'

where the underlined residues are those that were altered to encode a stop codon. Initial screening was performed by slot blot hybridization where the mutagenic oligonucleotide was labelled at the 5' end using T4 polynucleotide kinase and [γ - ^{32}P] ATP, described in materials and methods, and was used to probe for successful mutants. Due to the destabilizing effect of the mismatched base pairing between the oligonucleotide probe and a wild type target sequence, the probe could be washed off at a lower temperature than that required to remove the probe from mutant DNA. Thus by sequentially washing the membrane filter at progressively higher temperatures, starting at 5°C below the calculated melting temperature (T_m), the successful mutants can be identified. For each of the mutant oligonucleotides, the T_m the was calculated according to the Wallace rule (see materials and methods), these were: Gag p45 - 58°C, Gag p44 - 62°C and Gag p42 - 52°C. Figure 8A shows a typical set of autoradiographs of the filter (Gag p42 in this case), following successive washing steps. Controls included were wild type template DNA (slot 11), and non - homologous DNA from M13 KO7 phage (slot 12). The initial wash was at 5°C below the calculated melting temperature of the heteroduplex, then increased by 2°C steps until the probe washed off the wild type control (slot 11). Successful mutants were identified in slots 1, 4, 6, 7, and 9, and one of these was selected for confirmation of the change by DNA sequencing (figure 8B). Based on their primary amino acid sequence the constructs were expected to produce proteins

Figure 8.

Panel A. Site directed mutagenesis

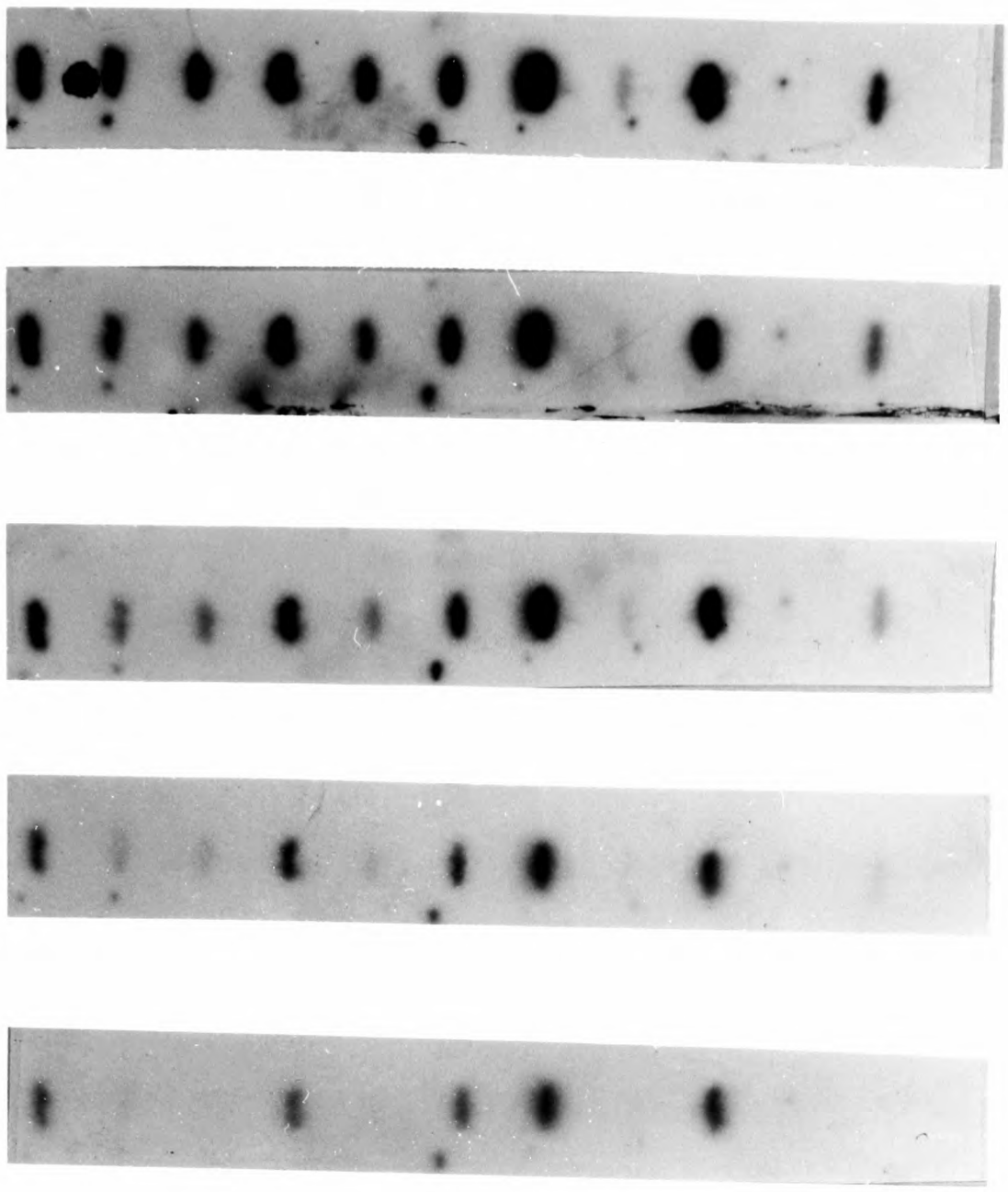
Photograph of autoradiographs for screening of successful site directed mutagenesis mutants in Gag p42. Slots 1 - 9 contain 9 separate test samples, while slot 11 contains wild type parent plasmid DNA (pAc CL29.1 - HIV*gag p46*), and slot 12 non - specific DNA derived from the M13 KO7 bacteriophage. On the right side, the washing temperature of the membrane is given in degrees °C above (+), or below (-), the calculated melting temperature (T_m), of the DNA heteroduplex. The lowest panel (T_m + 5 °C), revealed that the radiolabelled probe remained bound to the DNA from samples 1, 4, 6, 7 and 9, while being washed off the parent DNA sample (slot 11). This indicates that samples 1, 4, 6, 7 and 9 are successful mutants. Autoradiography was for 2 hrs at - 70 °C with one intensifying screen.

Panel B. Confirmatory dideoxynucleotide DNA sequencing

This panel shows a section from an autoradiograph of a sequencing gel confirming the mutation made in the Gag p42 truncation mutant. The letters above the panel indicate the samples in which the respective dideoxynucleotides were present, ie ddGTP was in the left most lane. Shown on the right is the sequence as read from the gel (- strand), and next to it the inferred complementary strand (+). The position of the base change (T to A), is indicated by an arrow. Autoradiography was for 16 hrs at - 70 °C with one intensifying screen.

A

Slot 1 2 3 4 5 6 7 8 9 10 11 12



Tm −5°c

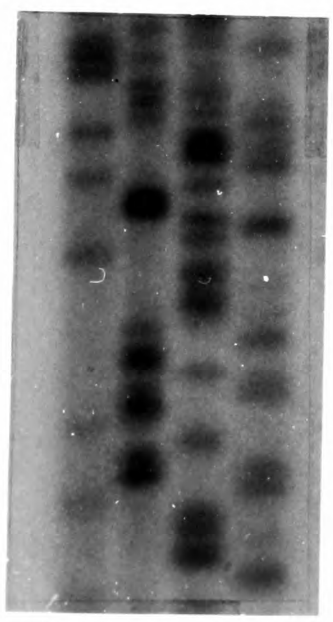
Tm −3°c

Tm +1°c

Tm +3°c

Tm +5°c

B



G A T C

−	+	Strand
3'	5'	
C	G	
T	A	
T	A	
T	A	
C	G	
T	A	
A	T	
C	G	
C	G	
A	T	
A	T	
A	T	← Altered Base
T	A	(T to A on − strand)
C	G	
A	T	
C	G	
A	T	
A	T	
A	T	
G	C	
T	A	
T	A	
A	T	
A	T	
5'	3'	

of molecular weights 46, 45, 44 and 42 kDa, and were named accordingly Gag p46, Gag p45, Gag p44 and Gag p42 respectively.

Analysis of Gag protein expression

Purified high concentration stocks of each of these plasmid DNAs containing the truncated *gag* genes were prepared. Approximately 6 - 10 μg of plasmid DNA was mixed with 1 μg of linearized AcRP - 6 SC genomic DNA and co - transfected into *Spodoptera frugiperda* (Sf) cells. Culture medium was harvested at 4 - 6 days post infection and titrated by plaque assay. Individual plaques were picked and amplified by infecting small cultures of Sf cells (5 x 10^5 cells / 32 mm dish). Total cell extracts of these cultures, harvested at 4 - 6 days post infection, were analyzed by separating the proteins by SDS/PAGE and western blotting. Recombinant Gag protein production was detected by a mixture of monoclonal antibodies specific to the Gag p17 and p24 domains, comprising the first of a three step antibody conjugate detection protocol for maximum sensitivity. The 1° antibodies were mouse monoclonal antibodies directed against HIV Gag p17 and p24 (Holmes, 1991 Cat. No. ADP 316 and ADP 307 respectively), 2° antibody was anti mouse biotin conjugate (Amersham Cat. No. RPN 1021), and the 3° antibody ExtrAvidin alkaline phosphatase conjugate (Sigma Cat. No. E - 2636). Cultures found to be producing recombinant Gag protein were subjected to 3 additional rounds of plaque assay purification. Finally, high titre stocks of the recombinant viruses were prepared and stored for further use.

A recombinant baculovirus was made available to me by Dr. Ian Jones that contained the full length HIV *gag* gene sequence. This construct was prepared

by PCR amplification, using the HIV - 1_{LAI} R3 DNA stock, cloned into an expression vector and recombinant baculoviruses prepared and purified. This was termed Gag p55, and was used to express the full length Gag protein as a control in this study.

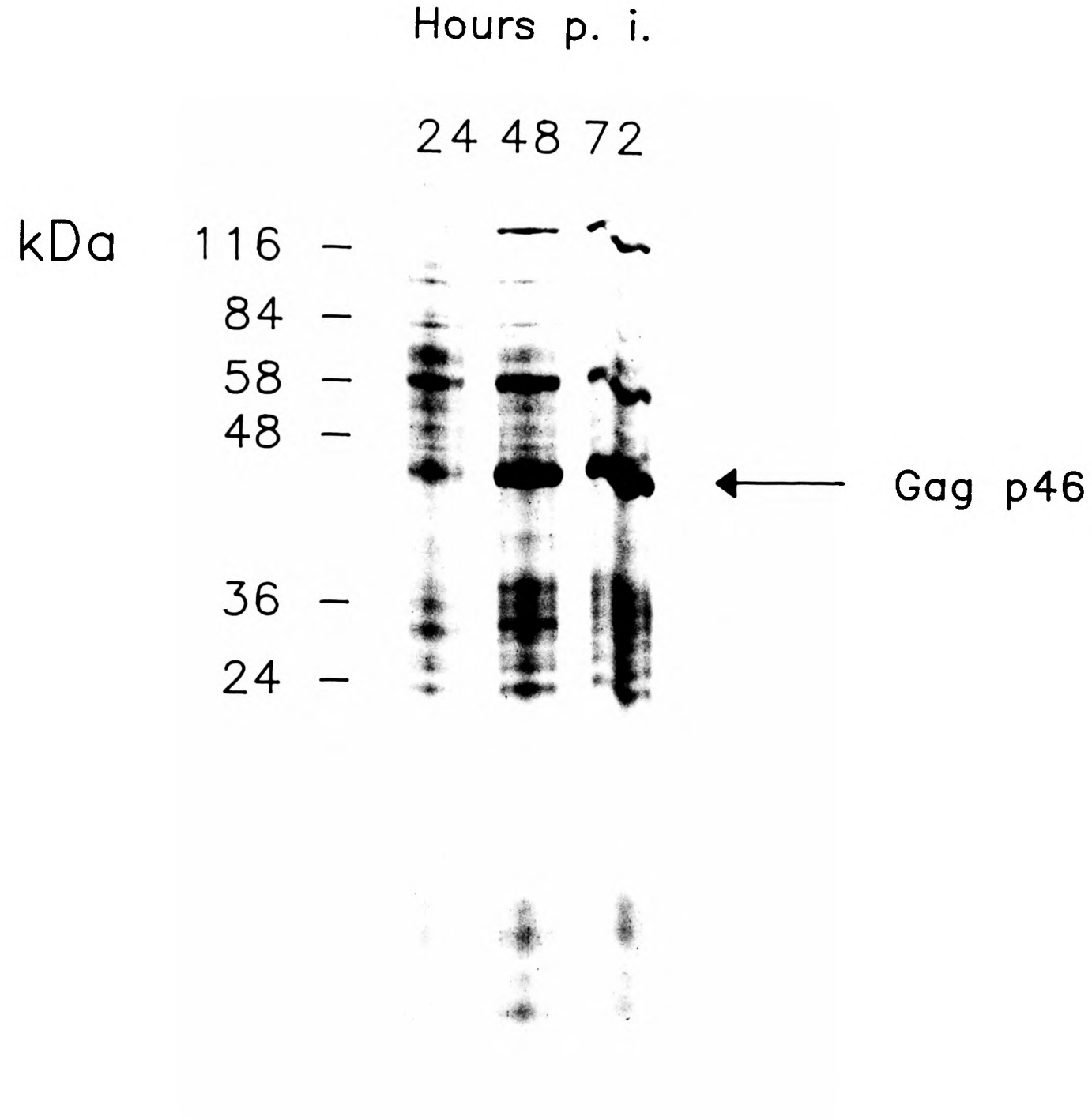
A time course experiment was used to demonstrate production of the recombinant protein. A culture of Sf cells was infected with the recombinant virus (data shown for Gag p46), at a multiplicity of infection (moi) of 10. At the time points 24, 48 and 72 hr post infection, 10^6 cells were harvested from the culture, washed in PBS and denatured (100°C, 5 min), in 50 μl of SDS gel loading buffer. These extracts were analyzed by SDS/PAGE, where the equivalent of 10^5 cells were loaded per lane for each time point (figure 9). The gel was stained for total protein by coomassie blue. A band of protein of about 46 kDa was seen that increased with intensity over time. This was subsequently confirmed as Gag in origin by western blot (data not shown). Additionally this experiment demonstrated that 2 days post infection was optimal for recombinant protein production while cellular degradation was at a minimum.

To confirm that each of the recombinant proteins reflected their predicted molecular weights and demonstrate efficient production in Sf cells, the following experiments were carried out. Cultures of 1.6 x 10^6 Sf cells (32 mm dishes), were infected with each recombinant (moi = 10), including the full length *gag* gene expressing recombinant for reference (Gag p55). At 2 days post infection, the cultures were harvested, the cells washed in PBS, and lysed in SDS gel loading buffer. Samples of the extracts (equivalent to 2 x 10^5 cells) were separated by SDS/PAGE in duplicate. One gel was stained for total protein content with coomassie blue, while the other was western blotted onto nitrocellulose

Figure 9.

Time course of Gag p46 protein production

Coomassie blue stained SDS/PAGE gel of samples harvested at 24, 48 and 72 hours post infection (p. i.), from a culture of Sf cells infected with the HIV Gag p46 recombinant virus. The position of the Gag p46 protein, indicated by an arrow, corresponds to a molecular weight of 46 kDa.


Hours p. i.
24 48 72
kDa
116
84
58
48
36
24
Gag p46

membrane and probed for Gag antigen by a 2 stage detection method, the 1° antibody was as above, while the 2° antibody was an anti mouse alkaline phosphatase conjugate (Sigma Cat. No. A - 0162). Wild type AcRP6 infected and mock infected Sf cells were included as negative controls. In figure 10, an increase in electrophoretic mobility of the recombinant proteins corresponding to the increasing size of the truncations made can be seen. Additionally, good correlation with the predicted molecular weights was noted.

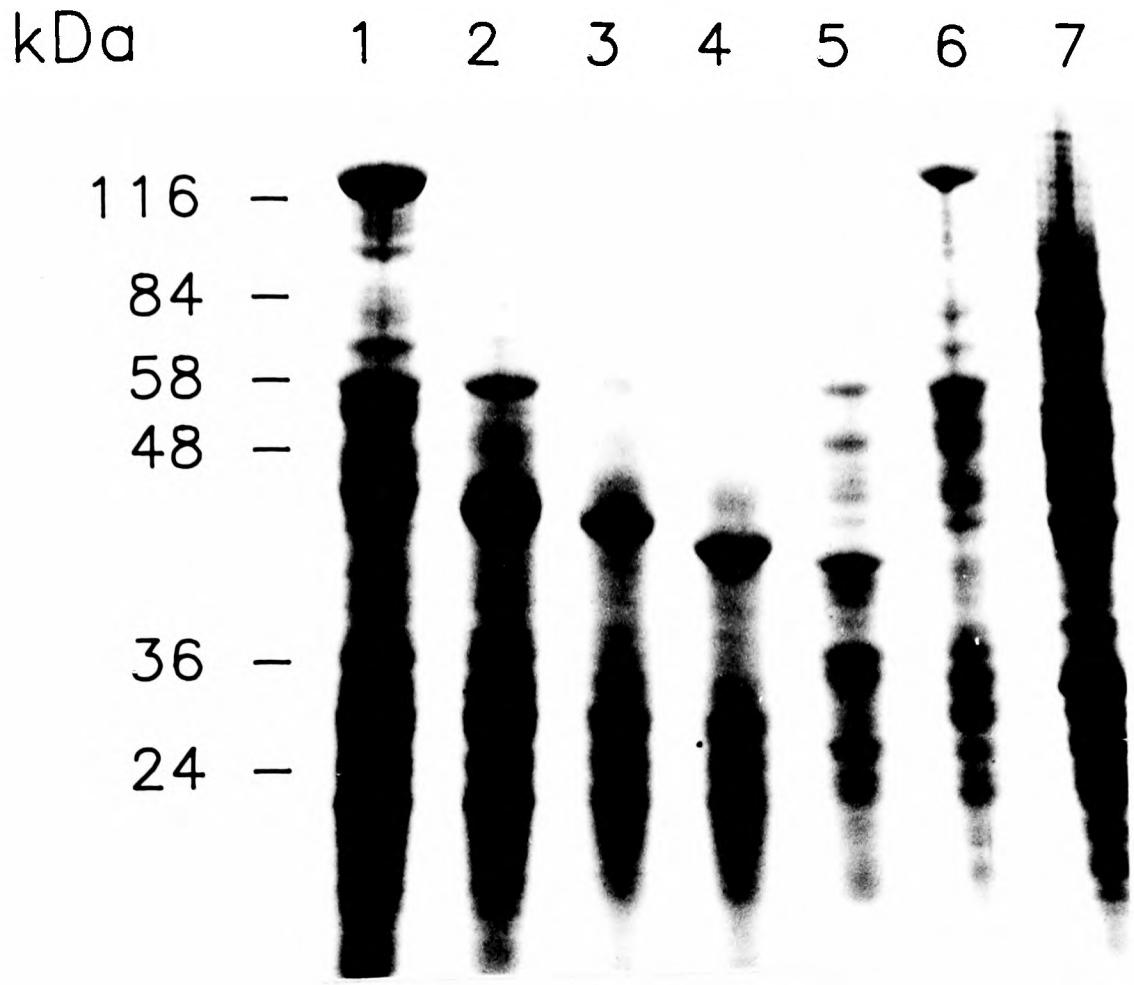
Assessment of particle forming ability of these mutants was done using the following technique which allowed detection of particulate antigen released into the culture medium by the infected cells. For each of the recombinant viruses a 75 cm^2 tissue culture flask containing 12 x 10^6 Sf cells was infected at an moi of 10. At 2 days post infection, the cultures were harvested, subjected to low speed centrifugation (185*xg*, 10 min), to remove the cells, and the supernatant loaded onto a continuous sucrose gradient of 20% to 60% in an ultracentrifuge tube. The gradient was centrifuged at 164,000*xg* for 2 hr, the supernatant carefully aspirated and the column fractionated into ten 0.5 ml aliquots. The fractionation was performed by hand using a wide bore pipette, and removing each layer consecutively. The densities of each fraction were determined by weighing each sample. This technique using narrow bore tubes was found to cause minimal mixing between fractions and able to provide reproducible results. Equal proportions of each fraction were mixed with SDS gel loading buffer and analyzed by western blot as described previously. The same protocol was followed for each of the five recombinant Gag producing viruses. The results shown in figure 11 indicate that Gag antigen sedimented to approximately 45% sucrose and was found in the medium of cultures expressing Gag p55, Gag p46, Gag p45 and Gag p44. However, in the case of Gag p42, a shift in the relative

Figure 10.

Expression of Gag precursor truncation mutants

Coomassie blue stained SDS/PAGE gel (panel A), and western blot probed with monoclonal antibodies to HIV Gag protein (panel B), of cell extracts of cultures infected with Gag p55 (lane 1), Gag p46 (lane 2), Gag p45 (lane 3), Gag p44 (lane 4), and Gag p42 (lane 5). Negative controls included were AcRP6 infected (lane 6), and mock infected (lane 7), Sf culture extracts. Molecular weight markers are indicated on the left side of the panel in kDa.

A



B

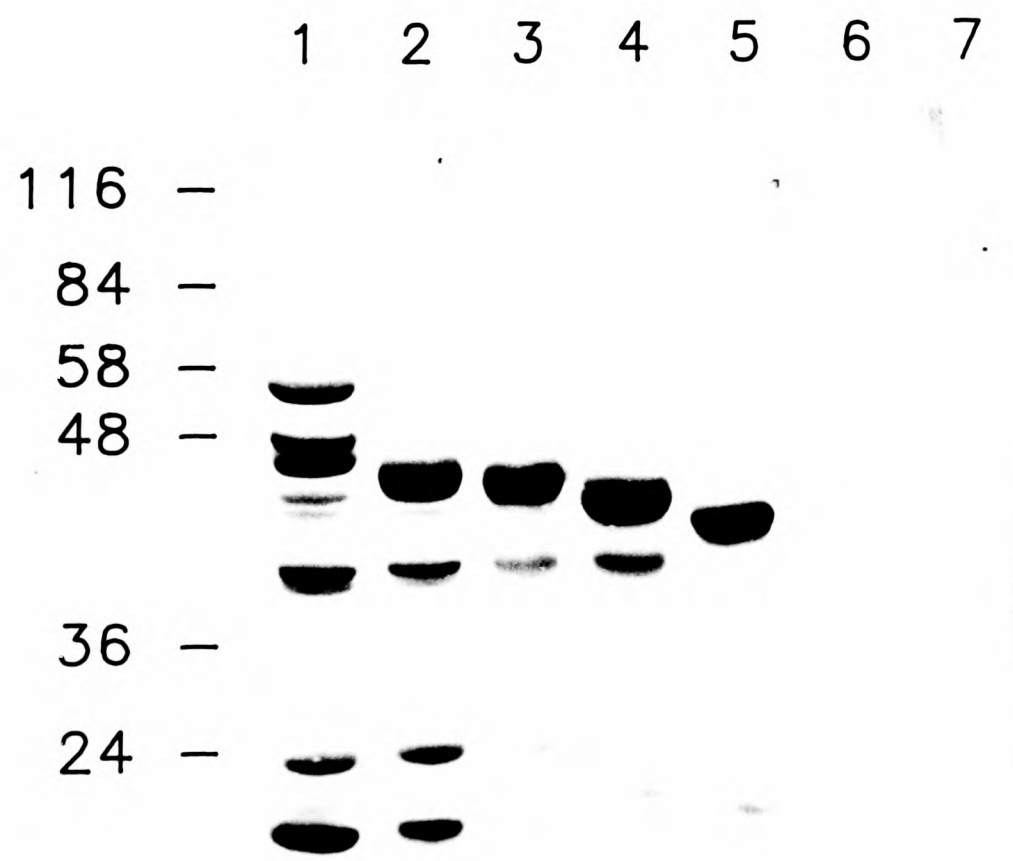


Figure 11.

Sucrose density gradient analysis of particle production of the Gag truncation mutants

Western blots of fractions taken from a continuous sucrose density gradient loaded with culture supernatants from cells infected with Gag p55, Gag p46, Gag p45, Gag p44 and Gag p42. The fractions were loaded onto the SDS/PAGE gel, from the top of the gradient (left side; 20% sucrose), to the bottom of the gradient (right side; 60% sucrose), and probed with monoclonal antibodies to HIV Gag protein.

Fraction number

(20%) 2 3 4 5 6 7 8 9 (60%)

Gag p55

Gag p46

Gag p45

Gag p44

Gag p42

position of this antigen was noted indicating altered sedimentation properties (lower density), equivalent to 30% sucrose, additionally further spread through the gradient was noted.

Electron microscopy analysis

The formation of particles was studied by thin section electron microscopy. Sf cells infected at an moi of 10 and harvested at 2 days post infection were fixed in glutaraldehyde buffer and prepared for electron microscopy as described in materials and methods. In cells infected with the full length Gag p55 expressing recombinant, immature core like particles could be seen forming at and budding from the plasma membrane (figures 12 and 13), as has been previously described (Gheysen *et al.*, 1989), while they were absent in wild type AcNPV infected cells (figure 14). Similar observations of immature core like particles were made in cells expressing Gag p46 (figure 15). In cells expressing Gag p45, particles were seen budding from the plasma membrane in abundance (figure 16), also interestingly, the formation of intracytoplasmic "Gag rings" were noted. These rings appeared to bud into intracellular vacuoles in the main, but were also seen to bud from the plasma membrane occasionally (figure 17). Additionally some normal budding into intracellular vacuoles was observed in contrast to the Gag p55 construct where this event was uncommon. Gag p44 expression of antigen was very similar to that of Gag p45 with extensive budding from the plasma membrane and some budding into intracellular vacuoles (figure 18). The intracytoplasmic Gag rings were also seen, but these only budded into the vacuoles and not at all from the plasma membrane. Approximately 10% of the cells expressing the Gag p42 truncation mutant exhibited surface plasma

Figure 12.

Electron micrograph - Gag p55

Thin section electron microscopy of an Sf cell infected with the Gag p55 recombinant virus. Budding particles are seen in abundance at the plasma membrane. The size bar = 1 μm.

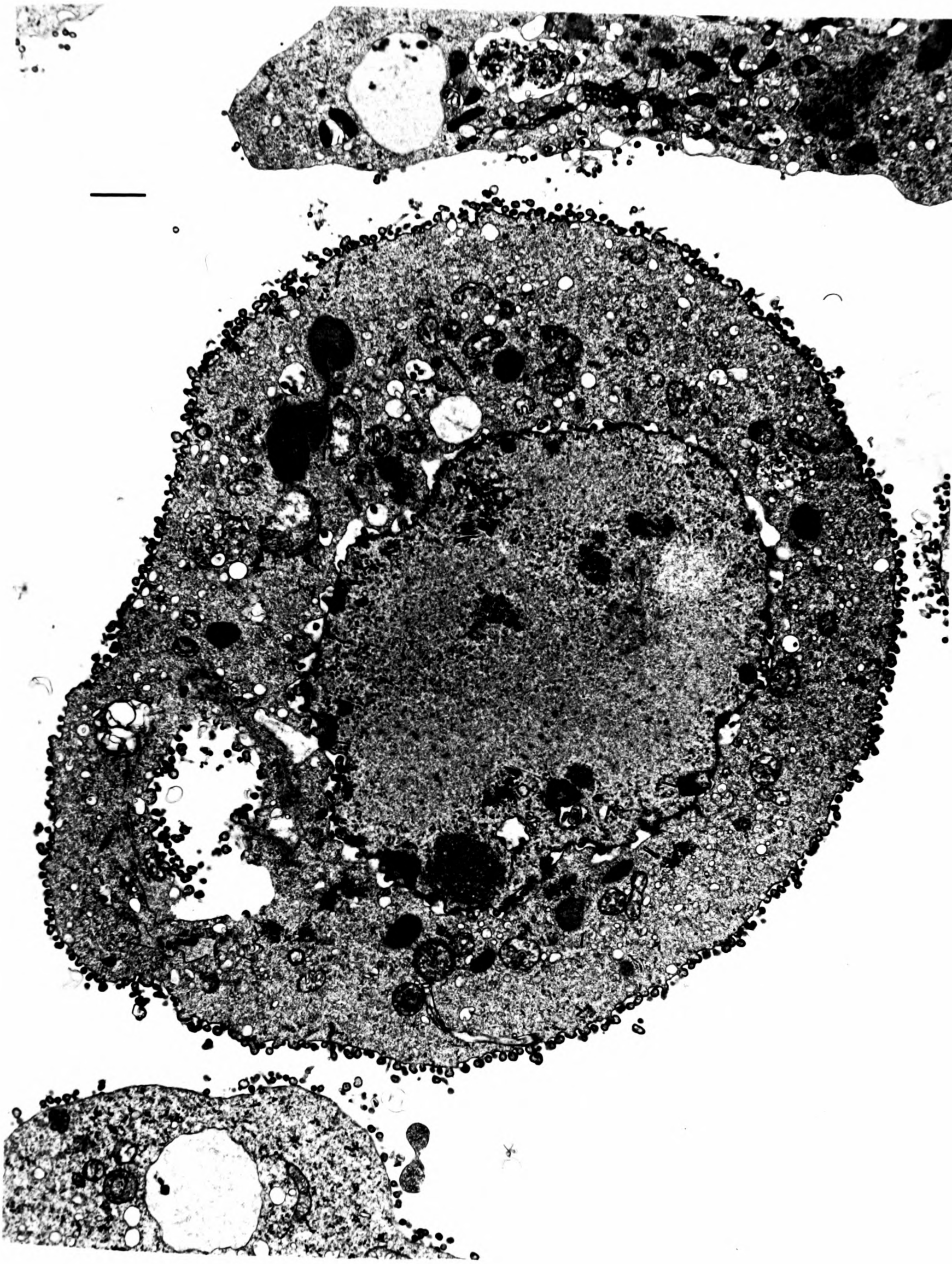


Figure 13.

Electron micrograph - Gag p55

Thin section electron microscopy of the surface of an Sf cell infected with the Gag p55 recombinant virus. Detail showing the budding of an immature HIV - 1 core like particle form the surface of the cell. Bar = 100 nm.

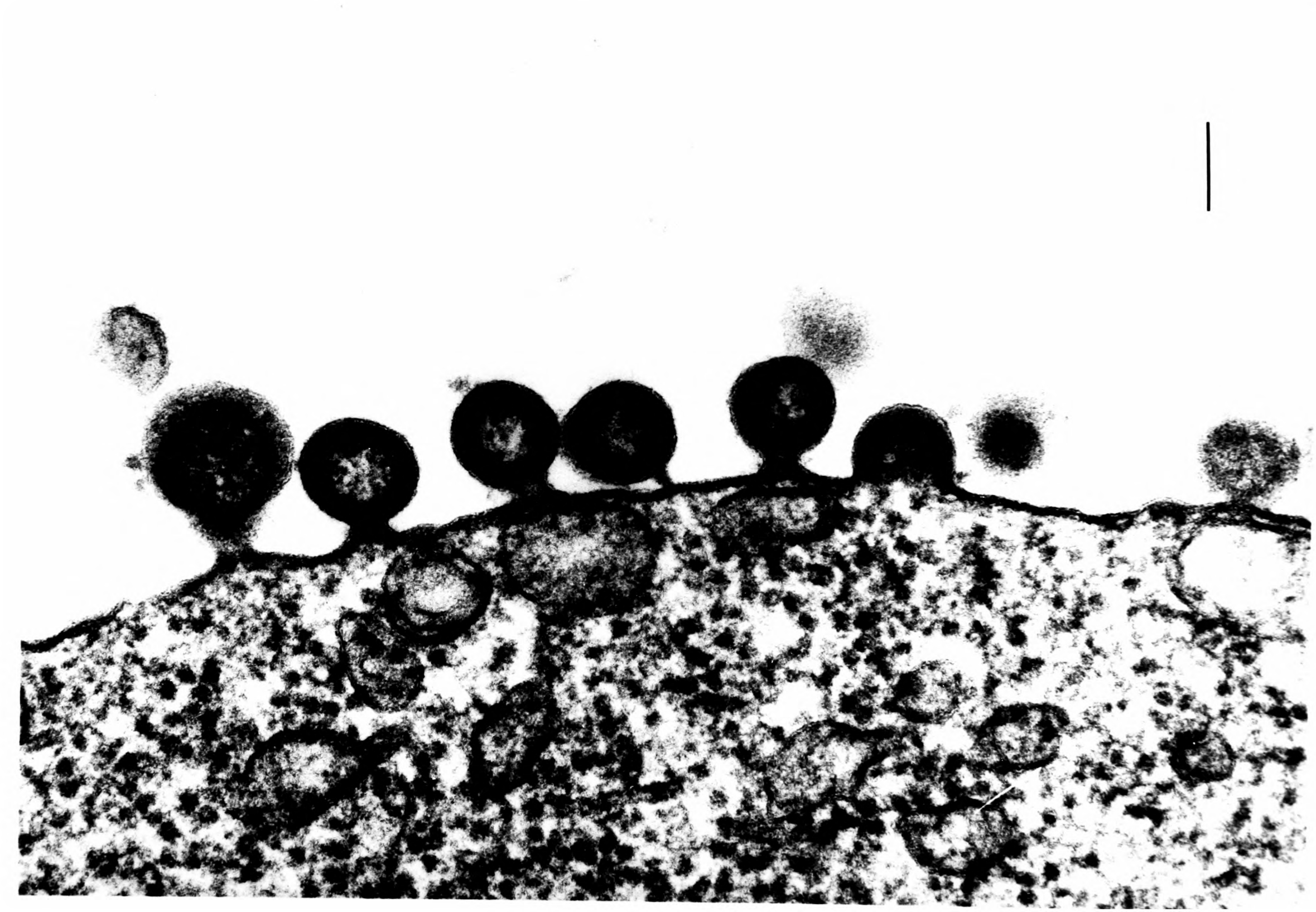


Figure 14.

Electron micrograph - AcNPV infected cell

Thin section electron microscopy of an Sf cell infected with the AcNPV wild type recombinant baculovirus. Bar = 1 μm.

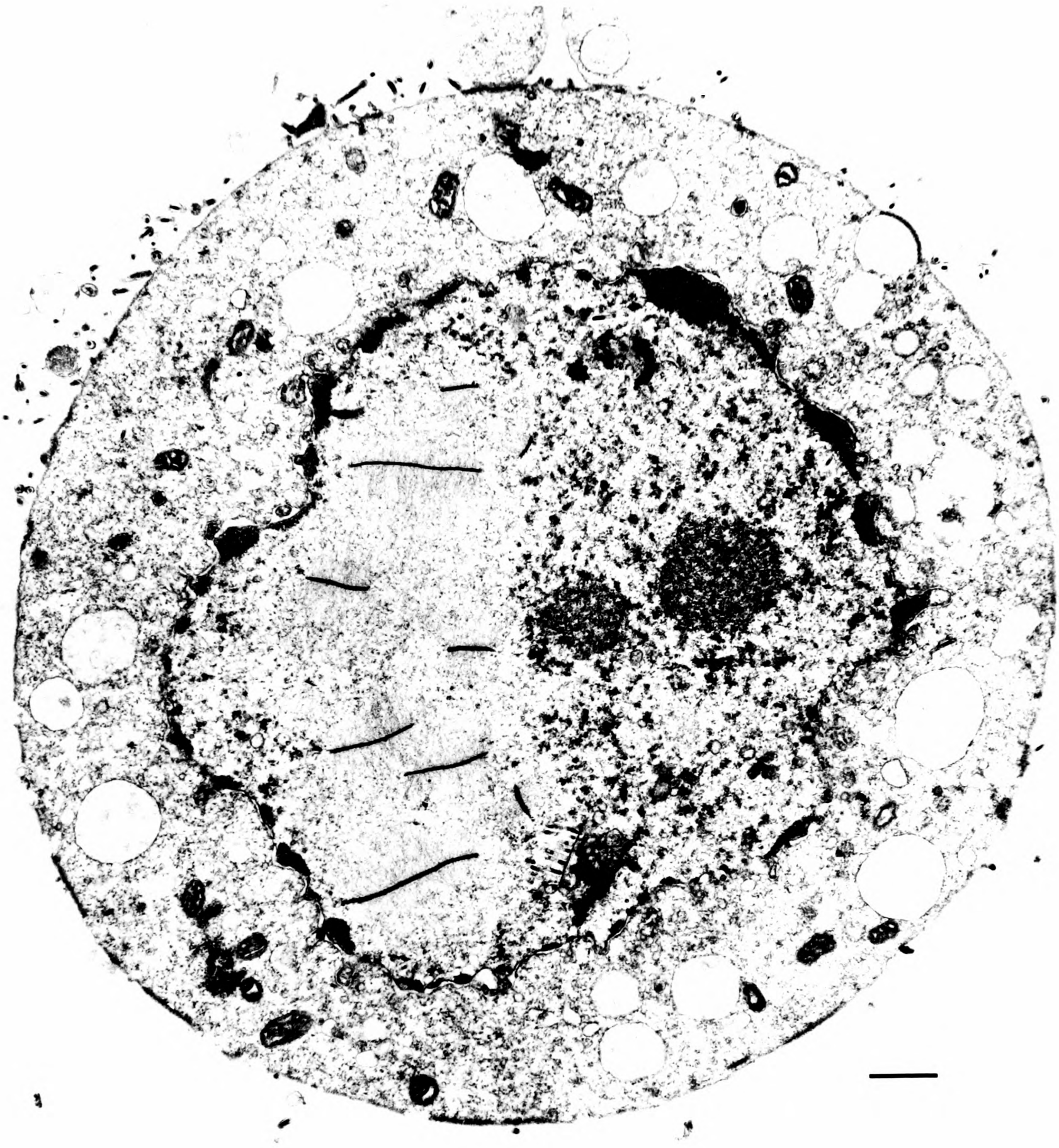


Figure 15.

Electron micrograph - Gag p46

Thin section electron microscopy of an Sf cell infected with the Gag p46 recombinant virus. Shows immature HIV - 1 core like particles budding from the plasma membrane. Bar = 100 nm.

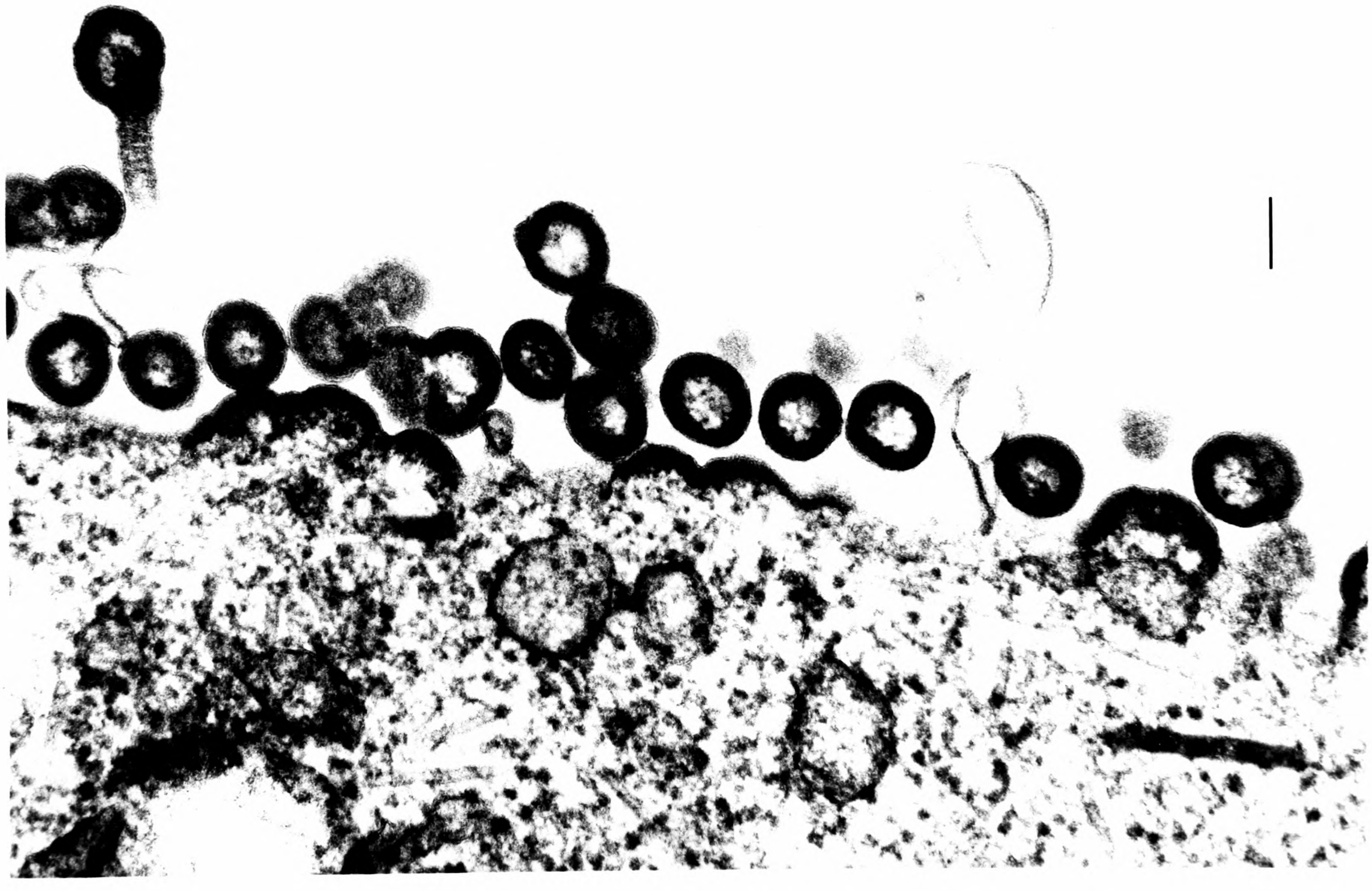


Figure 16.

Electron micrograph - Gag p45

Thin section electron microscopy of an Sf cell infected with the Gag p45 recombinant virus. Shows immature HIV - 1 core like particles budding from the surface of the cell. Bar = 100 nm.

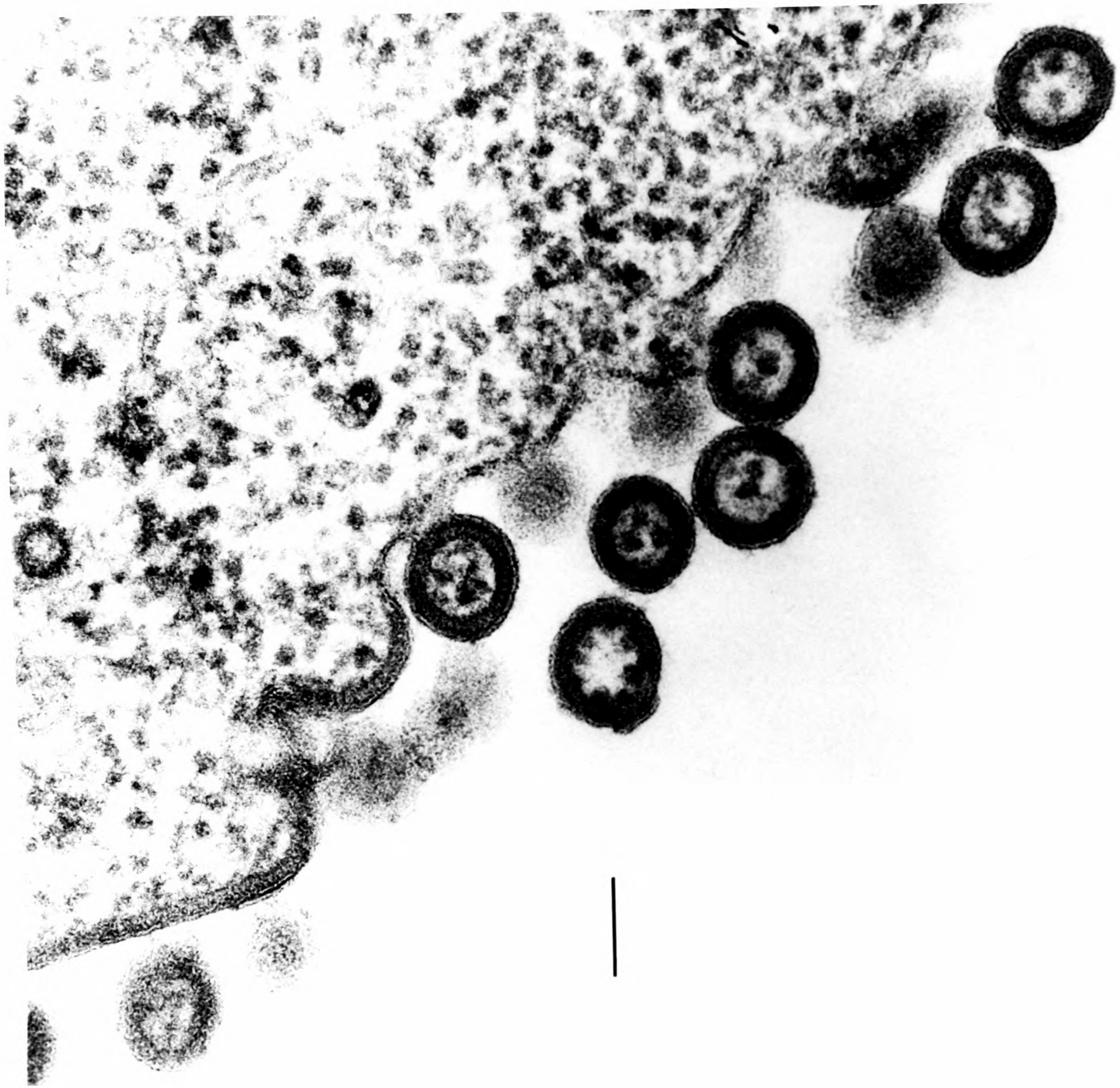


Figure 17.

Electron micrograph - Gag p45

Thin section electron microscopy of an Sf cell infected with the Gag p45 recombinant virus, showing budding of particles into intracellular vacuoles in addition to intracytoplasmic "Gag rings" (white arrow). Bar = 100 nm.

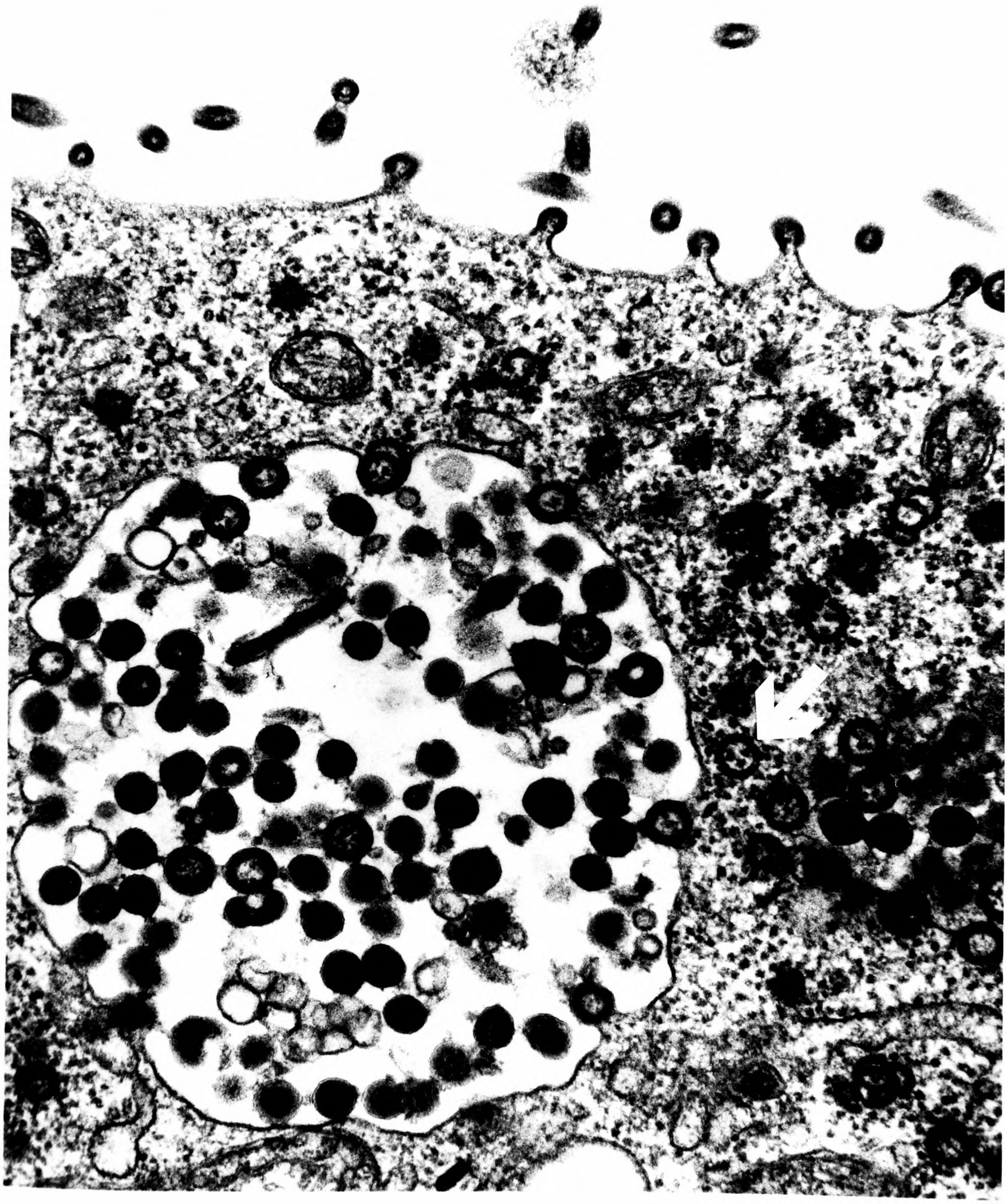
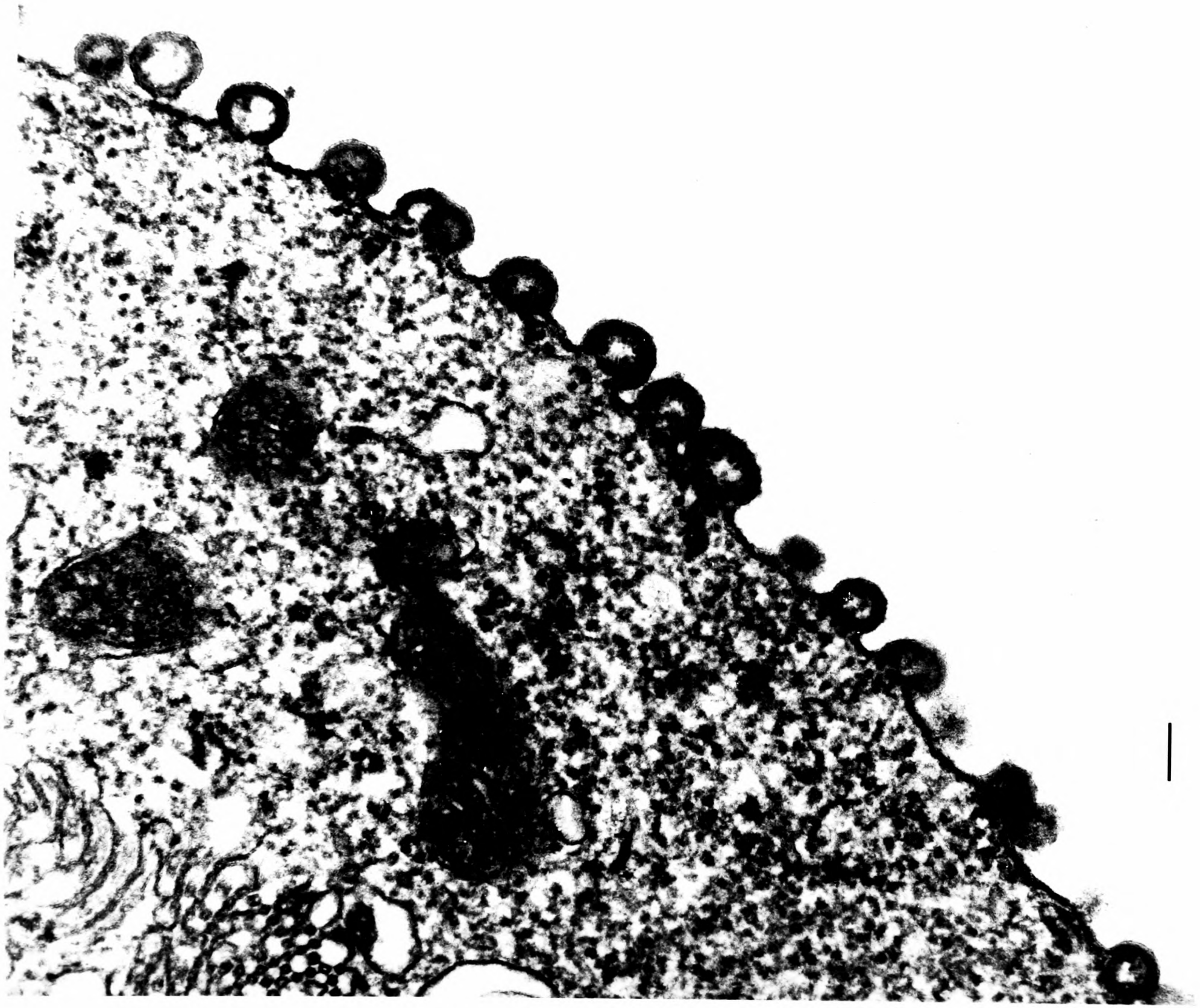


Figure 18.

Electron micrograph - Gag p44

Thin section electron microscopy of an Sf cell infected with the Gag p44 recombinant virus, showing the budding of immature HIV - 1 core like particles from the plasma membrane of the infected cell. Bar = 100 nm.



membrane budding and some budding into intracellular vacuoles. Intracytoplasmic Gag rings were rare in this group. The remaining 90% of cells showed no budding from the plasma membrane, only into intracellular vacuoles (figure 19). These vacuoles presumably formed deep in the cytoplasm then appeared to progress to the cells surface and break open releasing large numbers of Gag particles (figure 20). Another observation of interest was that particles within these vacuoles often but not always showed evidence of an inner membrane (figure 21). Intracytoplasmic Gag rings were more numerous in these cells than in the cells showing surface budding and were only seen budding into the vacuoles deep in the cytoplasm and not at all into those near or on the surface of the cell. The particles produced by Gag p42 expression also appeared to be 10% larger in size and less uniformly circular than those produced by the other truncation mutants.

Delineation of a Gag assembly domain boundary

As alluded to earlier, two additional truncation mutants were prepared to address a question raised by a report that was published during the course of this research (Royer *et al.*, 1991). The report suggested that deletion of the p7 and p6 domains of the HIV Gag precursor still allowed the protein to self assemble into particles, in apparent contradiction to previously published work (Gheysen *et al.*, 1989). It would seem likely that the apparent discrepancy had arisen here due to confusion over the exact position of the junction between the p24 and the p7 domains. These domains are delineated by the cleavage site of the virally encoded protease. There are in fact 3 possible protease cleavage sites at amino acid positions 363, 367 and 377 of Gag all of which are cleaved, but at differing

Figure 19.

Electron micrograph - Gag p42

Thin section electron microscopy of an Sf cell infected with the Gag p42 recombinant virus. Shows particles budding from the plasma membrane of the cell (10% of cells), and more commonly seen (90% of cells), particles enclosed within vacuoles. Bar = 100 nm.

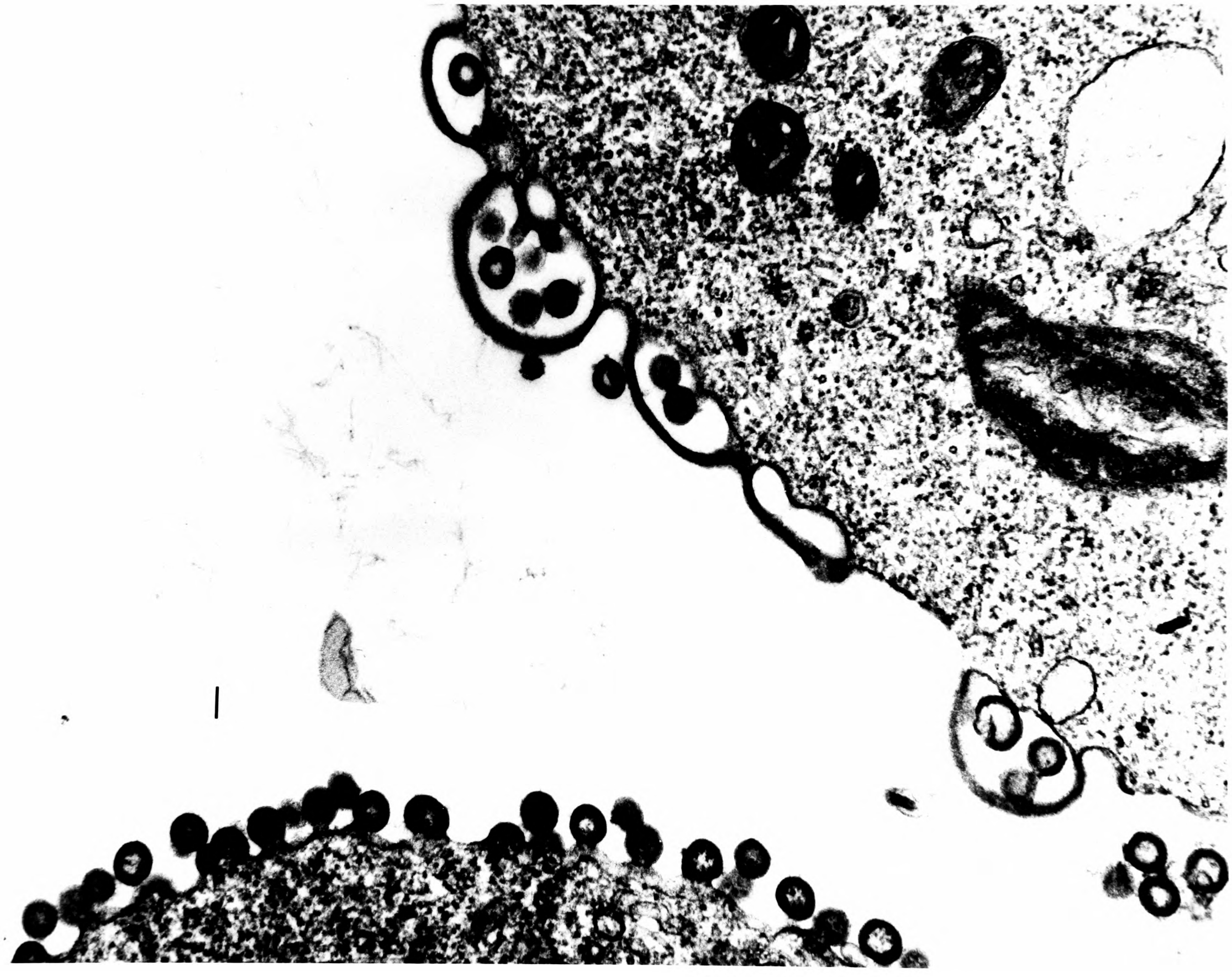


Figure 20.

Electron micrograph - Gag p42

Thin section electron microscopy of an Sf cell infected with the Gag p42 truncation mutant, showing abundant release of immature HIV - 1 core like particles from vacuoles at the cell surface. The particles were less uniform in their dimensions and approximately 10% larger, than particles produced by the other HIV Gag truncation mutants. Bar = 100 nm.

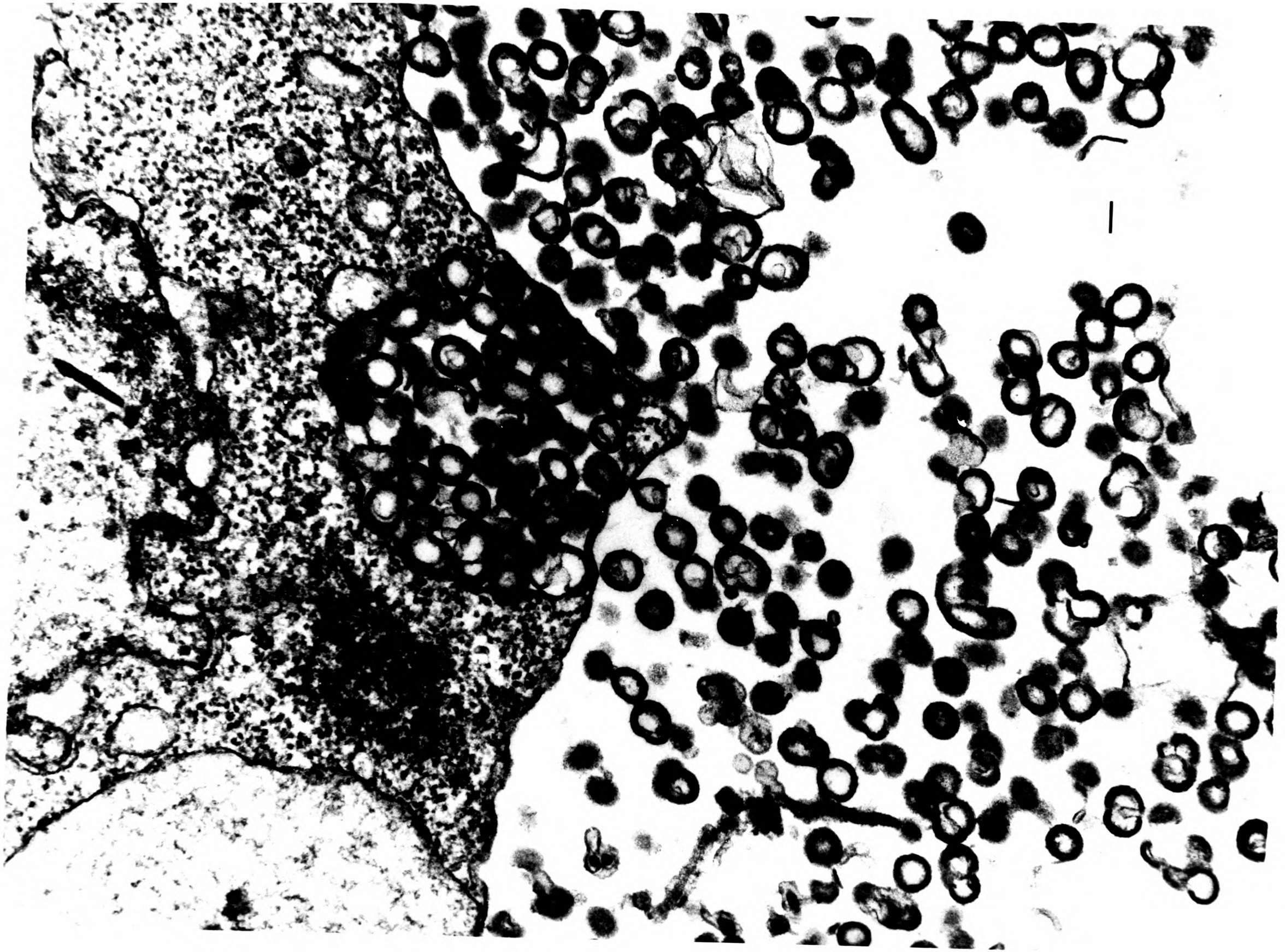
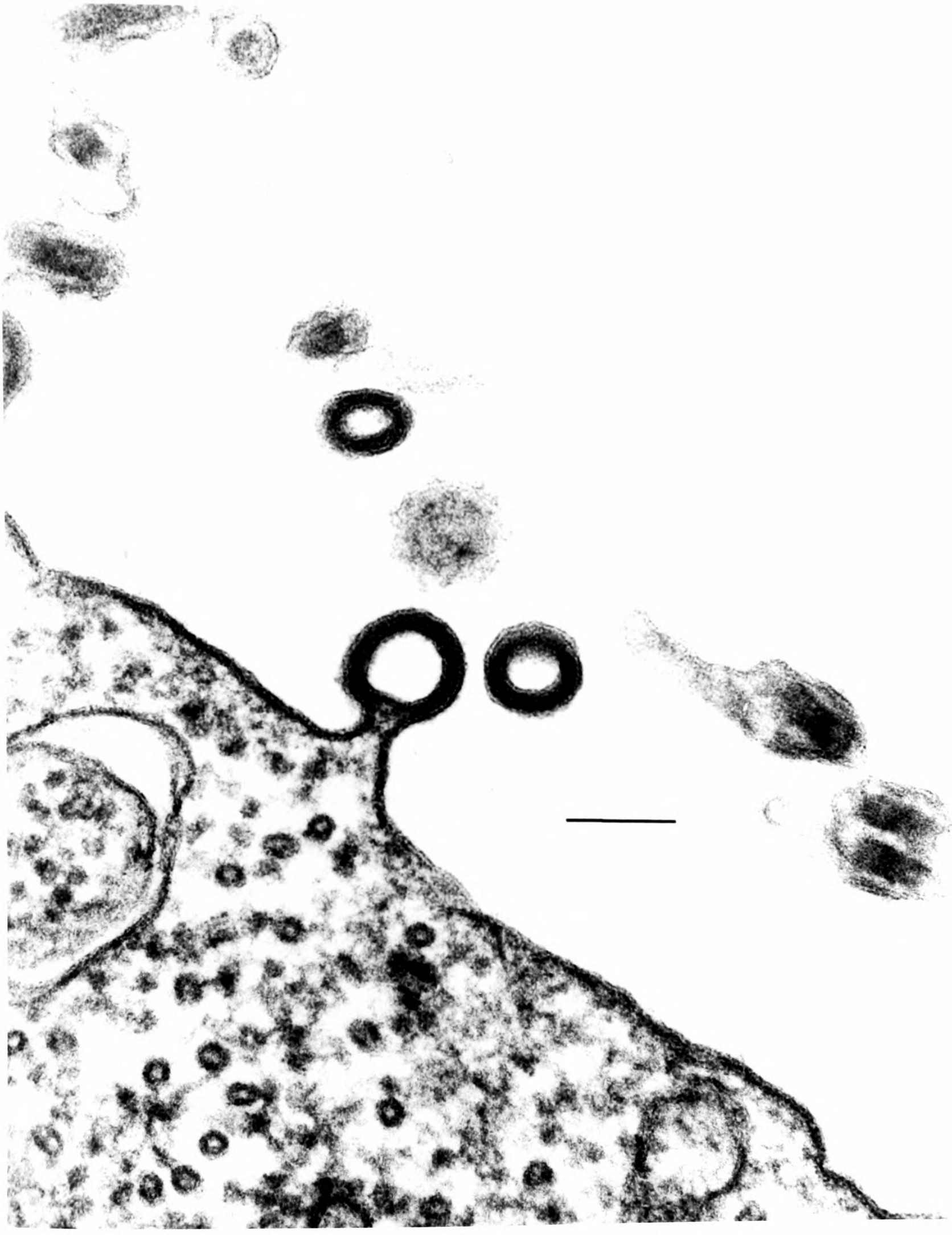


Figure 21.

Electron micrograph - Gag p42

Thin section electron microscopy of an Sf cell infected with the Gag p55 recombinant virus. Shows a particle at the surface of the cell that appears to have an internal membrane. Bar = 100 nm.



rates, by the HIV protease. The report noting abolition of particle formation (Gheysen *et al.*, 1989), deleted all sequences downstream of the cleavage site nearest the amino terminus of the precursor (amino acid 363). The subsequent publication (Royer *et al.*, 1991), stated there was particle formation observed if all sequences were deleted from amino acid 380, downstream of the cleavage site nearest the carboxy terminal of the protein, however the evidence presented to support self assembly and release of this truncated Gag antigen was scant.

In addition to clarifying this issue, the mutants were also prepared to map an end point for sequences necessary for particle self assembly. The truncation mutants prepared (figure 22), deleted all sequences downstream of amino acid lysine 363, (same as the construct vAcGag *Cfr* I prepared by Gheysen and colleagues), and amino acid tryptophan 373, a point which lies between the former construct and the construct (AcNPVgag14) prepared by Royer *et al.* (1991).

These mutants were constructed by a different method than previously used. This involved using polymerase chain reaction (PCR) amplification of the desired segment of the *gag* gene, with specific oligonucleotide primers designed to facilitate the subsequent cloning of the fragment into the pAc CL29.1 transfer vector. The PCR primers used were as follows:

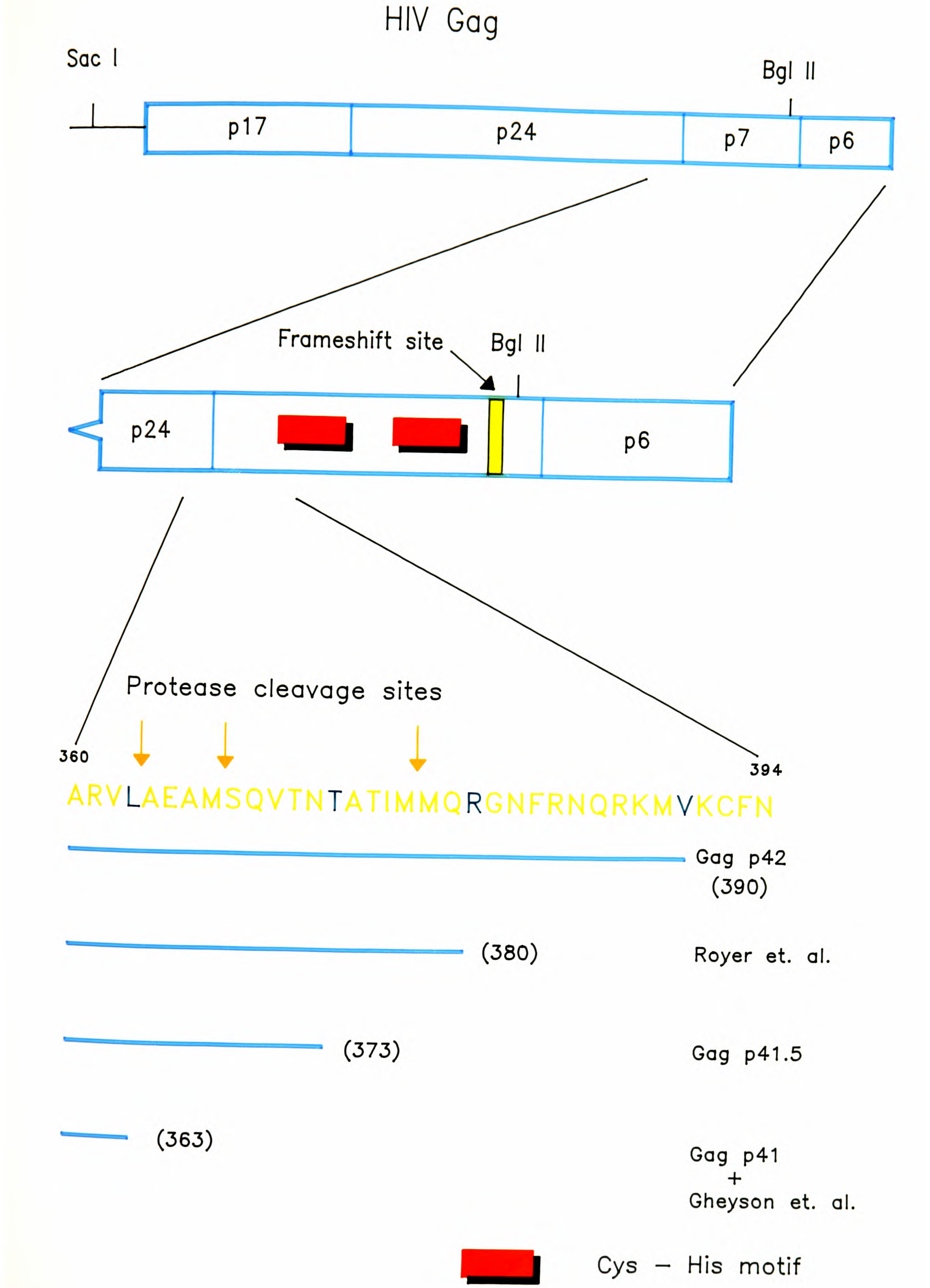
Gag p41F 5' - CGCGGAGCTCAGAGATGGGTGCGAGAGCGTC - 3'
Gag p41R 5' - CGCGTCTAGACTACAAAACTCTTGCCTTATGGC - 3'
Gag p41.5R 5' - CGCGTCTAGACTATGTATTTGTTACTTGGCTCAT - 3'

These were used in pairs as forward and reverse primers, Gag p41F and Gag p41R to create the first fragment, and Gag p41F and Gag p41.5R together to

Figure 22.

HIV Gag - detail of amino terminus of the p7 domain

The top level shows the HIV - 1 Gag coding region which has been expanded to give more detail of the p7 and p6 domains (middle section). The amino terminus of the p7 domain has been further expanded in the lower section, where the amino acid sequence is listed in single letter code (yellow). The number immediately above each end of the amino acid sequence corresponds the position of the residue in the Gag precursor. The orange arrows indicate the three protease cleavage sites at amino acid numbers 363, 367 and 377. Below the sequence, the carboxy terminal boundaries of the 2 additional HIV Gag truncation mutants (Gag p41.5 and Gag p41), are delineated. Additionally, the mutant prepared by Royer and colleagues (1991), has been shown. The number in parentheses at the end of the line indicates the last amino acid in the sequence, which correspondingly appears in dark blue in the above amino acid sequence.


HIV Gag
Sac I
Bgl II
p17
p24
p7
p6
Frameshift site
Bgl II
p24
p6
Protease cleavage sites
360
394
ARVLAEAMSQVTNTATIMMQRGNFRNQRKMVKCFN
Gag p42
(390)
(380)
Royer et. al.
(373)
Gag p41.5
(363)
Gag p41
+
Gheyson et. al.
Cys – His motif

create the second fragment from linearized template DNA derived from the plasmid pAc CL29.1 HIV$^{gag\ p46}$, using the following conditions:

Annealing temperature	-	45°C 1 min
Primer extension temperature	-	70°C 3 min
Dissociation temperature	-	95°C 1 min
Number of Cycles	-	30

The fragments were cloned into the expression vector pAc CL29.1 as previously described. According to the convention used earlier, the constructs were named based on the expected size of the recombinant protein in kDa calculated from the primary amino acid sequence, Gag p41 and Gag p41.5. Once the cloning step was complete, recombinant baculoviruses were prepared and purified as for the other truncation mutants.

To compare the molecular weights of the recombinant proteins produced by Gag p41 and Gag p41.5, cell extracts from infected cultures were harvested, prepared as before, and separated by SDS/PAGE. The experiment that demonstrated the expression of the recombinant proteins, was duplicated, as before, with one gel stained for total protein with coomassie blue, while the other was western blotted and probed with the 2 stage antibody protocol specific for the Gag protein as described above. The results shown alongside cellular extracts from the other truncation mutants (figure 23), indicate the smaller sizes of these proteins corresponding to their predicted molecular weights.

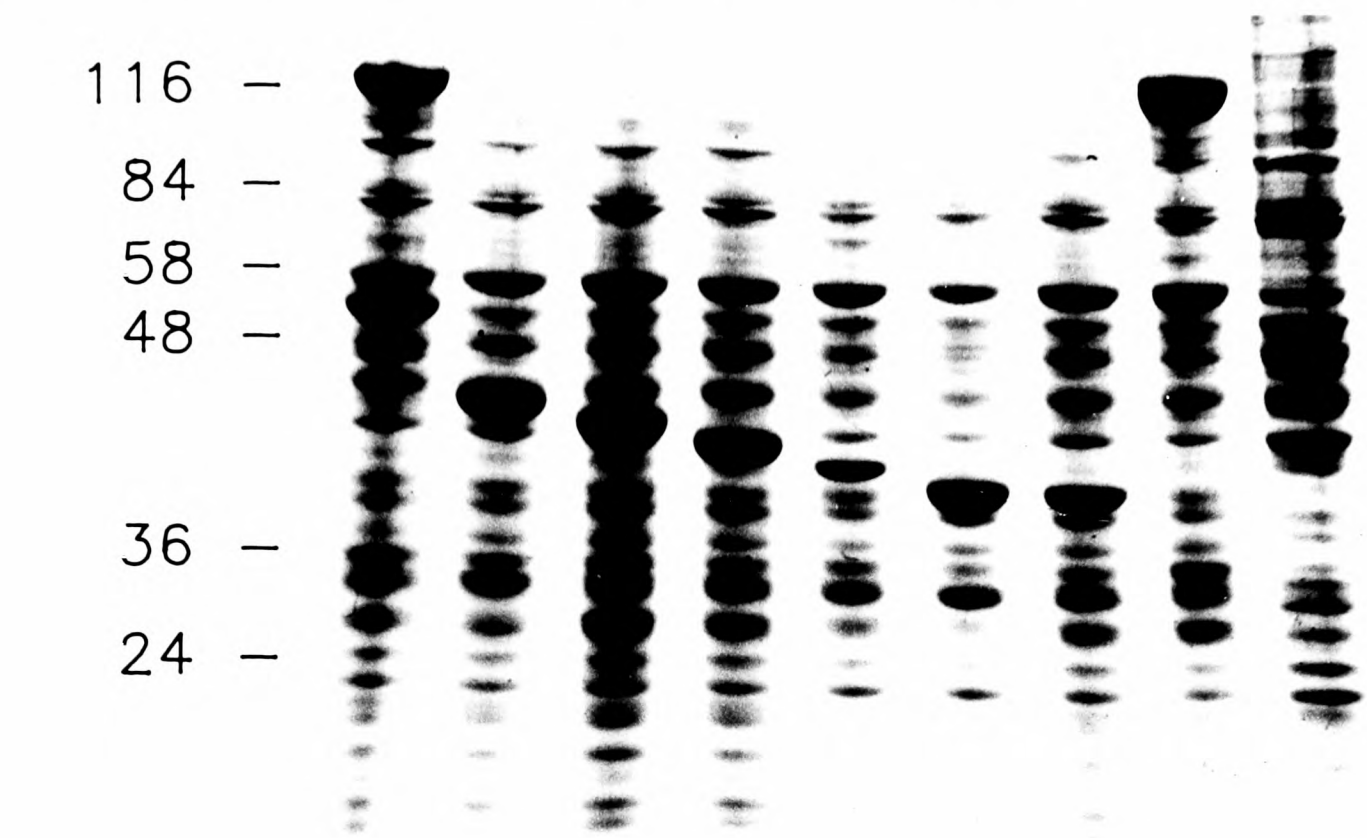
The particle forming ability of Gag p41 and Gag p41.5 were assessed in the same way as for the other truncation mutants analyzed. Figure 24 shows the sucrose

Figure 23.

Expression of the recombinant truncated HIV Gag mutants

Coomassie blue stained SDS/PAGE gel (panel A), and western blot probed with monoclonal antibodies to HIV Gag protein (panel B), of cell extracts of cultures infected with Gag p55 (lane 1), Gag p46 (lane 2), Gag p45 (lane 3), Gag p44 (lane 4), Gag p42 (lane 5), Gag p41.5 (lane 6), and Gag p41 (lane 7). Negative controls included were AcRP6 infected (lane 8), and mock infected (lane 9), Sf culture extracts. Molecular weight markers are indicated on the left side of the panel in kDa.

A



B

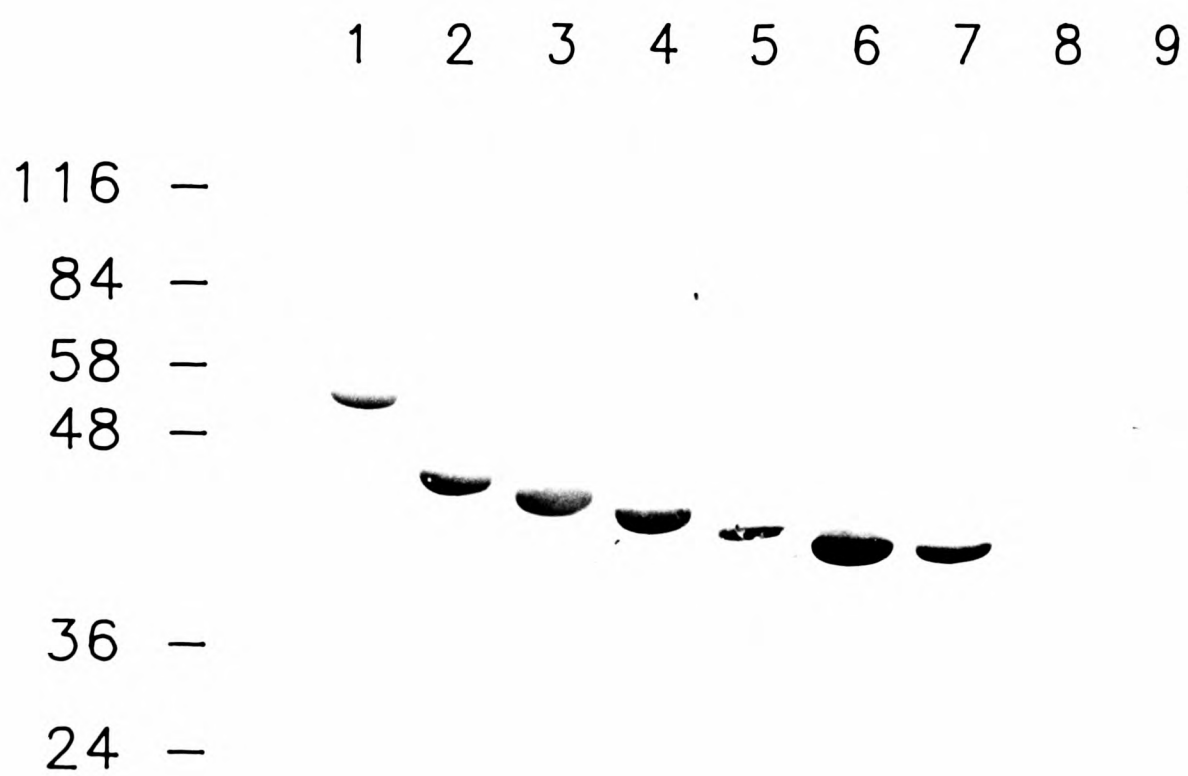


Figure 24.

Sucrose density gradient analysis of particle production of the Gag truncation mutants, Gag p41.5 and Gag p41

Western blots of fractions taken from a continuous sucrose density gradient loaded with culture supernatants from cells infected with Gag p55 for reference (top panel), with Gag p41.5 and Gag p41 in the 2 lower panels. The fractions were loaded onto the SDS/PAGE gel, from the top of the gradient (left side; 20% sucrose), to the bottom of the gradient (right side; 60% sucrose), and probed with monoclonal antibodies to the HIV Gag protein.

Fraction number

(20%) 1 2 3 4 5 6 7 8 9 (60%)

Gag p55

Gag p41.5

Gag p41

density gradient profile, and it can be seen that Gag p41.5 infected cells produced extracellular antigen that sedimented to about 30% sucrose, with a spread over several fractions of the gradient. Additionally Gag p41 also produced extracellular antigen, however the spread through the gradient was more pronounced than that seen in Gag p41.5. Thin section electron microscopy of both Gag p41 and Gag p41.5 showed no evidence for particle formation (figure 25). Unlike Gag p42 and others, there was no budding seen from the plasma membrane or into intracellular vacuoles. Additionally the intracytoplasmic Gag rings were absent. However extensive vacuolization was observed together with an unusual dense layer of protein beneath the plasma membrane of the cell (figures 26 and 27), that was not present in mock infected cells. These effects were not unlike those observed by Gheysen and colleagues in cells expressing the vAcGag *Cfr* I construct (Gheysen *et al.*, 1989). It was inferred that the structures seen were due to Gag derived antigen accumulating underneath the surface of the cellular plasma membrane, but being unable to self assemble into particles. The diffuse spread of antigen seen within the sucrose density gradient was therefore most likely to be due to fragments of Gag coated membrane derived from the surface of the infected cell.

A model for particle assembly

These results taken together with those of Royer and colleagues (1991), suggested that the addition of 7 more amino acids (ATIMMQR) to the carboxy terminus of the Gag p41.5 truncation mutant would restore particle self assembly proficiency. If this is the case, then the delineation of the carboxy terminal end of this putative Gag assembly domain is remarkable in that it is in juxtaposition

Figure 25.

Electron micrograph - Gag p41

Thin section electron microscopy of an Sf cell infected with the Gag p41 recombinant virus. Shows whole Sf cell with surface vacuolization visible. Similar vacuolization was seen in cells infected with the Gag p41.5 recombinant. Bar = 1 μm.

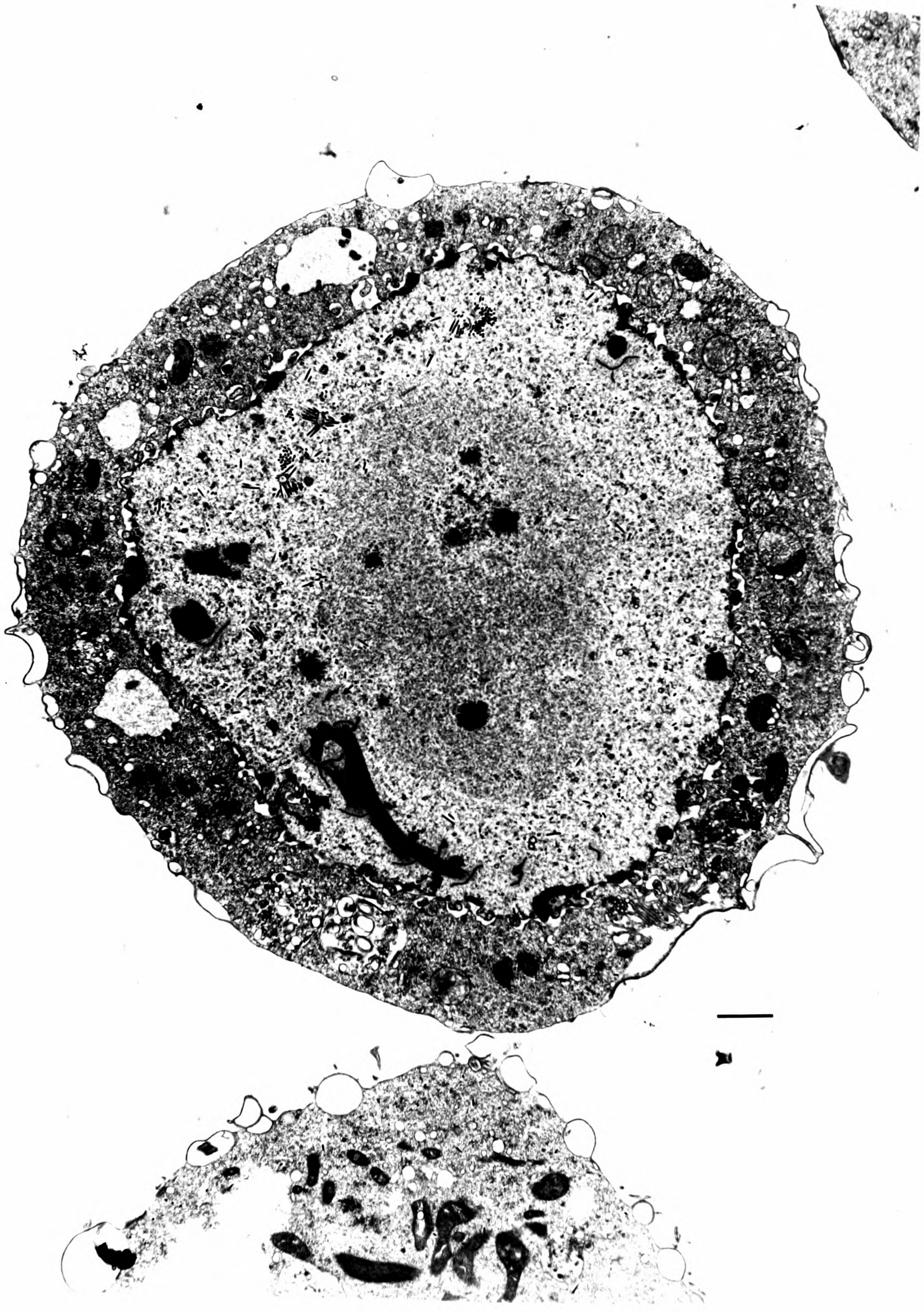


Figure 26.

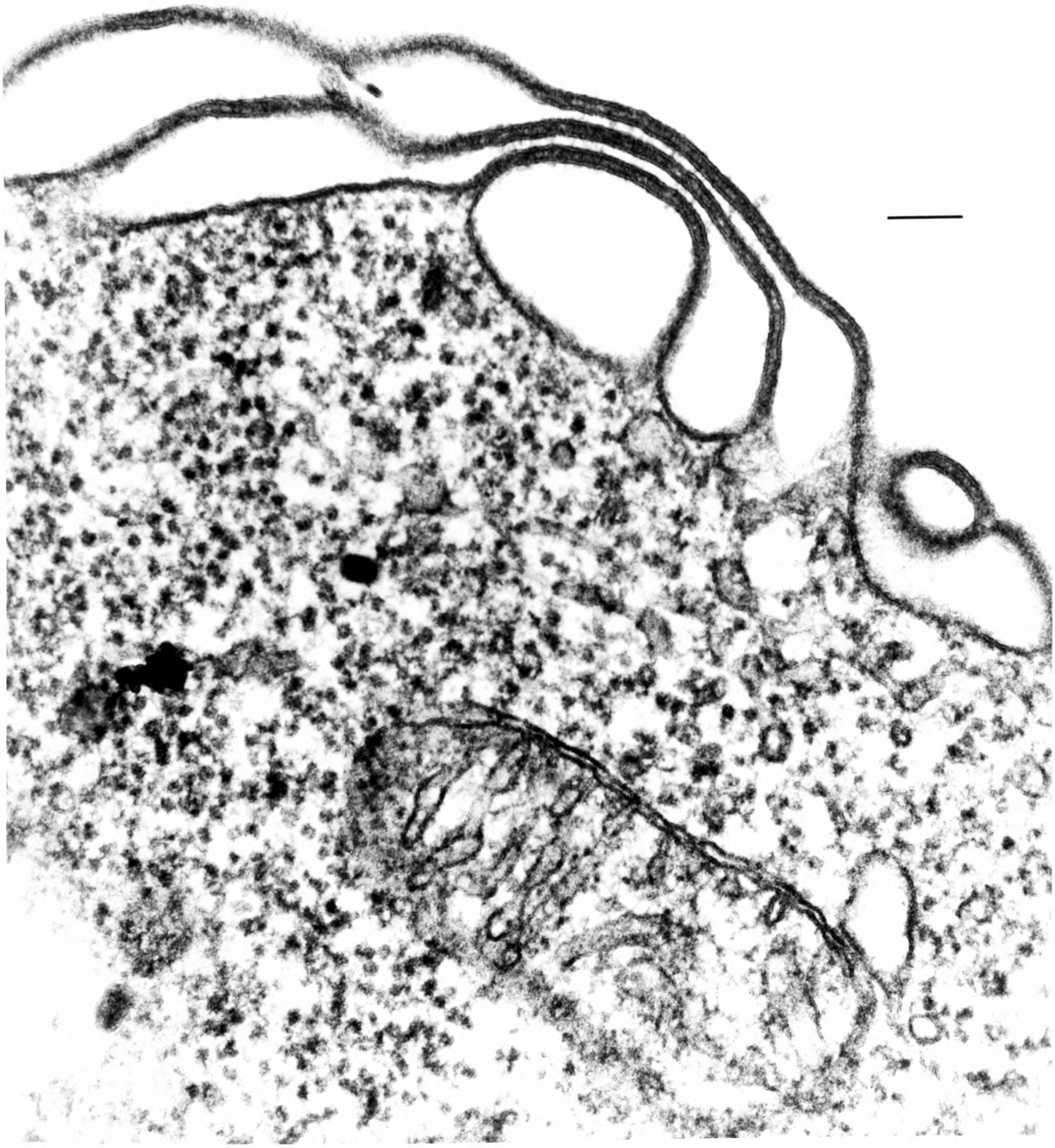
Electron micrograph - Gag p41.5

Thin section electron microscopy of an Sf cell infected with the Gag p41.5 recombinant virus. Detail of cell surface vacuolization. Bar = 100 nm.

Figure 27.

Electron micrograph - Gag p41

Thin section electron microscopy of an Sf cell infected with the Gag p41 recombinant virus. Detail of cell surface showing vacuolization. Bar = 100 nm.



with one of the known protease cleavage sites at the carboxy terminus of p24 (figure 22). This observation led to the proposal of a particle assembly mechanism that would ensure that prematurely cleaved Gag precursors, cut at any of the three sites at the p24 / p7 junction, would be excluded from the forming virion as they would lack the sequences necessary for particle self assembly. This model, depicted in figure 28, shows that, in the case of premature activation of the HIV protease, the cleaved precursors would be unavailable for incorporation into a forming particle and assembly would be aborted. The model would help to explain the observations, made by electron microscopy, that newly budded virus particles contain a doughnut shaped core (Gelderblom *et al.*, 1988; Gelderblom, 1991), which is indistinguishable from the cores of particles seen when the Gag precursor (p55) is expressed in the absence of all other virally encoded proteins (Gheysen *et al.*, 1989; Hu *et al.*, 1990), inferring that newly budded virions are made up predominantly of full length Gag precursor.

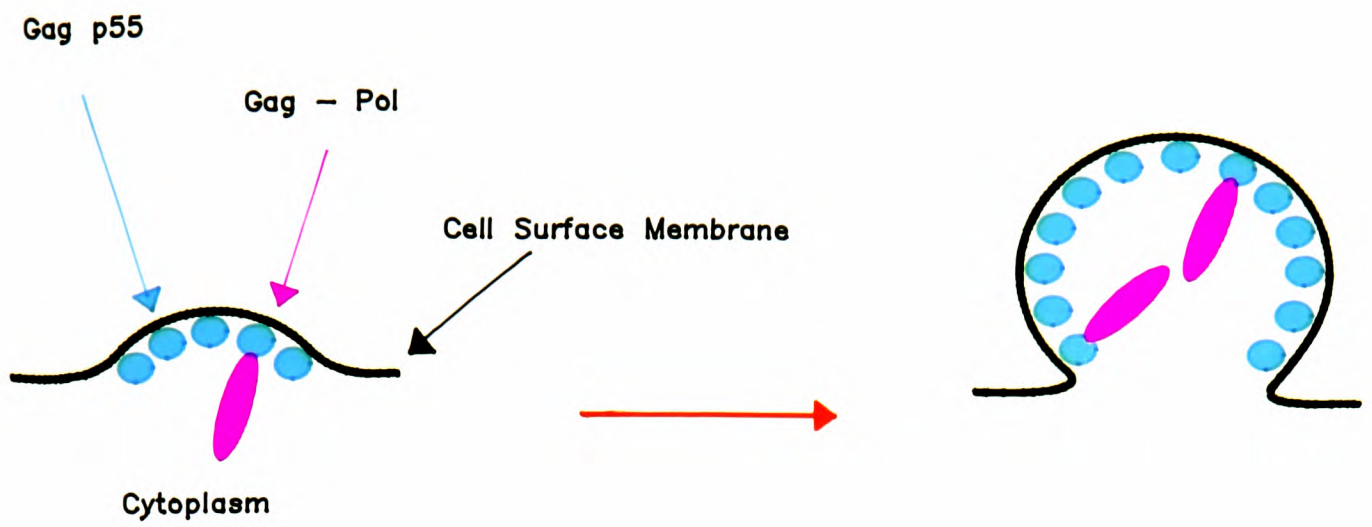
If this model were correct, truncated Gag antigens such as Gag p41.5 and Gag p41 should be incapable of incorporation into particles even if present with fully functional Gag antigen. Therefore, in order to test this, each of the Gag p41 and Gag p41.5 recombinants were co - expressed with the assembly proficient Gag p55 construct and the resulting particles assessed for the co - incorporation of truncated and full length Gag antigen.

Separate cultures of Sf cells (15 x 10^6 cells / 80 cm^2 flask), were co - infected with either Gag p41.5 and Gag p55 or Gag p41 and Gag p55 recombinant viruses (each at an moi = 10). Following 2 days incubation (28°C), the cultures were harvested and the supernatant applied to a sucrose density gradient as described above. Fractions from the gradient were separated by SDS/PAGE, western

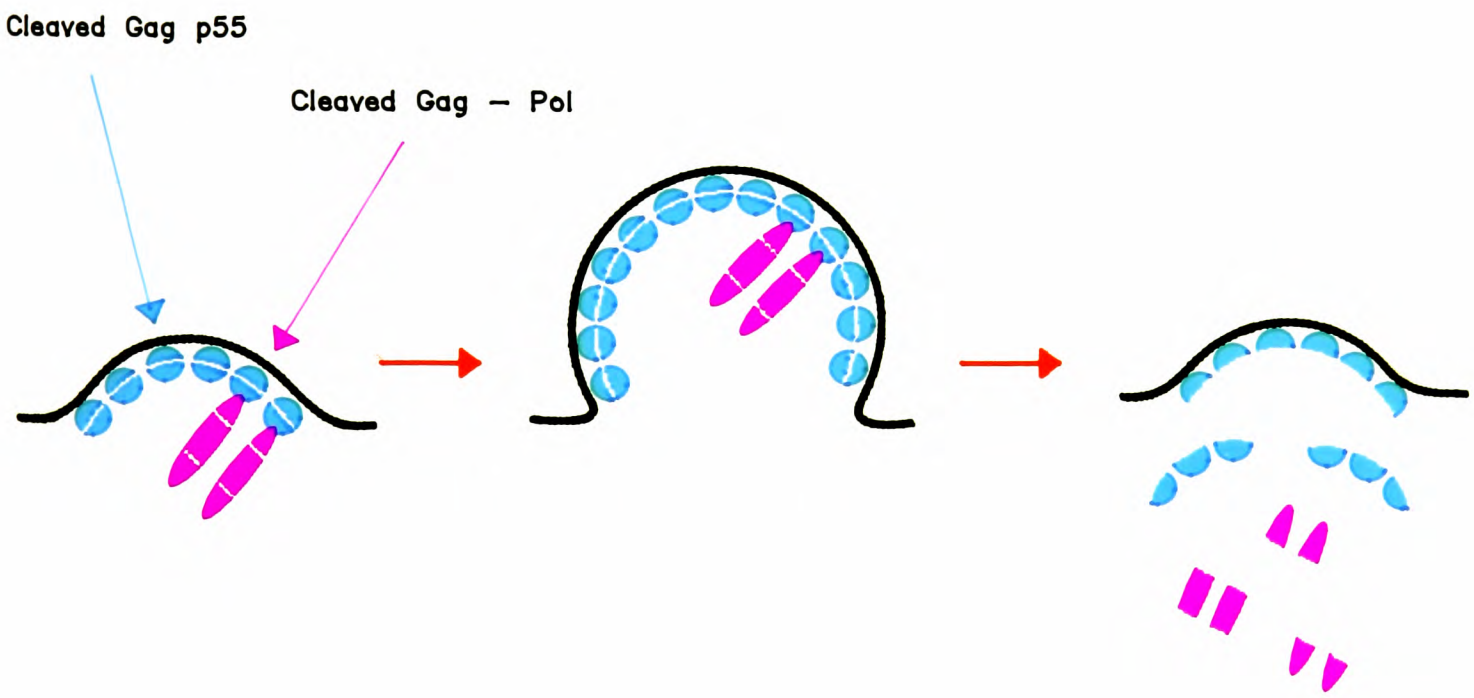
Figure 28.

Proposed model of Gag particle assembly

In the upper half of the diagram, the proposed model for normal Gag particle assembly is depicted. The Gag precursors (blue) associate to form a bud at the membrane of the cell, with the inclusion of the occasional Gag - Pol precursor (blue and magenta). The protease becomes active late in particle development, triggered by the coming together of 2 copies of the Pol domain (magenta). However, if the protease is activated prematurely by the juxtaposition of 2 Pol precursors early in particle development (lower half of diagram), the Gag and Pol precursors are cleaved and particle assembly is aborted.



Protease activates late – normal particle development



Protease activates early – cleaved Gag antigens cannot assemble and particle development is aborted

blotted and probed with monoclonal antibodies to Gag (2 stage protocol as described above). The results shown in figure 29, indicate that in both cases, the Gag p55 antigen migrated within the gradient to fractions 6 and 7, while the truncated antigen was spread throughout the gradient in a similar way to that observed when each truncation mutant was expressed singly (figure 24).

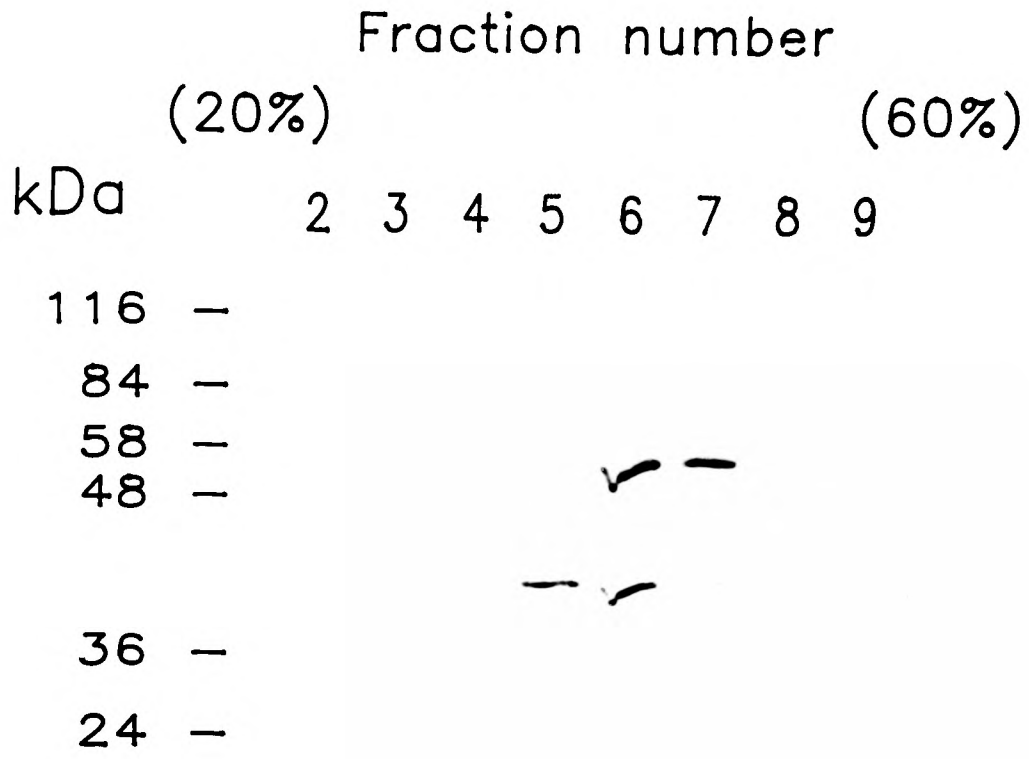
The relative position of the truncated Gag antigen within the gradient, being similar whether expressed with or without the Gag p55 precursor, suggested that there was no co - incorporation of the truncated and full length Gag molecules. In summary, these results are in support of the proposed model of Gag particle assembly, where prematurely cleaved precursors are excluded from the forming virion.

Figure 29.

Sucrose density gradient analysis of particle production by co - expression of the Gag p41.5 or Gag p41 truncation mutants with the Gag p55 recombinant virus

Western blots of fractions taken from a continuous sucrose density gradient loaded with culture supernatants from cells co - infected with either Gag p41.5 and Gag p55 (panel A), or with Gag p41 and Gag p55 (panel B). The fractions were loaded onto the SDS/PAGE gel, from the top of the gradient (left side; 20% sucrose), to the bottom of the gradient (right side; 60% sucrose), and probed with monoclonal antibodies to the HIV Gag protein. Molecular weight markers have been included on the left side of the diagram (kDa).

A


Fraction number
(20%)
(60%)
kDa
2 3 4 5 6 7 8 9
116
84
58
48
36
24

B


kDa
116
84
58
48
36
24

Discussion

The observation that the Gag p46 truncation mutant can still self assemble into particles, suggests that the p6 domain is not required for particle formation. Thus it may be inferred that the abolition of particle assembly seen in the Gag - Pol frameshift mutant constructed by Shioda and colleagues (1990), is likely to be due to steric hindrance of alignment of sequences in the Gag precursor that would otherwise promote oligomerization and self assembly. This finding is consistent with similar Gag - Pol fusion proteins studied in the retrovirus MoMuLV (Felsenstein and Goff, 1988).

Furthermore, it can be concluded that neither Cys - His motif is required for particle formation *per se* by virtue of the Gag p42 truncation mutant that has both of these regions deleted yet retained its particle forming ability. However it was noted that the nature and localization of particle assembly of the mutants that were assembly proficient, varied with each of the truncation mutants studied. This is perhaps not surprising as removal of parts of the carboxy terminal of this precursor may result in the exposure of other domains and signals that alter the location and assembly characteristics of the particle. This was particularly apparent in the Gag p45 and Gag p44 truncation mutants where some preformed intracytoplasmic Gag rings were observed that budded either into intracellular vacuoles or from the plasma membrane. Furthermore some variation was seen within one mutant itself (Gag p42), where some cells exhibited particles budding from the plasma membrane and others did not. As the preparation of the infectious recombinant virus was known to be homogeneous, this result suggested that some intercellular variation must occur.

This could be accounted for simply by levels of expression of the recombinant protein between different cells, otherwise there could be more complex differences between the cells such as influences from cellular factors that rise and fall in concentration as a result of the age, stress or phase of the cells growth cycle. Additionally the possibility of baculovirus derived proteins exerting some influence over these events cannot be ruled out.

Analysis of the two additional truncation mutants Gag p41 and Gag p41.5, revealed that they were both deficient in self assembly of particles. In the case of Gag p41, the result was in agreement with findings published by Gheysen and colleagues (1989). Additionally, structures appearing at and beneath the membrane of infected cells, together with extensive vacuolization, were noted in Gag p41, that were similar to those observed by Gheysen and colleagues. The finding that the Gag p41.5 truncation mutant was also assembly deficient (deleted from amino acid 373), implied that by the addition of the amino acid sequence ATIMMQRGNFRNQRKMV in single letter code, constituting the difference between Gag p41.5 (no particle assembly) and Gag p42 (particles formed), would be sufficient to restore precursor self assembly competence. It may be concluded then that within this sequence lie some essential signals for particle formation. The results presented by Royer and colleagues, of their assembly proficient construct AcNPVgag14 (Royer *et al.*, 1991), suggest that within the above sequence only the first 7 amino acids (ATIMMQR) need be present at the carboxy terminus of the Gag p41.5 truncation mutant to restore its self assembly proficiency. Consequently, the carboxy terminal boundary of this Gag assembly domain must lie within this sequence. It was found that this particular region was only moderately conserved across the isolates of HIV - 1

sequenced to date. However the amino acid substitutions seen were usually conservative, suggestive of a functional role for the region (Myers *et al.*, 1991).

On the basis of the observations made in delineation of the carboxy terminal boundary of a Gag assembly domain above, it is unlikely that a critically defined end point would be found, but rather a gradual destabilization of the required interactions between the appropriate assembly domains as they are progressively deleted. This would have the result of making particle formation increasingly unlikely within the cell, and could account for the rare occurrence of particles shown in the electron micrographs of the AcNPVgag14myr construct published by Royer and colleagues (1991). Additionally this might also account for the oligomerization of the Gag antigen in Gag p41.5 and Gag p41, that aggregate but do not form particles as such.

It may be postulated that self assembly of the Gag particle is a concentration dependant event. Once a critical level of precursor, containing the required assembly domains, has accumulated at a given point, primarily at the plasma membrane, the subunits oligomerize and the ring structure begins to form, dragging the plasma membrane around itself to form the bud. In the case of the observed intracytoplasmic Gag rings, it may be that these were formed when there was a sufficiently high concentration of Gag precursor that had become detached from the lipid membrane with which it would normally be found associated *via* an interaction with the amino terminal myristoylated glycine residue. Such intracytoplasmic preformed core structures that bud from the plasma membrane are typical of type B and D oncovirinae. Interestingly a report has recently been published where a single amino acid substitution near the amino terminus of the Gag protein of a type D oncovirus changed its

assembly pattern to that of a type C oncovirus (Rhee and Hunter, 1990). This suggested that the mechanisms involved in driving these different types of assembly protocols are not widely divergent from one another.

As the Gag precursor was progressively deleted, there was an increasing tendency for it to oligomerize into filaments beneath the surface of the plasma membrane. It appeared that signals were removed from the carboxy terminus that drove circularization of the subunit assembly process. Where particles were seen with an internal as well as an external lipid membrane, it was possible that this arose as a small vacuole budded from the surface of the cell that had a strip of Gag antigen beneath its surface, that circularized to form the particle. Seen in cells infected with Gag p42 expressing recombinant, these aberrant particles appeared both larger and less dense than full length Gag derived particles, which may account for their increased spread seen in the sucrose density gradient. This spread, also seen in the sucrose gradient of Gag p41.5 infected culture supernatant, may have been due to different sized sections of membrane coated Gag oligomers becoming detached from the cell surface. Extending this hypothesis further, oligomerization would be even more disrupted in the Gag p41 mutant, creating a more diverse range of these fragments of Gag coated membrane that could account for the even wider spread of antigen seen in its sucrose density gradient. In summary, a narrow band of concentrated antigen in the sucrose gradient would imply particles of uniform density and structure, however a diffuse spread of antigen through the gradient would appear to suggest that assembly is essentially disordered, with a range of densities of Gag coated membrane fragments in the gradient. Accordingly, it is important that assessment of particle formation by sucrose density gradient sedimentation be supported by electron microscopy.

It was noted that the carboxy terminal boundary of the putative Gag assembly domain was in juxtaposition to the viral protease cleavage sites at the junction of the p24 and p7 domains. This observation led to the proposal of a model whereby prematurely cleaved Gag precursors would be excluded from the forming virion as they would have had the required sequences for particle assembly removed. The proposed mechanism would predict abortion of particle assembly if the virally encoded protease became active too early in the Gag precursor assembly process. The model also suggested that even if prematurely cleaved Gag precursors were present with full length assembly competent Gag precursors, that they should be incapable of being incorporated. This prediction was tested by co - expression of the truncated Gag precursors (Gag p41 and Gag p41.5), with the full length Gag p55 recombinant, and the particles analyzed for evidence of co - incorporation. The results revealed that no co - incorporation had occurred thus supporting the proposed model. This mechanism would provide an explanation for the observation that newly budded immature HIV virions are morphologically indistinguishable from particles produced by the expression of the *gag* gene alone, suggesting that they are made up of predominantly of full length Gag precursors (Luckow and Summers, 1988b; Gheysen *et al.*, 1989; Hu *et al.*, 1990; Gelderblom, 1991). Such a scenario may go some way toward elucidating the mechanism by which the virus modulates premature processing of the Gag precursor by the protease so as to allow particle morphogenesis.

In this study the carboxy terminal region of an assembly domain of the HIV Gag precursor has been delineated to a small stretch of amino acids. How far this domain extends in the amino terminal direction is not known. Further internal

deletion mapping of the Gag precursor would yield more data to address that question. In the process of delineating the assembly domains end points the existence of other assembly domains within the Gag precursor, that interact either with copies of themselves on neighbouring precursors (*trans* - acting), or with domains within the same precursor (*cis* - acting), to promote assembly, may become apparent. In support of the proposition that multiple domains exist within the Gag precursor was the finding that particle assembly was abolished in the Gag p41 truncation mutant while it still retained the ability to oligomerize. This might be explained by a model that proposes separable domains for oligomerization and circularization of the Gag precursor. The notion of multiple domains existing within the Gag precursor may also accommodate separable regions that have functions distinct from assembly. Once the assembly of the particle is complete with the encapsidation of RNA, the protease cleaves the precursor into its subunits. This event may result in the exposure of domains that were previously hidden by the conformation of the full length precursor. The functions of these domains may be in stabilization of the genomic RNA within the viral particle, when the precursor collapses to form the central conical core. Following infection the core is delivered into the cytoplasm of the target cell, where these now exposed domains may also play a role in promotion of reverse transcription and targeting of the product to the nucleus for integration into the host cell genome.

Some interesting results have been found in studies of Gag precursor assembly of related retroviruses. In MoMuLV, a type C oncovirus, the capsid domain was particularly sensitive to mutation where even small deletions were sufficient to abolish particle formation (Schwartzberg *et al.*, 1984). In contrast to this, reports of deletion mutagenesis of the capsid domain of RSV of the same sub - family,

showed the capsid region to be remarkably tolerant to deletion mutagenesis. This was illustrated by a mutant where the deletion of a 171 amino acid section, that included half of the capsid domain and almost all of the matrix domain of the precursor protein, still allowed self assembly to occur (Wills and Craven, 1991). So clearly the position and extent of assembly domains within retroviral Gag precursors varies widely and is a virus specific entity. Studies to determine the composition of the cytoplasmic pre - integration complex of MoMuLV established the presence of the integrase enzyme together with the capsid antigen, supporting the hypothesis that the retroviral capsid may play a role in post infection events (Bowerman *et al.*, 1989). Studies to examine the same in HIV - 1 found that the presence of the integrase alone was sufficient for *in vitro* integration of the viral DNA into a target strand. While *in vivo* no evidence was found for involvement of the capsid antigen, however, the authors could not rule out the possibility that it was present but below the level of detection of their assay (Farnet and Haseltine, 1991b).

Another point for discussion has arisen out of the demonstration within this study that the carboxy terminal p6 domain of the HIV Gag precursor is not essential for particle assembly and release. This is in apparent contradiction to a recent report that demonstrates a key role for p6 in efficient particle release (Gottlinger *et al.*, 1991). The report is based on observations made of a truncated *gag* gene expressed as provirus in Cos - 7 cells, where deletion of the p6 domain completely restricted release of particles from the cell surface. The apparent discrepancy with the results presented here, where no such inhibition was observed, may be explained by the differences in the expression systems used to study the mutant precursors. Gottlinger and colleagues used provirus (all HIV genes present), expression in Cos - 7 cells (a monkey cell line transformed

by SV40 virus), while the experiments presented here are based on expression of a single gene *via* baculovirus promoters in insect cells. The mechanism of particle retention that requires p6 for alleviation in mammalian cells may simply be absent in insect cells or swamped by the amount of protein produced which is significantly higher than that from provirus expression in mammalian cells. This dichotomy perhaps highlights the need for careful judgement in interpretation of functions of proteins that are expressed both in isolation of other virally encoded proteins and in foreign cells.

Chapter Two

RNA Binding Analysis of the Gag Nucleocapsid Domain

Introduction

Every sequenced member of the retrovirus family has been found to contain one or two copies of the highly conserved Cys - His motif, as described in chapter one. Similar motifs have been identified in eukaryotic proteins that have a known ability to bind nucleic acid as has been described in the general introduction. The eukaryotic motifs are known as "zinc fingers" due the finger - like structures that result from the chelation of zinc ions, an essential part of the "finger" being able to bind nucleic acid. The HIV - 1 nucleocapsid protein (p7), which contains two Cys - His motifs (figure 7), has been directly isolated from infectious virus particles and was shown to bind two zinc ions both tightly and stoichiometrically (South *et al.*, 1990; Bess *et al.*, 1992). The parallel between the HIV - 1 nucleocapsid Cys - His motif and the eukaryotic zinc finger, as a nucleic acid binding domain, was further supported by a study where point mutations were introduced such that the two amino terminal conserved cysteine residues of the HIV - 1 Cys - His motif were changed to serine. The study presented data from three separate mutants where the alterations were present in either the proximal only, the distal only or both the proximal and distal motifs together. Progeny virions of all these three mutants showed a significant

reduction in their genomic RNA content, between 2% and 6% of wild type virions, confirming the motif's role in the encapsidation of RNA. Furthermore the infectivity of these mutants was found to be reduced by more than 5 orders of magnitude when compared to wild type HIV virions (Gorelick *et al.*, 1990).

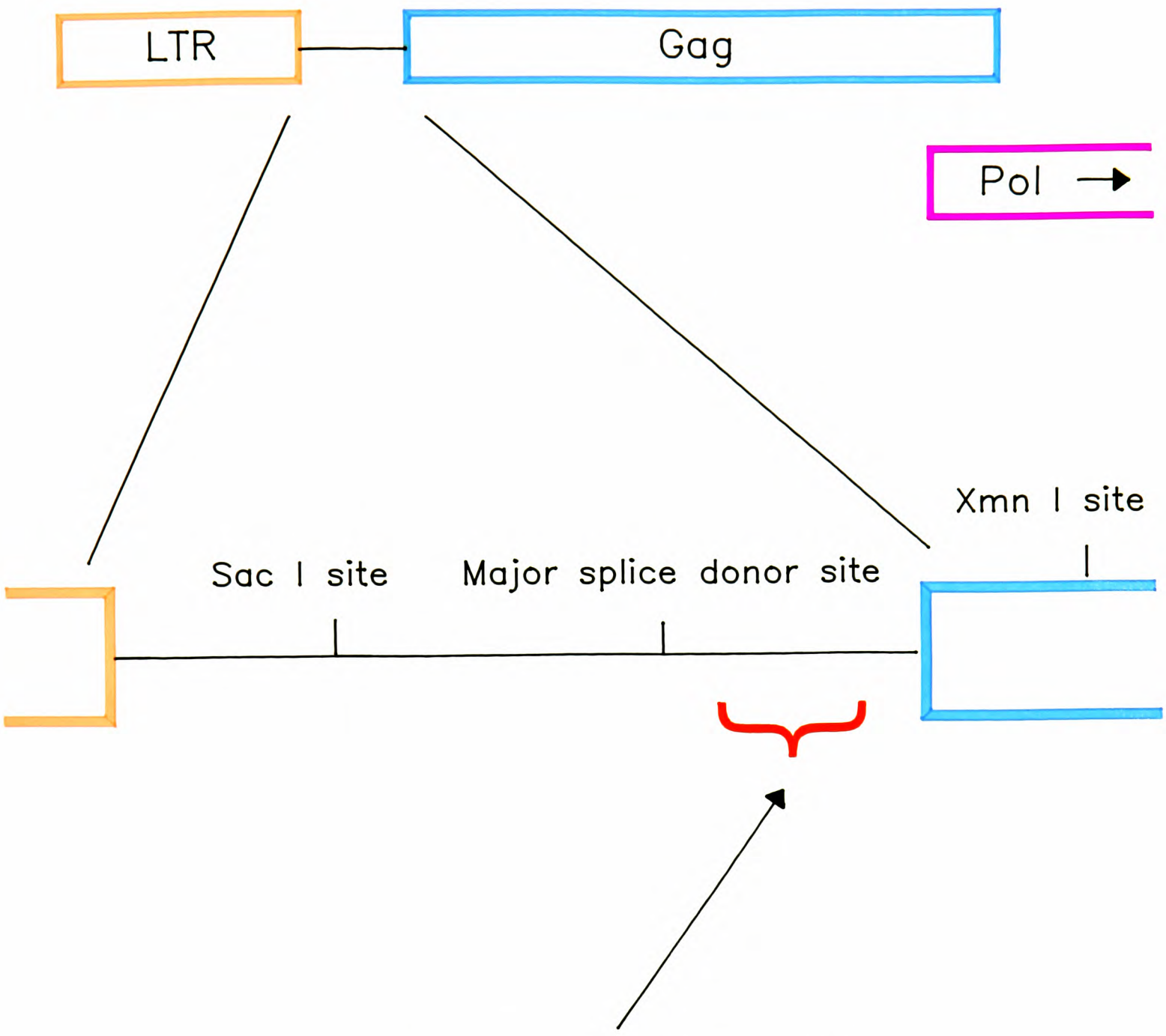
While these mutations significantly attenuated the virions infectivity, there was still evidence of RNA binding ability, albeit much reduced, suggesting that a residual amount of RNA binding could be mediated by non Cys - His Gag sequences. To address this possibility, that the above point mutations did not entirely abrogate the RNA binding ability of the Cys - His motif, the deletion mutants Gag p46, Gag p45 (both Cys - His motifs present), Gag p44 (distal motif deleted), and Gag p42 (both motifs deleted), were assessed for their ability to bind RNA.

In this chapter experiments were aimed at investigation of whether the deletion mutants could bind RNA *in vitro*. This was done by north western blot, where the recombinant Gag proteins were separated by SDS/PAGE, blotted onto a membrane filter, renatured and probed with a radiolabelled RNA probe that included the HIV - 1 *cis* - acting genomic RNA encapsidation signal (figure 30), known to be specific for the binding of RNA to the nucleocapsid protein (Lever *et al.*, 1989; Luban and Goff, 1991).

Figure 30.

Location of HIV - 1 genomic RNA encapsidation signal

The upper section of the drawing shows the 5' LTR (peach), the *gag* open reading frame (blue), of HIV - 1 and the 5' end of the *pol* open reading frame (magenta). The expanded section below shows detail of the intervening sequences, where the *Sac* I and *Xmn* I cloning sites together with the major splice donor site have been marked. The large red angular bracket delineates the region where the *cis* - acting genomic encapsidation signal lies.



Region containing *cis* – acting sequences that promote encapsidation of RNA

Results

Analysis of HIV Gag precursor RNA binding capability *in vitro*

Cultures of Sf cells (1.6 x 10^6 cells, 35 mm well), were infected with the recombinant viruses Gag p55, Gag p46, Gag p45, Gag p44 and Gag p42 at an moi of 10. At 2 days post infection the cultures were harvested, the cells pelleted (185*xg*, 10 min), washed in PBS (1.5 ml), and resuspended in 50 μl SDS gel loading buffer. Three identical gels were prepared and loaded with cell extracts from each of the cultures in adjacent lanes (approximately 10^5 cells / lane), one gel was stained for total protein content with coomassie blue, while another was western blotted and probed with antibodies specific to HIV - 1 Gag antigen (2 stage protocol detailed in chapter one). The third was electroblotted onto a 0.45 μm nitrocellulose membrane filter as for the western blot, except that after disassembly of the western blotting apparatus, the membrane was rinsed in PBS and incubated in north western buffer (4 °C, 12 hr) to allow renaturation of the proteins. A plasmid containing the *Sac* I - *Xmn* I fragment of the HIV - 1 genome (figure 30), downstream of the T7 RNA polymerase promoter sequence, was used as template for preparing an [α - ^{32}P] CTP (Amersham Cat. No. PB 20382), labelled RNA probe by run - off transcription (Titus, 1991). The RNA probe was precipitated with ethanol, washed once in cold 70% ethanol to remove unincorporated radio - nucleotides, added to 5 ml of north western buffer in a heat sealable plastic envelope containing the membrane and incubated (22 °C, 1 hour). The filter was washed twice in 100 ml north western buffer (22 °C, 10 min, shaking) and exposed to X - ray film (Kodak X - Omat S Film 100 Cat. No. 501 0145). In figure 10 can be seen the results of the coomassie blue stained gel and the western blotted filter in panels A and B

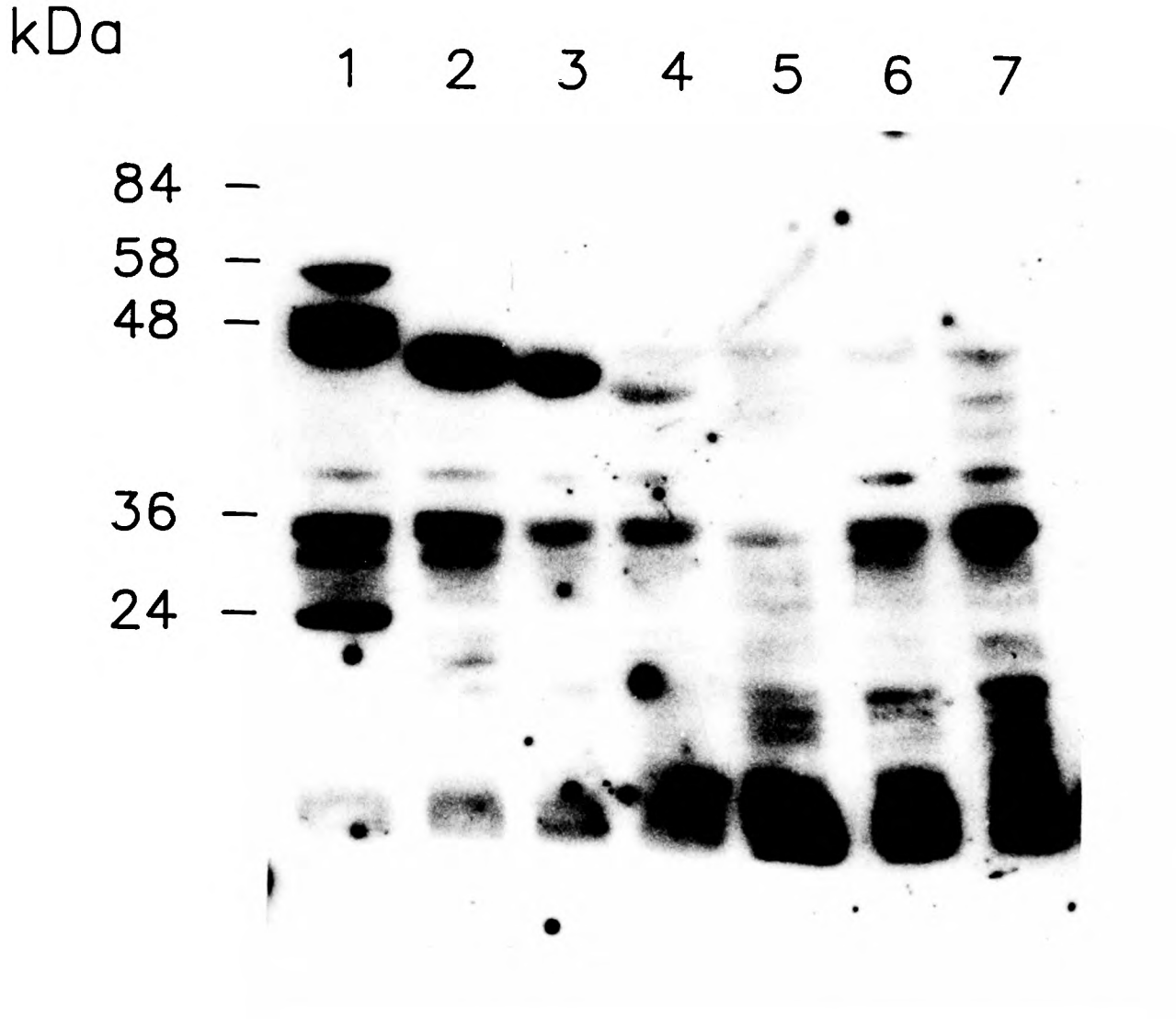
respectively, while the autoradiograph is shown in figure 31. In lane 1 (figure 31), strong binding is seen by the probe to the Gag p55 recombinant protein as well as its breakdown products at about 47 and 48 kDa molecular weight. Gag p46 and Gag p45 (figure 31; lanes 2 and 3 respectively), also show efficient binding by the probe while Gag p44 (figure 31; lane 4), with the distal Cys - His motif deleted shows significantly less RNA probe bound. The Gag p42 truncation mutant (figure 16; lane 5), with both proximal and distal motifs deleted showed little or no binding by the probe. Negative controls were included in lanes 6 and 7 (figure 31), these were wild type AcNPV infected and mock infected culture extracts respectively. Lower molecular weight bands were also visible in all lanes including both the negative controls suggesting the presence of nucleic acid binding proteins derived from the host cell. To verify that the binding was sequence specific, the experiment was repeated using a radioactively labelled probe, that was complementary to the RNA encapsidation signal. Autoradiography revealed only background binding of the probe, and not to any of the HIV Gag proteins (data not shown).

This result clearly demonstrated the ability of the renatured full length Gag precursor to specifically bind the *cis* - acting RNA encapsidation signal *in vitro*. Having established RNA binding *in vitro* for those truncation mutants that contain at least one Cys - His motif, the question, of whether particles produced from the expression of these mutants contain RNA specifically acquired during packaging, was addressed.

Figure 31.

North western blot

North western blot of cellular extracts from cultures of Sf cells infected with the full length Gag p55 recombinant, (lane 1), and the truncation mutants Gag p46 (lane 2), Gag p45 (lane 3), Gag p44 (lane 4), and Gag p42 (lane 5). Negative controls included were AcRP6 infected and mock infected Sf cell extracts (lanes 6 and 7 respectively). The blot was probed with a radiolabelled RNA sequence corresponding to the HIV - 1 genomic RNA encapsidation signal. Molecular weights are indicated in kDa on the left of the panel.


kDa
1
2
3
4
5
6
7
84
58
48
36
24

Discussion

The RNA binding capability of the HIV - 1 Gag nucleocapsid domain has been confirmed *in vitro* and was dependant on the presence of at least one Cys - His motif. Binding was observed to be significantly more efficient if both motifs were present and abrogated entirely when both motifs were deleted. These results are in agreement with published work showing that the disruption of these motifs by point mutagenesis lead to progeny virions with significantly reduced RNA content (Gorelick *et al.*, 1990). Furthermore, the observation that removal of one motif drastically reduces binding efficiency, more so than could be expected if each motif were to bind RNA equally, was supported, suggesting an interaction between the two motifs that increases the overall binding efficiency.

Experiments were done to identify RNA within the Gag particles that specifically included the HIV - 1 genomic RNA encapsidation signal. RNA extracted from these particles was tested for the presence of specific encapsidation signals by both PCR amplification and northern blot analysis, however, at the time of writing no clear evidence had been obtained to demonstrate the incorporation of RNA into the Gag particles. Thus the presence of specific RNA transcripts within the recombinant Gag produced particles that include the Cys - His motifs, remains suggested but not proven. Future experiments to determine this question may include the use of such methods as nuclease digestion protection and primer run off transcription analyses.

Chapter Two: *RNA binding analysis*

Considering the highly conserved nature of the Cys - His motif throughout the retrovirus family, a brief evaluation of published data relevant to RNA encapsidation in HIV - 1 is warranted. The best characterized members of the retrovirus family, in this respect, are the Rous sarcoma virus and the Moloney murine leukemia virus, both type C oncoviruses, which exhibit Gag particle assembly and budding concomitantly at the plasma membrane in a similar fashion to the lentivirinae. However, the type C oncovirinae differ in that the mature particles contain an isometric core, where the lentivirinae core is cone shaped (Gelderblom, 1991). The nucleocapsid domain of MoMuLV has been shown to have an affinity for zinc ions (Cornille *et al.*, 1990), as well as two additional functions in catalysis of dimerization of RNA with formation of the dimer linkage structure (DLS), and annealing of the transfer RNA (tRNA), primer onto the RNA genome (Prats *et al.*, 1990; Roy *et al.*, 1990). Data from studying mutations in the single Cys - His motif of the MoMuLV nucleocapsid domain, indicated the necessity of the motif for packaging viral RNA into the forming virion. Furthermore, evidence was found that suggested the amino acid residues between the conserved Cysteine and Histidine moieties of the Cys - His motif, were involved in determination of the specificity of the RNA encapsidation process (Gorelick *et al.*, 1988; Meric and Goff, 1989; Joshi *et al.*, 1990). The Rous sarcoma virus has two copies of the Cys - His motif within its nucleocapsid domain, and like MoMuLV has been attributed a role in encapsidation of genomic RNA during particle assembly (Meric and Spahr, 1986; Meric *et al.*, 1988). Similarly the domain was observed to catalyse dimerization of the viral genome and to promote the annealing of the primer tRNA onto the primer binding site (PBS), at the 5' end of the genome (Prats *et al.*, 1988; Bieth *et al.*, 1990; Oertle and Spahr, 1990; Aronoff and Linial, 1991).

Equivalent functions for the HIV nucleocapsid protein to those proposed for RSV and MoMuLV have also been demonstrated. Published work has verified that *in vitro* generated RNA molecules could form dimers spontaneously that contain a DLS similar to MoMuLV, a process that was accelerated by the presence of the nucleocapsid domain (Darlix *et al.*, 1990). Interestingly, the formation of the RNA heterodimers of HIV - 1 RNA with either MoMuLV RNA or RSV RNA have been reported, emphasizing the similarity of the RNA dimerization mechanism between these retroviruses (Marquet *et al.*, 1991). Additionally, the nucleocapsid domain was ascribed a role in the promotion of annealing the tRNA primer to the PBS of HIV - 1, a step that was found essential for initiation of reverse transcription that follows entry of the virus into the host cell (Barat *et al.*, 1989; Rhim *et al.*, 1991). In summary, the currently available data suggest that there are more characteristics of assembly in common between the type C oncovirinae and the lentivirinae, than there are differences. This was particularly apparent in a report where a single amino acid substitution near the amino terminus of the Gag precursor, altered the assembly characteristics from that of a type D to a type C oncovirus (Rhee and Hunter, 1990), emphasizing the similarities of the mechanisms of morphogenesis.

Further research in this area might address the question of whether dimerization of genomic RNA is a pre - requisite for binding and encapsidation by the nucleocapsid domain making its incorporation into the particle a structurally dependant interaction. Alternatively, the interaction may at first be a sequence specific phenomena for single stranded RNA which subsequently leads to dimerization and incorporation into the forming virion. Currently work is on going to create dimerization mutants of the HIV viral genome (MRC Aids Directed Program, Warwick, 1991), where the DLS, thought to exist as a purine

tetrad structure (Marquet *et al.*, 1991), is being modified so as to abolish its capacity to dimerize. In due course, the assessment of these mutants to bind to the nucleocapsid domain *in vitro*, and to be incorporated into particles *in vivo* would reveal more information on the mechanism of RNA encapsidation.

Chapter Three

Analysis of the transmembrane glycoprotein of SIV

Introduction

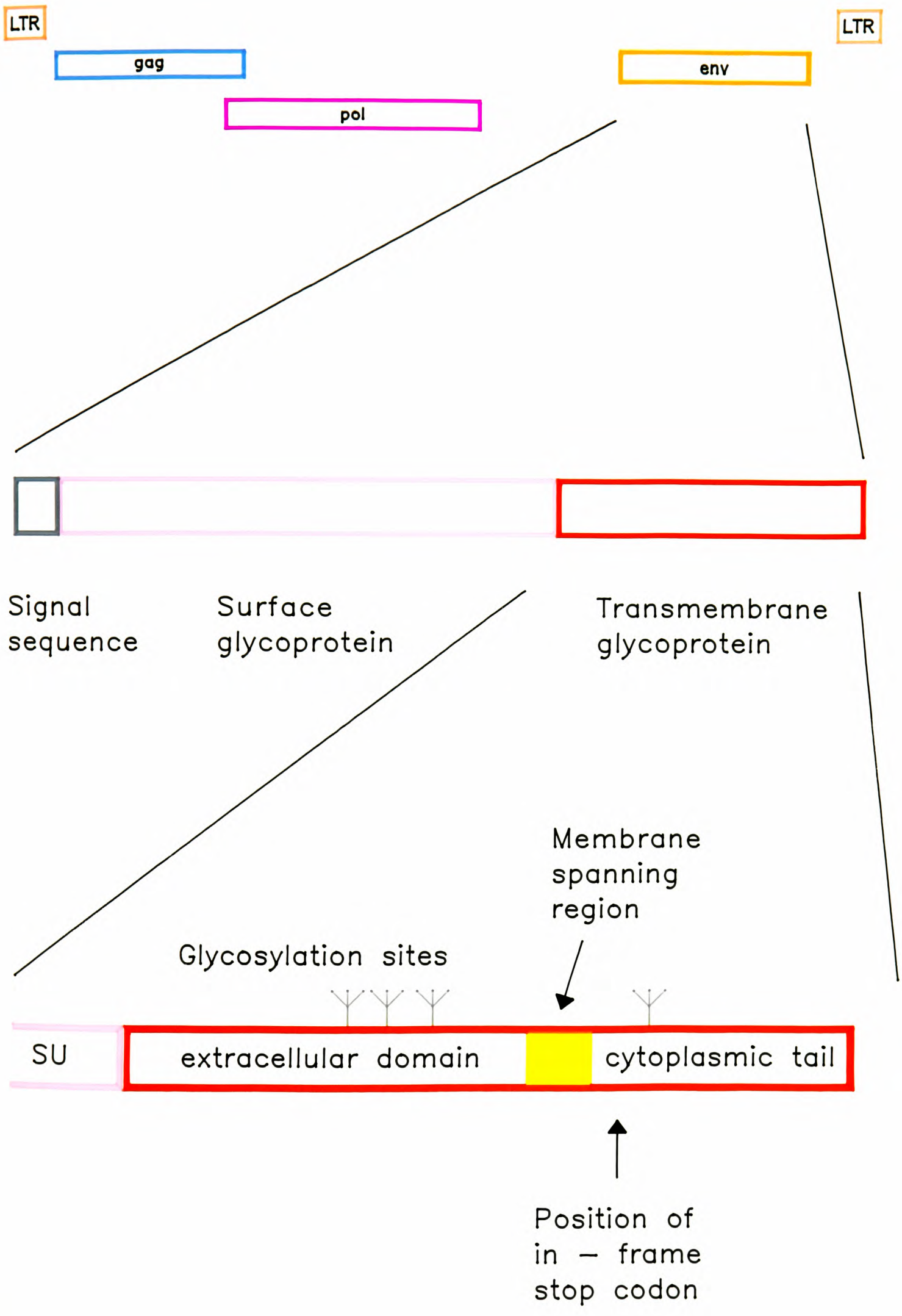
SIV was first isolated by co - cultivation of monkey peripheral blood lymphocytes (PBLs), splenic lymphocytes and cell free samples with a variety of human T - cell lines (Daniel *et al.*, 1985). After the virus was cloned, significant nucleic acid sequence homology to HIV was shown (Hirsch,V *et al.*, 1986; Kornfeld *et al.*, 1987), with subsequent sequencing experiments confirming the similarity in genomic organization of SIV to isolates of HIV (Chakrabarti *et al.*, 1987; Franchini *et al.*, 1987; Hirsch,V *et al.*, 1987). SIV was found to contain the *gag*, *pol* and *env* genes common to all retroviruses, together with five auxiliary genes *tat*, *rev*, *nef*, *vif* and *vpr* found in HIV - 1 and HIV - 2. However, SIV lacked an equivalent of the *vpu* gene found in HIV - 1, and encoded an additional gene, *vpx* that was similar to that found in the HIV - 2 genome, as described in the introduction.

The sequencing experiments revealed an in frame stop codon in the sequence encoding the transmembrane (TM), glycoprotein, located toward the 3' end of the *env* open reading frame in each of the isolates studied (figure 32). Without the stop codon, the TM glycoprotein would have a molecular weight of

Figure 32.

The SIV TM glycoprotein

In the top section, the SIV LTRs are shown (peach), flanking the *gag* gene (blue), *pol* gene (magenta) and the *env* gene (orange). The auxiliary genes have been omitted in this diagram for clarity. The middle section shows an expanded view of the *env* encoded protein showing the signal sequence (grey), surface glycoprotein (pink) and transmembrane glycoprotein (red). Below this, a further expanded view of the transmembrane glycoprotein is shown with the glycosylation sites (grey), the membrane spanning region (yellow), and the position of the in - frame stop codon indicated.


LTR
gag
pol
env
LTR
Signal sequence
Surface glycoprotein
Transmembrane glycoprotein
Membrane spanning region
Glycosylation sites
SU
extracellular domain
cytoplasmic tail
Position of in – frame stop codon

approximately 41 kDa, similar to the TM proteins of HIV - 1 and HIV - 2, however its presence resulted in the truncation of the glycoprotein to approximately 32 kDa. Interestingly, it was later reported that the stop codon was not present in clones directly isolated from SIV infected monkeys by PCR analysis, suggesting the naturally occurring form is the full length 41 kDa glycoprotein. Furthermore, it was found that the stop codon arose as a result of selective pressure for the truncated TM glycoprotein when the virus was grown in human cells, accounting for its presence in the first isolates of SIV (Hirsch,VM *et al.*, 1989a). This was demonstrated *in vitro*, where the kinetics of viral replication and infectivity varied significantly with the size of its TM glycoprotein. Specifically, when the virions were tested for their infectivity in human derived HUT - 78 cells, those bearing the full length TM protein were 5 orders of magnitude less infectious (in $TCID_{50}$ assays), than those with the truncated TM. Conversely, when the target cells used were rhesus monkey PBLs, viruses with the full length TM, displayed a growth advantage over the truncated TM virus. However, following 17 days incubation, revertants were found in the culture infected with the truncated TM virus, that had lost the stop codon, restoring the TM protein to its full length. Thus a clear preference for the full length TM glycoprotein in rhesus PBLs *in vitro* was demonstrated. Further experiments *in vivo* revealed the dominant genotype isolated from SIV infected macaques at 10 months post inoculation, contained no stop codons in the *env* open reading frame, regardless of whether the monkeys were infected with virus that harboured the truncated TM or the full length TM glycoprotein (Hirsch,VM *et al.*, 1989a). Similar observations were made in a variety of SIV isolates grown both in a number of human cell lines and in live primates (Kodama *et al.*, 1989, 1990).

The position of the stop codon, although varying from one isolate to the next, was invariably within a short stretch of approximately 6 amino acids immediately to the carboxy terminal side of the putative hydrophobic membrane spanning domain of the TM glycoprotein (figure 32). The position of the stop codon resulted in the deletion of the cytoplasmic tail. Thus it was concluded that the cytoplasmic domain of the SIV *env* encoded glycoprotein was likely to mediate an interaction with host cell derived factors, which could occur at the point of virus entry and uncoating and / or during the transport and assembly processes of virus production (Kodama *et al.*, 1989, 1990). The phenomena of truncation of the SIV TM glycoprotein when propagated in human cells, was qualified in a later publication, where growth of a variant of SIV in a particular human cell line, showed no low level replication kinetics or TM glycoprotein truncation (Werner *et al.*, 1990). The authors found that the growth of a number of isolates of the SIV_{agm} strain plus an SIV_{abm} strain in human Molt 4 / 8 T - lymphoma cell lines was stable over a long period of time (more than 1 year), and yielded high titres of infectious virus progeny. This finding could be taken to support the hypothesis that there are cellular factors, present in some cells and absent in others, that interact unfavourably with the cytoplasmic domain of the SIV TM glycoprotein during the replication cycle of the virus. On the other hand, the differences could be attributed to the strain of SIV. A better understanding of the mechanisms at work here could yield useful information on the virus's interaction with its host cell, and may in the longer term enable means to limit or prevent the replication of the immunodeficiency virus.

The following investigation was directed at understanding the differences between the truncated and full length forms of the TM glycoprotein in $SIV_{mac}251$ strains that constituted the differing growth characteristics and

infectivity of progeny virus propagated in human and monkey derived cell lines. Recombinant baculoviruses were prepared that expressed both the truncated and full length TM glycoproteins in insect cells. The recombinant glycoproteins were then compared biochemically to identify any differences that might be attributable to the presence or absence the cytoplasmic domain.

Results

The $SIV_{mac}251$ strain, isolated in 1985 at the New England Regional Primate Research Centre (Daniel *et al.*, 1985), was obtained by Almond and colleagues at the National Institute of Biological Standards and Control. This strain was introduced into a macaque monkey (32H), giving rise to a persistent infection. Virus isolated from this monkey was passaged twice through human derived C 8166 cells, whereupon cellular DNA from the culture was harvested and used as template for PCR amplification of the *env* gene. The PCR product was cloned into the M13 bacteriophage cloning vector and stored as pNIBSC - 2 in the AIDS directed programme reagent repository (Holmes, 1991 ADP Cat. No. 207). This clone was the source of genetic material used in these experiments.

Construction of recombinant baculoviruses expressing the truncated and full length TM glycoproteins

The PCR product, pNIBSC - 2, was amplified from the carboxy terminal region of the *env* open reading frame, and consequently has no ATG initiation codon as normally it is expressed as a fusion product with the surface glycoprotein. To overcome this problem, a transfer vector pAc RP14, described in detail elsewhere (Overton *et al.*, 1989), that contained an ATG initiation codon downstream of the polyhedrin gene promoter sequence, was used to provide the necessary initiation signal for expression in Sf cells using recombinant AcNPV virus. pNIBSC - 2 contained an in frame stop codon at amino acid residue 222, which corresponded to amino acid 740 in the original $SIV_{mac}251$ sequence. It was noted that the original amber stop codon (UAG) in $SIV_{mac}251$ (amino acid

736) was replaced by a CAG codon encoding glutamine (Franchini *et al.*, 1987). A Bam HI to Kpn I fragment of pNIBSC - 2 was cloned into the pAc RP14 transfer vector. Reading frame correction was necessary to align the TM glycoprotein open reading frame with the ATG transcription initiation codon. This was achieved by cleavage of the plasmid DNA with Bam HI and removal of the "overhanging" single stranded DNA by digestion with 15 units of mung bean nuclease (Amersham Cat. No. T 2420 Y) in mung bean nuclease buffer (30˚C, 30 min), re - ligation and transformation. Double stranded DNA sequencing over this junction confirmed that the appropriate changes had been made (figure 33; panel A).

To generate the full length SIV TM glycoprotein, the in frame ochre stop codon, UAA, at amino acid residue position 222, was altered to CAA. This change restored the glutamine residue found at this position in the original $SIV_{mac}251$ strain. The alteration required an additional cloning step where the pNIBSC - 2 Bam HI - Kpn I fragment was initially cloned into the pUC 118 transfer vector (Vieira and Messing, 1982, 1987), which, having single stranded DNA production capability, allowed site directed mutagenesis of the stop codon using the oligonucleotide primer SIV gp42 M1 detailed below:

SIV gp42 M1 5' - ACCCATATC<u>C</u>AACAGGACC - 3'

Calculated T_m = 54˚C

where the altered base is underlined. After initial screening by DNA hybridization as has been previously detailed (chapter one), the region was sequenced to confirm that the correct alteration had been made (figure 33; panel

Figure 33.

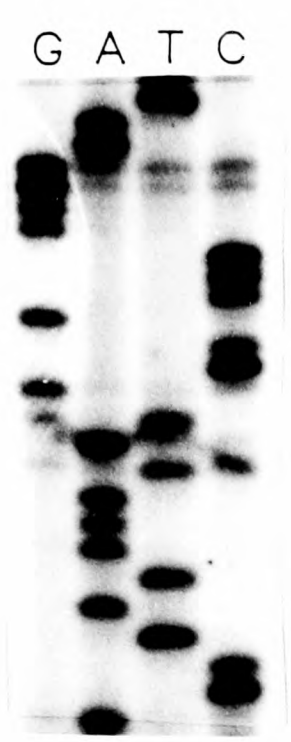
Panel A. DNA sequencing over junction of pAc RP14 and SIV gp26 coding sequence

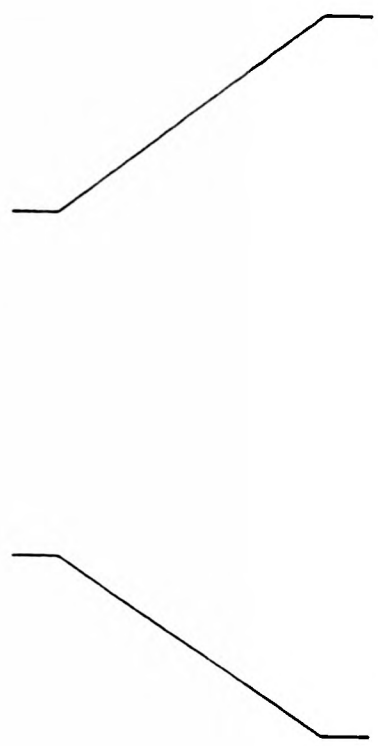
Section of a double stranded DNA sequencing gel autoradiograph, with the lanes containing the dideoxynucleotides marked above (ie ddGTP in left most lane). On the right is the sequence as read from the autoradiograph, with the initiation codon ATG of pAc RP14 indicated by a large angular bracket. Also shown is the deleted Bam HI site at the junction of the pAc RP14 and SIV gp26 coding sequences bringing them into the same reading frame. Autoradiography was at 20 ° C for 18 hrs.

Panel B. DNA sequencing to confirm the alteration of the stop codon in SIV gp26

Section of a single stranded DNA sequencing gel autoradiograph, with the lanes containing the dideoxynucleotides marked above (ie ddGTP in left most lane). On the right is the sequence as read from the autoradiograph, with the altered base (T to C) indicated by an arrow. Autoradiography was at - 70 ° C for 19 hrs with one intensifying screen.

A





+ Strand

3'

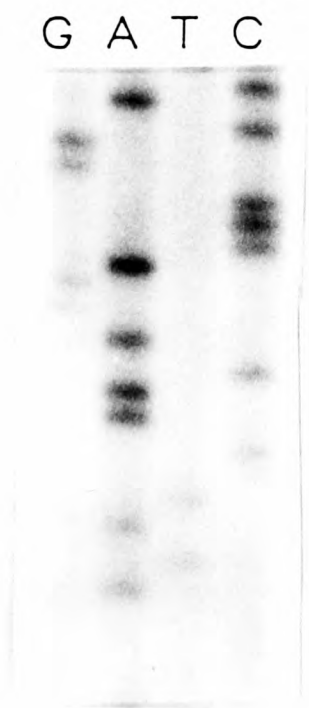
G G C C C G C C C G T A C T A A

C T A G Deleted Bam HI site

} Initiation codon

5'

B



+ Strand

3'

C C C A G G A C A A C C T A T A

← Altered Base (T to C)

5'

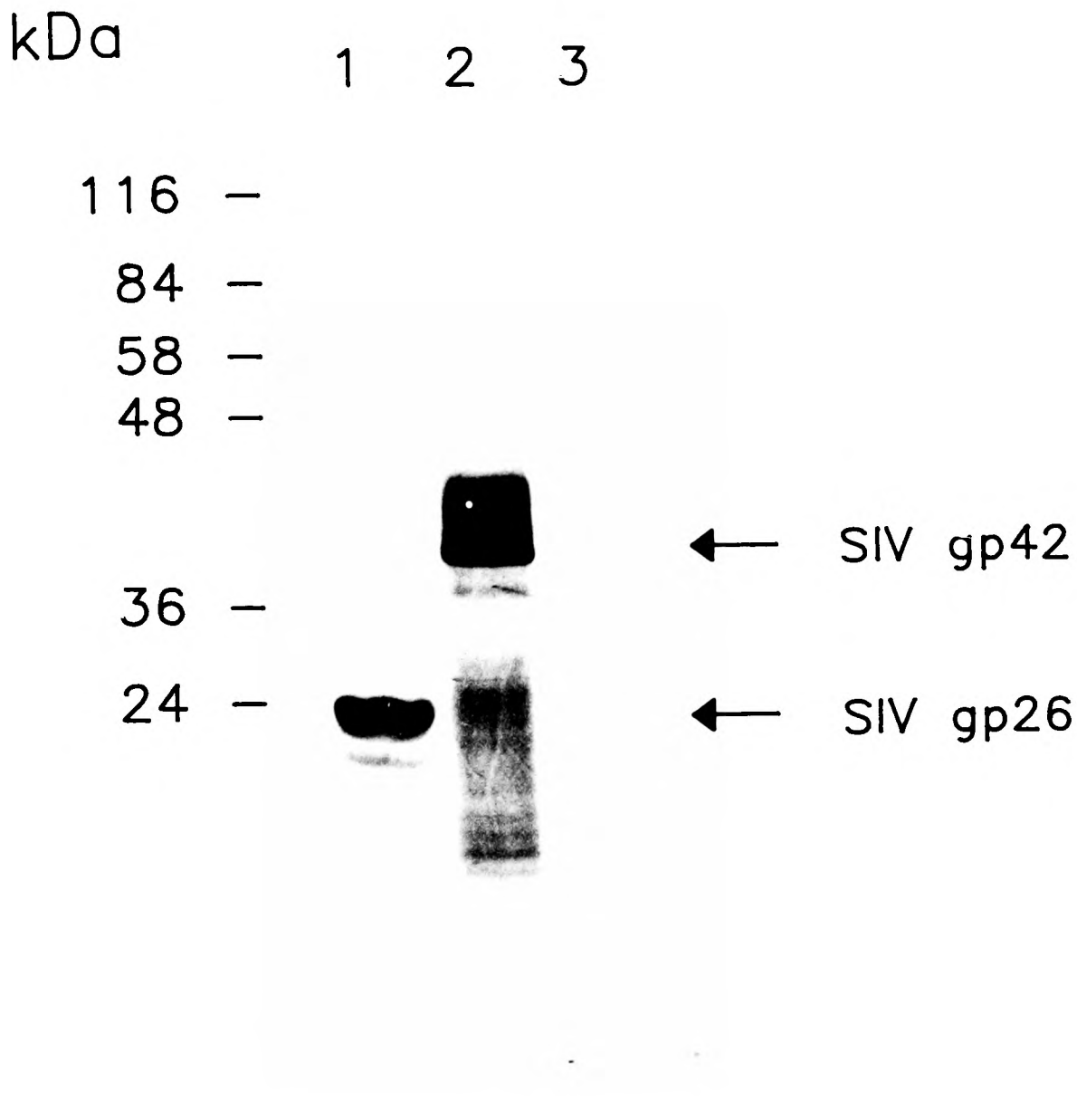
B). The fragment was cloned into pAc RP14 and the reading frame altered as described above for the SIV gp26 recombinant.

Recombinant baculoviruses were prepared expressing each of these proteins as described in chapter one. Successful recombinants producing the glycoproteins were detected by SDS/PAGE and western blot probed with a 3 stage antibody detection protocol as follows. The 1° antibody was heat inactivated serum from an $SIV_{mac}251$ infected monkey (Holmes, 1991 ADP Cat. No. 416), the 2° stage was anti monkey IgG generated in rabbits (Nordic Immunology Cat. No. 14 - 978), and the 3° antibody was anti rabbit IgG alkaline phosphatase conjugate (Sigma Cat. No. A - 8025). High titre stocks of virus were prepared (2×10^7 pfu / ml), and stored for further use. The predicted molecular weights of the truncated and full length TM glycoproteins based on their primary amino acid sequence, were 26 kDa and 42 kDa and were termed SIV gp26 and SIV gp42 respectively. The recombinant proteins were analyzed by separating cell extracts of Sf cultures infected (moi = 10), with each recombinant virus, harvested at 2 days post infection by SDS/PAGE. The gel was western blotted and the proteins detected using the 3 stage detection protocol. The results (figure 34), revealed a distinct band migrating at approximately 26 kDa in cell extracts from the culture infected with the SIV gp26 recombinant (lane 1), while in SIV gp42 infected cell extracts (lane 2), a band of approximately 42 kDa was noted, together with a smear of reactive antigen extending from approximately 42 kDa up to 45 kDa in size. A negative control of AcRP6 infected cell extracts was included (figure 34; lane 3). The appearance of glycosylated proteins, typically seen as smears when analyzed by SDS/PAGE, raised the possibility that the SIV gp42 recombinant glycoprotein was glycosylated while SIV gp26 was not.

Figure 34.

Expression of SIV gp26 and SIV gp42 recombinant proteins

Western blot analysis of cellular extracts from SIV gp26 (lane 1), SIV gp42 (lane 2) and AcRP6 (lane 3), infected Sf cultures. On the right side, the reactive SIV antigen has been indicated by an arrow, SIV gp42 above and SIV gp26 below, while on the left the molecular weight markers are indicated (kDa).


kDa
1
2
3
116
84
58
48
36
24
SIV gp42
SIV gp26

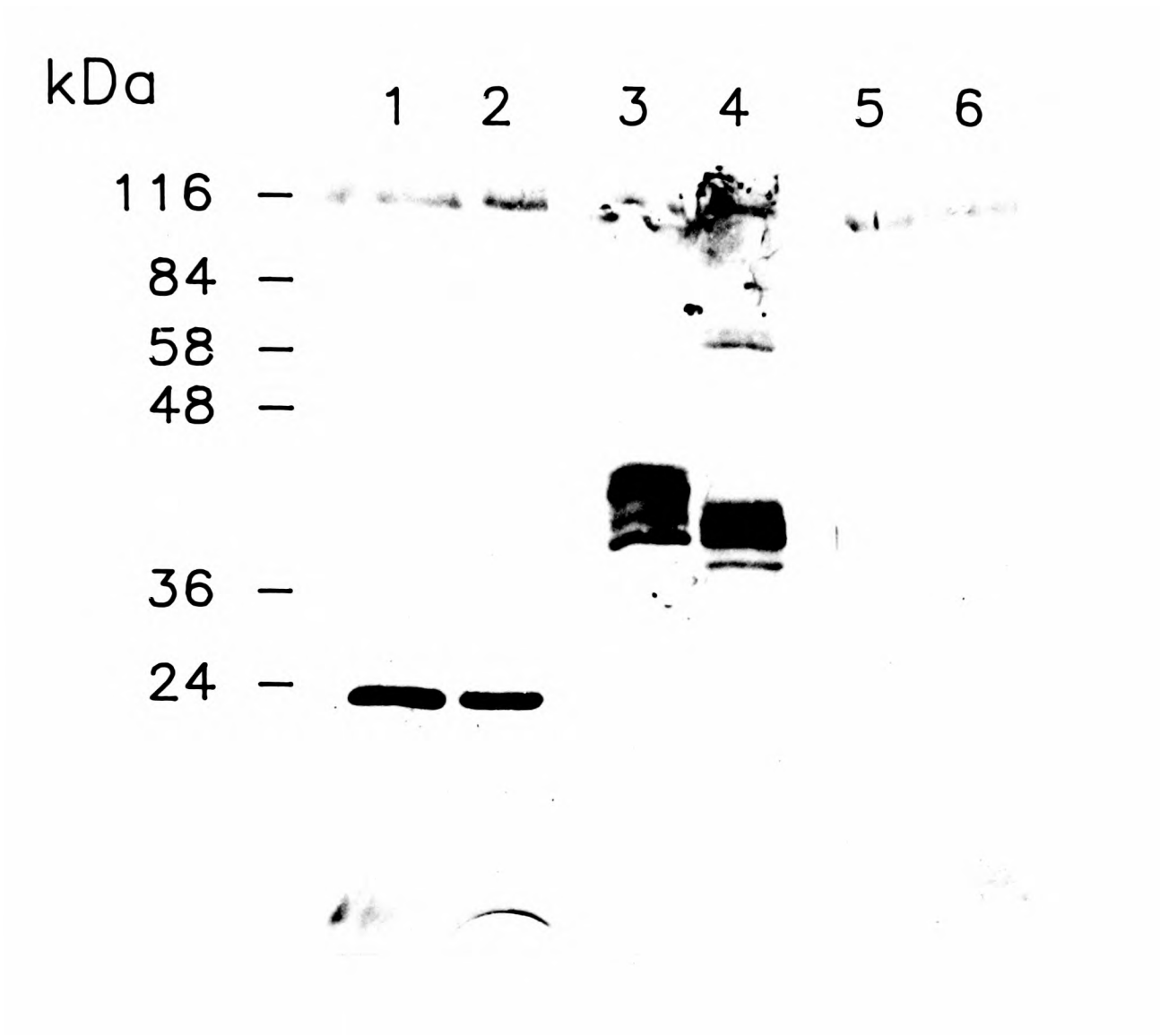
Analysis of glycosylation of expressed proteins

To confirm or not the glycosylation of SIV gp26 and SIV gp42, the proteins were treated with an endoglycosidase that cleaves all accessible glycan moieties from the protein (Fenouillet *et al.*, 1990), and the resulting molecular weights compared to untreated samples. Cultures of Sf cells (1.6 x 10^6 cells / 35 mm dish), were infected (moi = 10), with the SIV gp26 and SIV gp42 expressing recombinant baculoviruses and harvested at 2 days post infection. The cells were pelleted (185*xg*, 10 min), washed in PBS and repelleted, then taken up finally in 250 μl of PBS. The non - ionic detergent Nonidet P40 (BDH Cat. No. 56009), was added to a final concentration of 0.5% and the mixture incubated on ice (0 ° C, 20 min) to lyse the plasma membrane and leave the nuclear membrane intact. The nuclei were removed by centrifugation (1000*xg*, 10 min), and the supernatant used in the subsequent experiment. An aliquot of this preparation was taken, added to SDS gel loading buffer and stored as an untreated control. To the remainder 300 mU of endoglycosidase F / N - glycosidase F enzyme (Boehringer - Mannheim Cat. No. 878 - 740), was added and the mixture incubated at 37 ° C. Aliquots were withdrawn from the incubation after 2, 6 and 24 hr, mixed with SDS gel loading buffer and stored on ice. The reaction was boosted by the addition of 300 mU of endoglycosidase after 6 hr of incubation. Equal aliquots of each time point for each protein were separated by SDS/PAGE, western blotted and detected by the 3 stage antibody protocol detailed above. The cleavage of accessible glycans proceeded faster than expected and was complete after 2 hr incubation as judged by no further change from 2 to 24 hr. The results, presented in figure 35, show samples from untreated SIV gp26 and SIV gp42 extracts adjacent to those withdrawn after 2 hr incubation with the endoglycosidase. Wild type AcRP6 infected and mock

Figure 35.

De - glycosylation of SIV gp26 and SIV gp42

Western blot analysis of cell extracts derived from Sf cultures infected with SIV gp26 and SIV gp42 recombinant viruses. Untreated SIV gp26 and SIV gp42 samples are shown in lanes 1 and 3 respectively, while the same treated with endoglycosidase (37°C, 2 hrs), are shown in the adjacent lanes 2 and 4 respectively. Controls of AcRP6 infected (lane 5), and mock infected (lane 6), Sf cell extracts are shown. Molecular weight markers are indicated on the left side (kDa).


kDa
1
2
3
4
5
6
116
84
58
48
36
24

infected culture extracts were included in parallel as negative controls. There was no change in size observed in samples from SIV gp26 even after the extended incubation period (24 hr). SIV gp42, however, showed a reduction in the size of the 44 to 45 kDa antigen smear, leaving several discrete bands at approximately 43 kDa molecular weight, which were likely to be endoglycosidase resistant forms. This observation demonstrated that the endoglycosidase enzyme was able to remove glycan moieties from SIV gp42. Untreated samples showed no reduction in the molecular weight of antigen after extended incubation, ruling out the possibility of instability of the protein or the action of cellular derived glycosidases. It was concluded that the SIV gp26 glycoprotein showed no evidence of glycosylation, while SIV gp42 glycoprotein was glycosylated in insect cells, accounting for up to 4 kDa of its total molecular weight.

Localization of the expressed proteins

Viral glycoproteins are typically transported to the surface of the cell following modification in the endoplasmic reticulum and golgi apparatus by cellular enzymes. During the final phase of post translational modification, the cellular location of the proteins is determined (reviewed in Rothman and Orci, 1992). The SIV glycoproteins are directed to the plasma membrane in their natural host cells. Having shown that the removal of the cytoplasmic tail of the SIV TM glycoprotein altered the glycosylation pattern of the protein, an investigation to determine whether there was any consequential effects on targeting of the modified proteins within the insect cell was carried out. To examine the location of the proteins within the cell the following experiment was carried out.

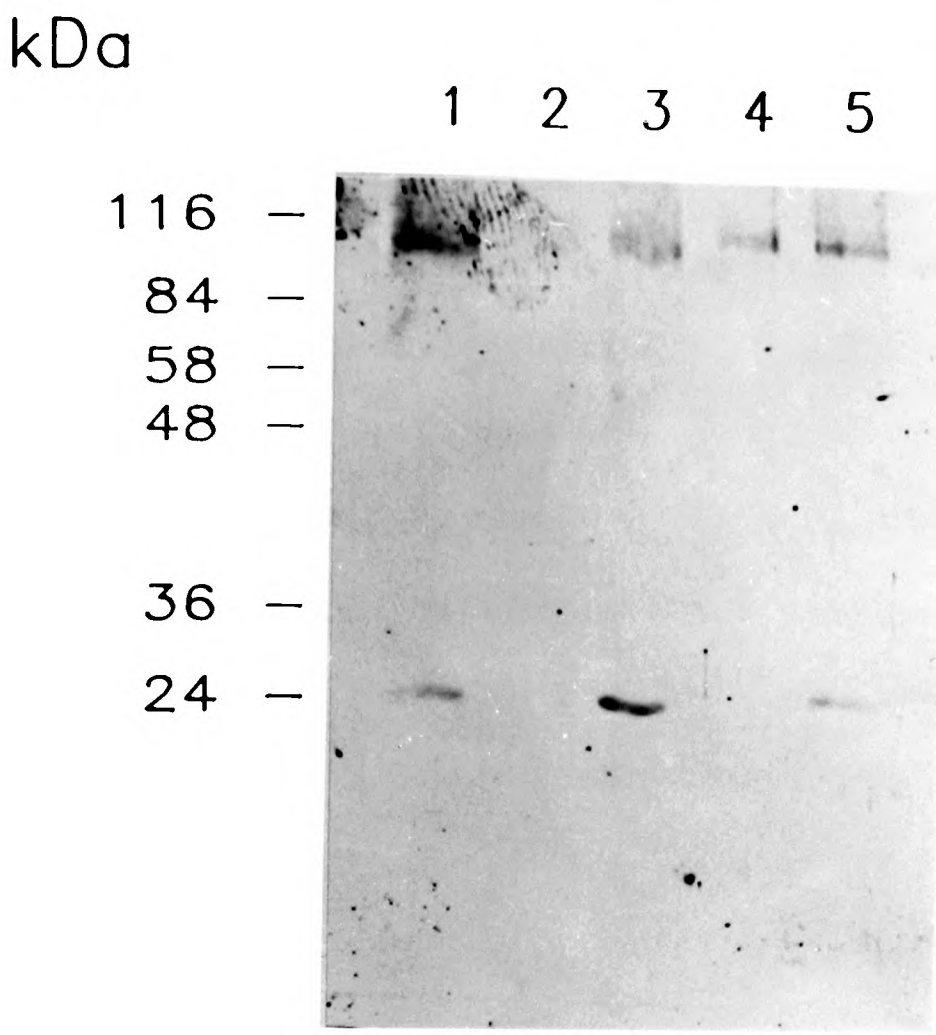
Suspension cultures of Sf cells (1 x 10^8 cells total), were infected by resuspension of the pelleted cells (185*xg*, 10 min), in SIV gp26 and SIV gp42 virus stock (22 ° C, 1 hour). The cells were diluted with TC - 100 culture medium to a density of 1 x 10^6 cells / ml and incubated at 28 ° C for 2 days. The cultures were harvested, the cells washed twice in PBS, pelleted (185*xg*, 10 min), and resuspended finally in PBS to a cell density of between 1 - 2 x 10^7 cells / ml. This slurry was sonicated on ice using an MSE soniprep sonicator (model 150) with 5 periods of sonication of 30 sec each (power level = 24), interspersed with 15 sec cooling off periods. Cellular debris was pelleted (9,000*xg*, 10 min), resuspended in PBS and an aliquot taken and mixed with SDS gel loading buffer in preparation for SDS/PAGE analysis. Likewise aliquots of the supernatant were also extracted and mixed with loading buffer. The samples, together with recombinant protein expressing whole cells lysed in loading buffer as positive controls, were analyzed by SDS/PAGE and western blotted where the standard 3 stage antibody detection protocol was used. SIV gp26 antigen was detected entirely within the sonicate supernatant fraction (figure 36), and not in the pelleted fraction of the sonicated cell slurry. This suggested that the protein was largely in a soluble form, being easily released upon disruption of the cell. Analysis of the SIV gp42 results revealed a different situation where antigen was detected both within the cellular debris pellet and the sonicate supernatant (figure 36), in approximately equal quantities. A possible explanation for this observation, was that small fractions of cellular membranes were generated during sonication that contain embedded SIV gp42 antigen. These fragments, on account of their small size, would not be pelleted by the low speed centrifugation step, but remain in the supernatant, giving the impression of being in a water soluble form. To test this hypothesis, the sonicate supernatant was subjected to high speed centrifugation

Figure 36.

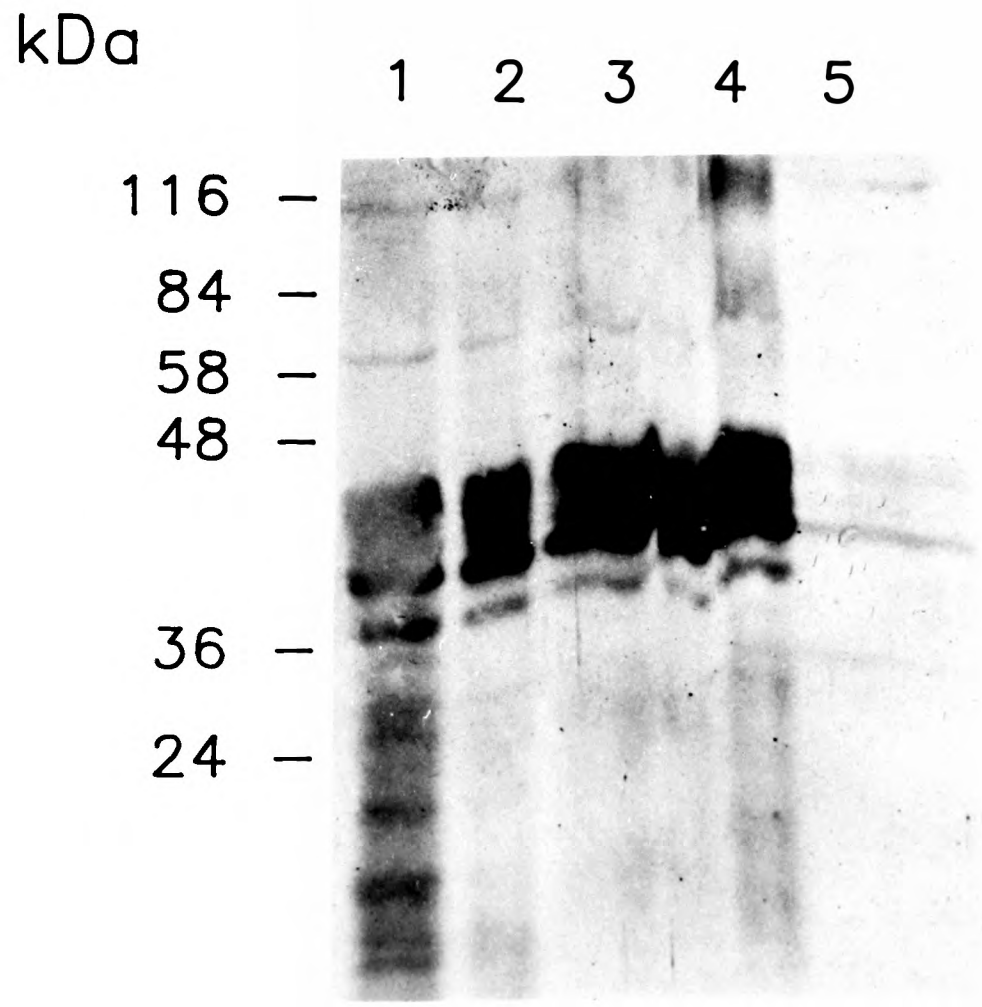
Localization of SIV gp26 and SIV gp42

Western blot analysis of SIV gp26 (top panel), and SIV gp42 (bottom panel), infected Sf cell extracts (lane 1). Sonicated infected cell extracts were centrifuged (9,000*xg*, 10 min), and the pellet and supernatant fractions analyzed in lanes 2 and 3 respectively. The supernatant fraction (lane 3) was subjected to further high speed centrifugation (164,000*xg*, 90 min), and the pellet and supernatant fractions analyzed, lanes 4 and 5 respectively. Molecular weight markers are indicated on the left side (kDa).

SIV gp26



SIV gp42



(164,000*xg*, 90 min), sufficient to pellet any small membrane fractions. The results revealed that the glycoprotein was almost entirely seen in the high speed centrifugation pellet fraction, leaving the supernatant fraction virtually clear of antigen (figure 36). The same procedure of high speed centrifugation was applied to the sonicated cell supernatant of SIV gp26, which showed that the antigen remained in the supernatant (figure 36). It was concluded from this that the SIV gp42 glycoprotein was located within the cell, associated largely with cellular membranes, while the SIV gp26 antigen was found in a soluble form, not associated with membranes.

It has been demonstrated that several dominant antigenic sites are located within the amino terminal region of the HIV - 1 TM glycoprotein (Gnann *et al.*, 1987; Bugge *et al.*, 1990; Earl *et al.*, 1991a), including a highly conserved epitope capable of eliciting neutralizing antibodies (Bolognesi, 1990). Given that structural assembly domains are sufficiently conserved between the TM glycoproteins of HIV and SIV so as to allow heterodimer assembly (Doms *et al.*, 1990), it was possible that antigenic epitopes may be similarly conserved between the viruses. The ability of the recombinant SIV gp26 and SIV gp42 proteins to elicit neutralizing antibodies was assessed. Guinea pigs were inoculated with partially purified SIV gp26 and SIV gp42 protein, however, despite several attempts and booster inoculations, only weakly reactive antibodies were generated, which were unsuitable for further investigation.

Construction of SIV gp42^{SIG}

The TM glycoprotein is synthesized as a part of a precursor, fused to the SU glycoprotein, which has, at its amino terminus, a stretch of hydrophobic amino acids, that constitutes a signal sequence. This sequence directs the precursor protein to the start of the cellular secretory pathway where post translational modification takes place (Peterlin and Luciw, 1988; Kieny, 1990). To examine the effects the signal sequence may exert on the post translational modification of the recombinant expressed SIV TM glycoprotein within insect cells the following experiment was carried out. The HIV - 1 Env glycoprotein signal sequence was selected as it was known to efficiently direct the SU glycoprotein through the secretory pathway in insect cells, when expressed by a recombinant baculovirus (Morikawa,Y *et al.*, 1990; Wells and Compans, 1990). The construction involved PCR amplification of the DNA stretch encoding the HIV - 1 signal sequence and cloning of the fragment into an assembly vector observing the correct reading frame alignment, that contained the SIV gp42 coding sequence. The method has been detailed below.

PCR amplification was used to produce a 146 base pair fragment encoding the HIV - 1 *env* precursor signal sequence. The following primer pair and conditions were used:

SigF 5' - CGCGGGATCCGGCAATGAGAGTGAAGGAGA - 3'

SigR 5' - CGCGCCCGGGCCCATAATAGACTGTGACCC - 3'

Annealing temperature	-	45°C 1 min
Primer extension temperature	-	70°C 3 min
Dissociation temperature	-	95°C 1 min
Number of Cycles	-	30

together with linearized plasmid template containing the molecular clone of the HIV - 1_{LAI} strain. The primers were designed to include the initiation codon ATG (underlined), and to facilitate the introduction of the sequence, correctly aligned, into the pUC 118 vector containing the SIV gp42 coding sequence. The integrity of the junction between the 3' end of the signal sequence and the 5' end of the glycoprotein sequence was verified by restriction endonuclease digestion of a unique reconstituted *Sma* I site. Insertion of Bam HI linkers (Pharmacia Cat. No. 27 - 7725), at a Klenow repaired Asp 718 cleaved restriction site at the 3' end of the TM sequence, allowed transfer of a Bam HI - Bam HI cassette into the pAc CL29.1 transfer vector.

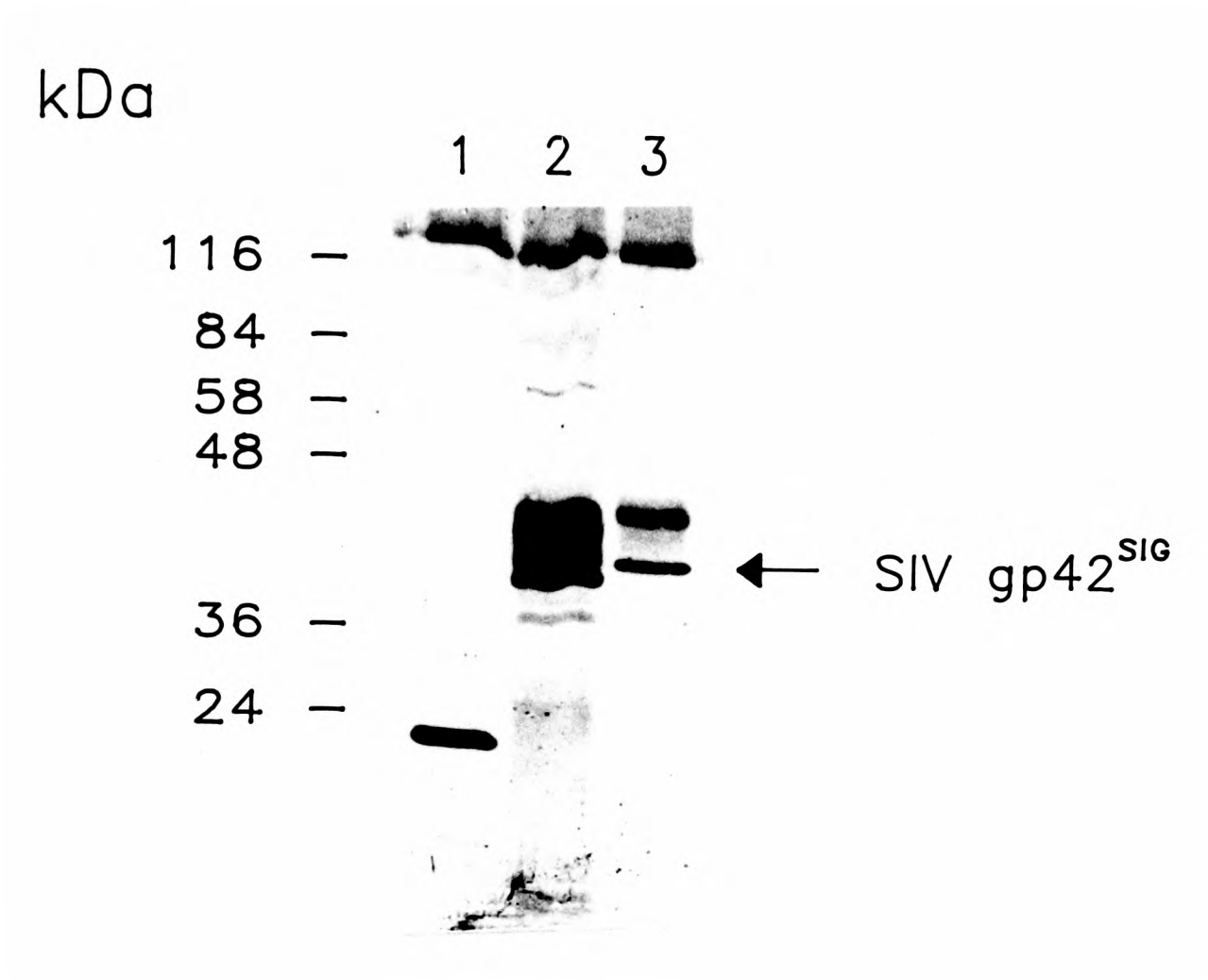
Characterization of SIV gp41^{SIG}

Recombinant viruses were generated and purified as previously described and high titre stocks prepared. Analysis by SDS/PAGE and western blot of the recombinant protein termed SIV gp42^{SIG} can be seen in figure 37 (lane 3), together with SIV gp26 and gp42 in adjacent lanes for reference (lanes 1 and 2 respectively). The predicted molecular weight based on the primary amino acid sequence was 48 kDa, however, after cleavage of the signal sequence, as would be expected during processing, the predicted size was reduced to approximately 43 kDa, which correlated well with the observed molecular weight of the

Figure 37.

Expression of SIV gp42^{SIG}

Western blot analysis of cell extracts from Sf cells infected with SIV gp26 (lane 1), SIV gp42 (lane 2), and SIV gp42^{SIG} (lane 3). The SIV gp42^{SIG} antigen is indicated by an arrow. Molecular weight markers are indicated on the left side (kDa).


kDa
1 2 3
116
84
58
48
36
24
SIV gp42SIG

glycoprotein. Additionally, the smear of antigen previously observed in the recombinant SIV gp42 glycoprotein, was reduced in the SIV gp42^{SIG} recombinant to a single band of about 46 kDa, that was likely to be the glycosylated form of the protein. This was confirmed in the following experiment, in which similar endoglycosidase treatment was carried out as was done to assess glycosylation of SIV gp26 and gp42. The results (figure 38; panel A), demonstrated a reduction in size of the larger 46 kDa band to a 44 kDa species, following endoglycosidase digestion, which confirmed that SIV gp42^{SIG} was glycosylated. As fewer partially glycosylated forms were seen in this construct than in the SIV gp42 recombinant protein, the addition of the signal sequence appeared to promote more efficient glycosylation. The cellular localization of SIV the gp42^{SIG} was done as described for figure 36. The results (figure 38; panel B), revealed a similar profile to that of SIV gp42 without the signal sequence. This showed that the glycoprotein was largely associated with cellular membranes.

Figure 38.

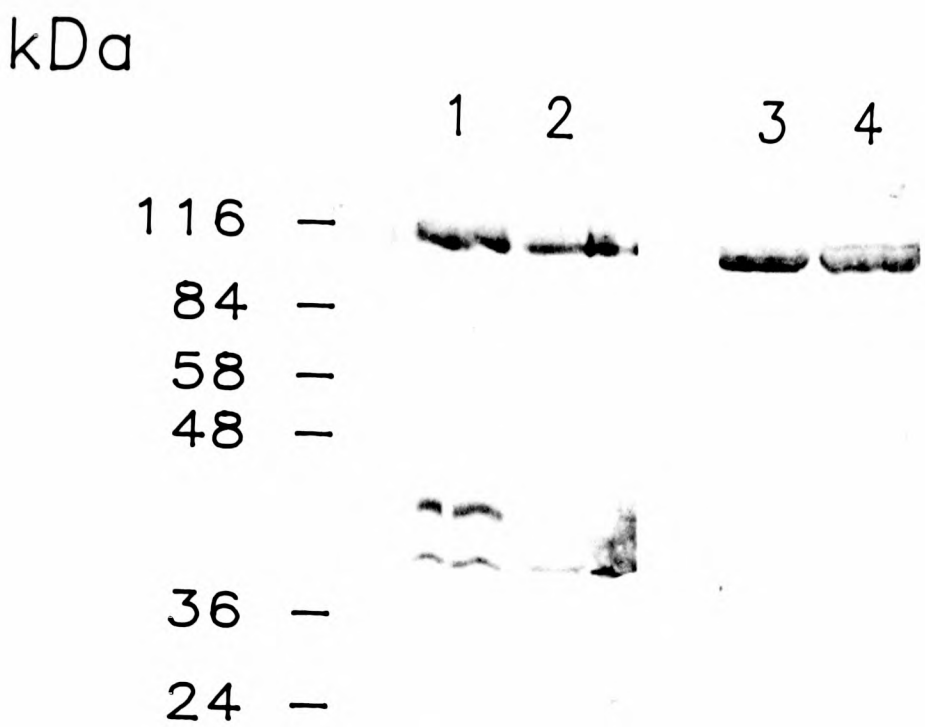
Panel A. De - glycosylation of SIV gp42^{SIG}

Western blot analysis of cell extracts derived from Sf cultures infected with SIV gp42^{SIG} recombinant virus, untreated (lane 1), and treated with endoglycosidase (37°C, 2 hrs), in lane 2. Controls of AcRP6 infected (lane 3), and mock infected (lane 4), Sf cell extracts are shown. Molecular weight markers are indicated on the left side (kDa).

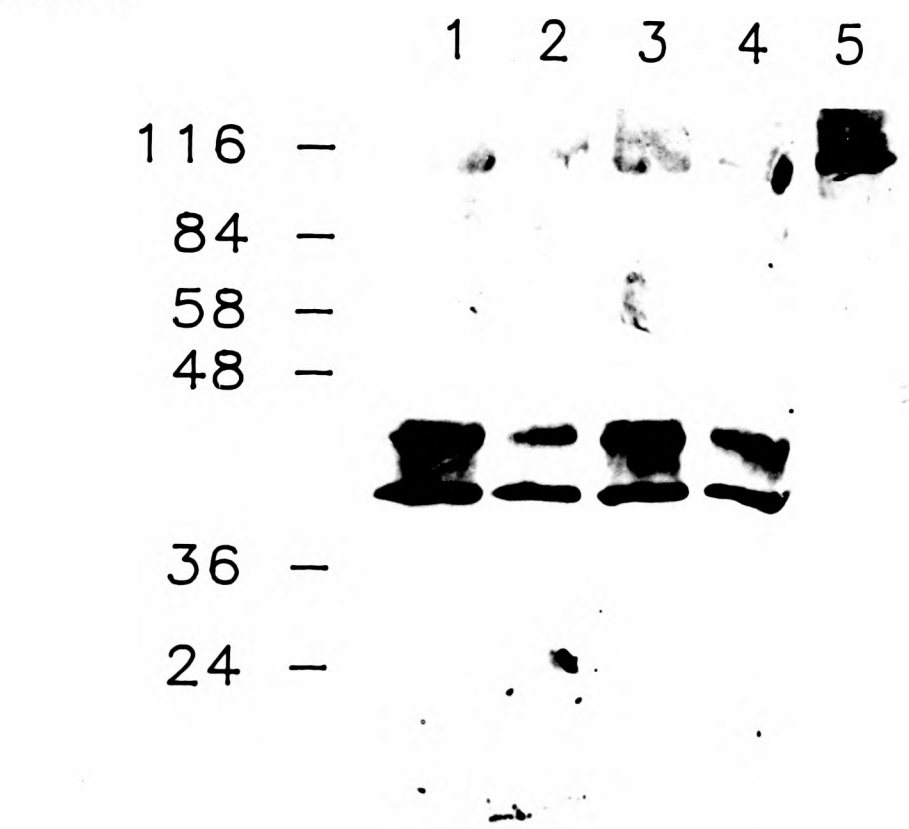
Panel B. Localization of SIV gp42^{SIG}

Western blot analysis of whole cell extracts infected with SIV gp42^{SIG} (lane 1). These cells were sonicated and centrifuged (9,000*xg*, 10 min), and the pellet and supernatant fractions analyzed in lanes 2 and 3 respectively. The supernatant fraction (lane 3), was subjected to further high speed centrifugation (164,000*xg*, 90 min), and the pellet and supernatant fractions also analyzed on the same blot in lanes 4 and 5 respectively. Molecular weight markers are indicated on the left side (kDa).

A



B



Discussion

The full length SIV gp42 TM glycoprotein contains 4 potential glycosylation sites, of which only 3 are widely conserved (Myers *et al.*, 1991). Each of the conserved sites are located in the region to the amino - terminal side of the putative hydrophobic membrane spanning domain (figure 32), and are thus present in both of the recombinant proteins studied here. The results presented here demonstrate that removal of the cytoplasmic tail of the TM glycoprotein alters the glycosylation potential of these sites, SIV gp26 (not glycosylated) and SIV gp42 (glycosylated). Addition of the HIV - 1 Env precursor signal peptide to the TM protein (SIV gp42^{SIG}), promoted more efficient glycosylation where a single band above the unglycosylated form was noted by SDS/PAGE and western blot analysis. This was contrasted with SIV gp42 alone which displayed several bands or a smear of antigen probably representing a series of partially glycosylated species.

The observation that SIV gp42, which lacks a signal sequence, was glycosylated at all, suggested a role for the cytoplasmic tail in having some influence on post translational modification as is discussed in more detail later. It was possible that the differences seen in the intracellular localization between the truncated and full length forms could be linked to the different glycosylation patterns. Both truncated and full length forms of the SIV TM protein contained the hydrophobic stretch of amino acids, which by analogy with the HIV - 1 TM protein, was designated the membrane spanning region. In HIV - 1, this stretch has been predicted to form an α - helical structure and was noted as being the only membrane spanning domain in the current model of the TM protein (Andreassen *et al.*, 1990). Deletions within this sequence have been shown to

disrupt membrane anchorage and thus support the predictions of the model (Gabuzda *et al.*, 1991). Although, in the SIV gp26 and SIV gp42 constructs, both these domains are present, SIV gp26 was soluble while SIV gp42 was associated with membranes.

The sub - family of lentivirinae have been noted for their unusually long TM protein cytoplasmic domains, by comparison to other members of the Retroviridae (Chakrabarti *et al.*, 1989; Gallaher *et al.*, 1989). For example, RSV has a short cytoplasmic tail of only 50 amino acids, while 150 to 160 amino acids is typical of the immunodeficiency viruses (Myers *et al.*, 1991). Evidence has been reported that the hydrophobic membrane spanning domain alone of the RSV TM glycoprotein is sufficient to anchor the molecule in the membrane. Furthermore, the signals that direct the intracellular processing and transport were localized to the extracellular domain of the glycoprotein (Perez *et al.*, 1987). In contrast to these findings, the results presented here suggest that the hydrophobic region of the SIV TM alone is not sufficient to anchor the protein within the membrane. Hence, additional factors were proposed, that either are intrinsic to or interact with the cytoplasmic domain of the SIV TM glycoprotein to form an efficient stop - transfer signal, and to stabilize the protein in a membrane spanning orientation.

Within the structural model proposed for the HIV - 1 TM glycoprotein, the C - terminal 90 amino acids of the domain folds into two amphipathic helices (Venable *et al.*, 1989), which have been proposed to form a second association with the lipid membrane (Haffar *et al.*, 1988, 1990b, 1991). Additionally, when the HIV - 1 TM glycoprotein was purposely truncated in a similar position to the SIV truncated TM, the progeny virions were non - infectious (Hunter and

Swanstrom, 1990), while partial deletions of the cytoplasmic tail produced progeny virions that were markedly attenuated in replication (Kowalski *et al.*, 1987; Lee,SJ *et al.*, 1989; Adachi *et al.*, 1991). These findings implied the roles of the SIV and HIV TM domains were functionally similar within their own natural host cells. This supported the hypothesis that the implied hydrophobic nature of the SIV TM cytoplasmic domain has the effect of stabilizing the integration of the protein within the lipid bi - layer. This would, conceivably, promote the appropriate glycosylation events as well as direct the protein through the secretory pathway.

An additional function for the HIV - 1 TM cytoplasmic tail has been identified. Recent evidence implicated the HIV - 1 TM cytoplasmic domain in directing both itself and the *gag* derived proteins to a specific location in polarized cells, where assembly and budding took place at the plasma membrane (Owens *et al.*, 1991). These results shed some light on a mechanism where the virus, in its natural host cell, can direct the localization of its proteins to a specific patch of the plasma membrane for assembly of the viral particles. The process, directed by the cytoplasmic tail of the TM protein and possibly mediated by factors derived from the host cell, would be severely disrupted if the domain were deleted. In this scenario, if the virus had infected certain types of polarized cells *in vivo*, the mechanism would allow release of progeny virions from regions of the cell where they may gain entry to the circulatory system, thus promoting their distribution throughout the body. Such a mechanism would confer a substantial overall growth advantage. The question of why the presence or absence of this domain alters the growth characteristics and the infectivity of progeny virions in cultured T - cells remains unanswered. There is little evidence available that addresses the question of whether polarized assembly, budding and release of

virions occurs in these cells. In the event that release is polarized, then the differences between propagation of wild type SIV in monkey cells and human cells, and between different human cells lines, could be accounted for by the presence or absence of cellular factors that interact with the cytoplasmic domain and direct the localization of virion assembly.

Although the cytoplasmic tail of sequenced SIV and HIV - 2 isolates display some variation, there are regions interspersed throughout the domain that are widely conserved. These observations strongly suggest a role for the cytoplasmic tail of the protein which, with current data, remains speculative. Analysis of mutants with deletions in these conserved regions may provide additional clues. The results presented here suggest the domain maybe involved in directing or manipulating the protein during post translational modification. Conceivably, this would be at the level of controlling trafficking through the appropriate compartments of the ER and Golgi apparatus, or by ensuring the protein assumes the correct orientation and association with the lipid membrane. The possibility that some cellular factors are involved in this process by binding to or interacting with the domain cannot be ruled out. Furthermore, the interaction with other virally derived proteins may help the virus to specifically acquire its glycoproteins during assembly and budding from the plasma membrane. The question of how the cytoplasmic domain of the SIV TM glycoprotein affects the growth characteristics of the virus in human or monkey cells is largely unanswered, however, the identification of a role of the region here, represents a starting point.

Two recent reports on mutagenesis of conserved glycosylation sites within the extracellular region of the HIV - 1 TM glycoprotein demonstrated that their

disruption could dramatically reduce the infectivity of the progeny virions (Dedera *et al.*, 1992; Lee,WR *et al.*, 1992). The cause for this reduction was assigned to impaired fusogenic activity of the TM glycoprotein while the synthesis, processing, folding and the multimerization properties were all found to be similar to wild type. The results presented in this chapter suggested that removal of the cytoplasmic tail of the SIV TM glycoprotein influenced the glycosylation potential of these sites. In an extension of these observations, because the glycosylation of the TM protein is important for bioactivity, the removal of the cytoplasmic tail could modulate the glycosylation of the molecule and hence its biological activity. Thus mutant virions harbouring a truncated TM glycoprotein may have altered infectivity, replication rate and host range properties when compared to wild type virus.

Chapter Four

Practical uses of Gag particles as carriers of immunogenic epitopes

Introduction

The HIV - 1 *gag* gene product has the ability to self assemble into immature virus - like particles when expressed in insect cells, the mechanisms of which have been examined in chapter one. The non - infectious particles bud from the cells surface, thus acquiring a lipid membrane envelope, and accumulate in the culture medium, making them easily purifiable by density gradient centrifugation. It is these properties that make the Gag particle particularly attractive for development in the design and construction of vaccines.

The replication and pathogenesis of HIV in humans is highly complex. The virus has as one of its major targets an important component of the host's immune system, the CD4 positive T - cell population. This poses particular difficulties in developing immunization strategies, while the highly variable nature of the outer *env* encoded glycoprotein creates further complications (reviewed Adler, 1987; Beverley and Sattentau, 1987; Gallo, 1990; McCune, 1991). Recent experiments using SIV in primates as an animal model, demonstrated the hazards of using whole killed virus or killed virus infected cell preparations for immunization, a contemporary approach for eliciting immunological protection against viral

pathogens (Hilleman, 1985; Langlois *et al.*, 1992). The immune response was initially found to be protective against challenge by homologous SIV, however, further analysis showed it was largely directed against host cell surface antigens derived from tissue culture in which the virus was propagated. These antigens were present in both the original vaccine preparation and the challenge dose (Stott, 1991; Cranage *et al.*, 1992; Le Grand *et al.*, 1992; Langlois *et al.*, 1992).

One of the possible practical uses of the Gag particles could be as carriers of foreign antigens. In this chapter, two potential means for loading the HIV - 1 Gag virus - like particle with additional antigens are investigated. The first of these is a development from the experiments described earlier in chapter one, where portions of the Gag precursor were deleted from the carboxy terminus of the protein. Replacement of these regions with a domain encoding a relevant epitope, while maintaining self assembly competence was investigated. It was apparent that a size limitation may exist as implied by the inability of the Gag - Pol fusion protein to self assemble into particles (chapter one, Shioda and Shibuta, 1990). However, in the case where large domains are required for the necessary immune response, it may be possible to rescue a self assembly deficient construct by co - expression with an assembly competent Gag only construct. Such a mechanism has been shown to operate in HIV infected cells where incorporation of the large Gag - Pol fusion precursor into the forming virion is driven by the Gag domain (see chapter one; Wills and Craven, 1991).

The second mechanism exploited the budding process at the plasma membrane when the forming virion concurrently acquires the lipid bi - layer envelope. If an epitope were expressed on the surface of the *gag* expressing cell, such as an integral membrane spanning glycoprotein, then it may be acquired by the

emerging particle as part of its membrane envelope. This mechanism of glycoprotein acquisition has also been demonstrated in the retroviral life cycle in its natural host cell (Peterlin and Luciw, 1988; Hunter and Swanstrom, 1990; Haseltine, 1991), however it is unclear whether this is a random event or if the glycoproteins are deliberately selected by some interaction between the cytoplasmic tail of the glycoprotein and the Gag precursor domain. While attempts to demonstrate the latter found no evidence for such a link with the cytoplasmic tail of the glycoprotein, preliminary experiments with RSV suggested that there was an interaction between the Gag protein and hydrophobic membrane spanning region of the TM glycoprotein (Perez *et al.*, 1987; Hunter and Swanstrom, 1990). In attempting to artificially load the *gag* derived particle with surface expressed glycoproteins some light may be shed on this question.

These two methods may allow the construction of easily purifiable particulate vaccines that contain several different epitopes. The feasibility of exploiting these mechanisms are investigated in this chapter using recombinant baculovirus expression of the proteins in insect cells.

Results

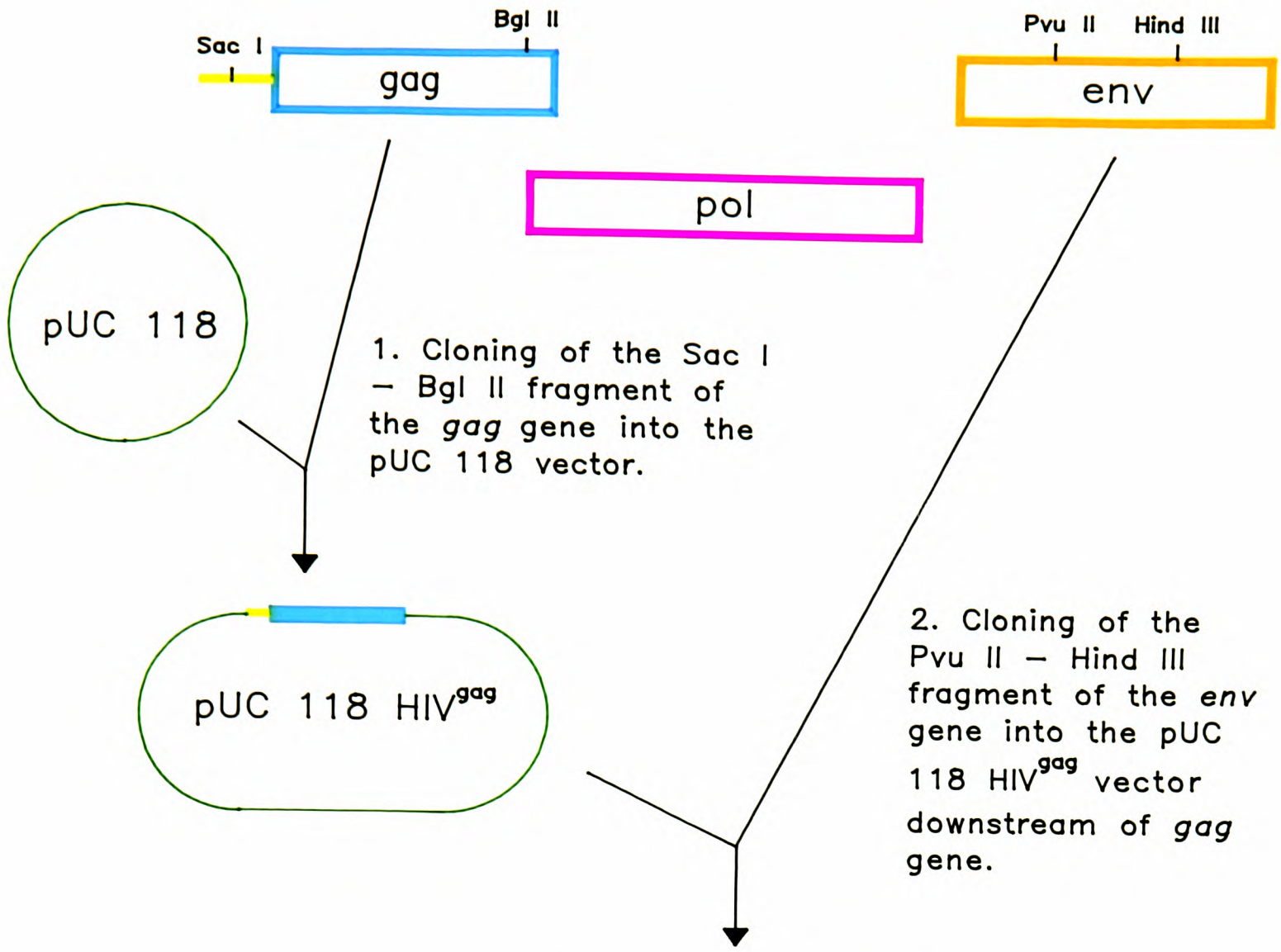
Fusion of foreign domains onto the carboxy terminus of the Gag protein

The fusion of immunodominant antigens to the carboxy terminus of the Gag precursor was first investigated. In early experiments, a region derived from the envelope protein of the HIV - 1_{LAI} strain, between amino acids 287 and 467 was found to contain epitopes capable of eliciting neutralizing antibodies (construct PB1 in Putney *et al.*, 1986). This 180 amino acid sequence included the V3 or GPGR loop hypervariable domain which was later confirmed as the principal neutralizing determinant of the HIV - 1 envelope glycoprotein (Javaherian *et al.*, 1989). For these reasons, the region was selected as a test fusion domain and the assembly of a transfer vector with the region fused to the Gag precursor is described here. The *Sac* I to *Bgl* II fragment of the HIV - 1 *gag* gene, detailed in chapter one, was cloned into the pUC 118 cloning vector digested with *Sac* I and Bam HI (Sambrook *et al.*, 1989). A *Pvu* II to *Hin* dIII segment of the *env* gene (encoding amino acids 287 through 640), which included the sequences mentioned above, was inserted into the vector cut with *Xba* I downstream of the *gag* open reading frame (figure 39). The restriction enzyme digestion generated 5' overhangs, which were filled in by incubation with 1 unit of the Klenow fragment of DNA polymerase I (Amersham Cat. No. T - 2141 Y), 0.5 μM each of dATP, dTTP, dCTP and dGTP (Pharmacia Cat. No. 27 - 2035 - 01), in the manufacturers recommended buffer (22°C, 30 min). A blunt ended ligation was done, bringing both the *gag* and *env* genes into the same reading frame. DNA sequencing over this junction revealed the loss of a single base pair, throwing the *env* sequence into the -1 reading frame with respect to *gag*.

Figure 39.

Construction of HIV Gag - Env fusion protein

Diagram showing the steps of construction of the Gag - Env fusion precursor. The uppermost level of the diagram shows the *gag* (blue), *pol* (magenta) and *env* (orange), genes of HIV - 1, with the restriction enzyme sites used in the cloning indicated, *gag* gene; *Sac* I and *Bgl* II, *env* gene; *Pvu* II and *Hin* dIII. 1. The *Sac* I - *Bgl* II fragment of the *gag* gene was cloned into the pUC 118 cloning vector (green). 2. The *Pvu* II - *Hin* dIII fragment of the *env* gene was cloned into the pUC 118 HIV^{gag} vector, downstream of the *gag* open reading frame. 3. Sequencing across the *gag* - *env* junction revealed a single base pair deletion had occurred during cloning, which resulted in the *gag* and *env* genes being out of frame. This was corrected by site directed mutagenesis. 4. The *gag* - *env* cassette was sub - cloned into the pAc CL29.1 transfer vector (red). 5. The purified plasmid DNA of the pAc CL29.1 HIV$^{gag\text{-}env}$ vector was co - transfected into insect cells with AcRP6 genomic DNA, and recombinant baculoviruses isolated and purified.



5. Co – transfection of the pAc CL29.1 HIV$^{gag-env}$ transfer vector with AcRP6 DNA into insect cells and recovery of recombinant virus.

This was corrected by site - directed mutagenesis using the oligonucleotide Gagenv f/s:

Gagenv f/s 5' - GTAGAGGATCCTTCCCTAAAA - 3'

Calculated T_m = 56°C

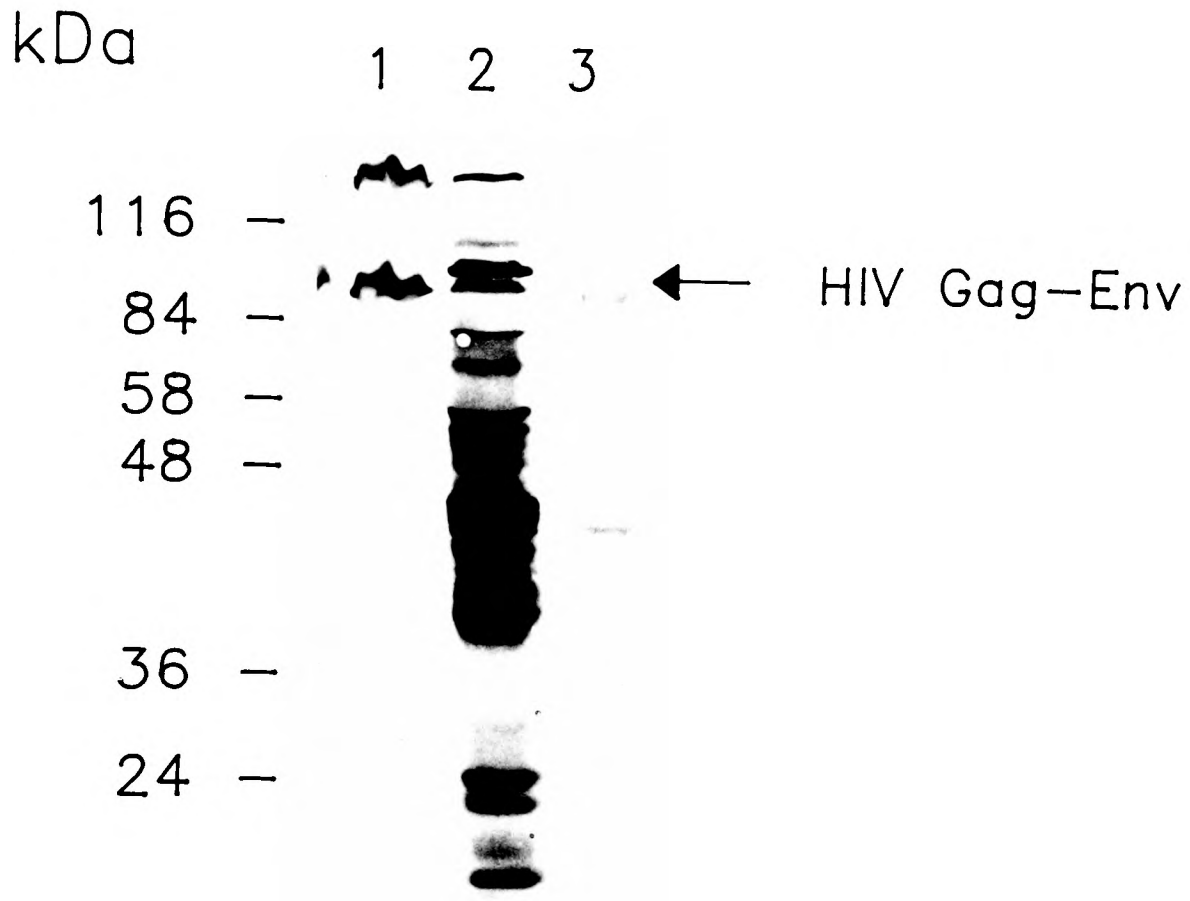
where the inserted base (C), is underlined. Mutants were screened for by DNA hybridization as previously described. The alteration reconstituted a Bam HI restriction enzyme site, which allowed confirmation of the correct change by successful restriction digestion analysis. The whole *gag - env* cassette was then transferred by *Sac* I and *Hin* cII digestion into the *Sac* I and *Sma* I cleaved sites of the pAc CL29.1 transfer vector, yielding the plasmid pAc CL29.1 HIV*gag-env*.

Recombinant viruses, termed Gag - Env, were produced as previously described and high titre stocks prepared. Sf cells taken from a culture at 2 day post infection (1.6 x 10^6 cells / 35 mm dish), infected at an moi of 10, were solubilized in SDS gel loading buffer. The extracts were separated by SDS/PAGE, western blotted and probed with monoclonal antibodies to HIV Gag p17 and p24 as described in chapter one (3 stage protocol). The results shown in figure 40, indicated a protein of approximately 90 kDa was present in the cellular extracts from the infected culture and absent in the extracts of mock infected Sf cells and wild type AcRP6 infected cells, that were included as negative controls. The predicted molecular weight of the protein based on the amino acid sequence was 89 kDa, which correlated well with the observed band reactive to anti Gag antibodies. The level of expression was lower than has been seen for other recombinant antigens produced and a number of breakdown products were

Figure 40.

Expression of HIV Gag - Env fusion protein

Western blot analysis of Sf cell extracts infected with the HIV Gag - Env expressing virus (lane 2), the position of the recombinant protein has been indicated by an arrow. The blot was probed with monoclonal antibodies to HIV Gag p24 and p17 domains. AcRP6 infected (lane 3), and mock infected (lane 1), Sf cell extracts were included as controls. Molecular weight markers are shown on the left of the panel (kDa).


kDa
1
2
3
116
84
58
48
36
24
HIV Gag–Env

observed, which suggested the molecule was either heat labile or subject to proteolysis by intracellular proteases. The ability of this construct to self assemble into particles was assessed by sucrose density gradient centrifugation, in the same way as detailed in chapter one. No evidence of particle formation was detected by western blot of the sucrose density gradient fractions. Given that a lower level of expression of recombinant antigen had been observed for this construct, it was suspected the inability to detect particulate antigen might have been explained by the levels of protein falling below the threshold of detection. However when medium from a large volume culture (50 x 10^6 cells / 175 cm^2 flask; infected at moi 10) was harvested at 2 days post infection, concentrated and applied to another sucrose density gradient, the analysis by western blot, using the 3 stage antibody detection protocol (for maximum sensitivity), revealed there was no particulate antigen detectable in the gradient. It was concluded that this construct was self assembly deficient.

The Gag - Env protein included approximately 43 kDa of the Env domain fused to the carboxy terminus of the Gag precursor. It is currently unknown what size of protein may be fused to the precursor before the protein loses the ability to self assemble. However, some idea can be gained from the self assembly deficient Gag - Pol fusion precursor, synthesized in mammalian cells by recombinant vaccinia viruses (Shioda and Shibuta, 1990). This molecule has a molecular weight of 160 kDa, with approximately 110 kDa derived from the *pol* open reading frame. While the Gag - Env construct was smaller than this, the additional Env protein may still have been sufficient in size to interfere with the assembly process. If this were true, then it may be possible to rescue the assembly defective Gag - Env molecules by co - expression with an assembly competent Gag only precursor, in the same manner as the Gag - Pol fusion

precursor is packaged with the Gag p55 precursor in the authentic HIV infected mammalian cells.

To test this, Sf cells (15 x 10^6 cells / 80 cm^2 flask) were co - infected with both the Gag p46 and the Gag - Env expressing viruses (moi = 10 / recombinant), and harvested at 2 days post infection. Culture medium was recovered and analyzed for evidence of particulate Gag derived antigen by sucrose density gradient centrifugation as described in chapter one. Duplicate western blots were prepared of SDS/PAGE separated samples from the gradient fractions. The first was probed using the three stage anti Gag antibody protocol, while the other, with sheep anti HIV - 1 gp120 antibody (Holmes, 1991, Cat. No. ADP 401 / S 216), followed by anti sheep whole IgG antibody (Sigma Cat. No. B - 7390), and ExtrAvidin alkaline phosphatase conjugate (Sigma Cat. No. E - 2636). Bands were seen on the blot probed with anti Gag antibody of 46 kDa molecular weight (as seen in chapter one, figure 10), that suggested there were particles being formed, however the absence of larger molecular weight bands co - migrating with the 46 kDa band indicated that the Gag - Env fusion protein was not being incorporated into these particles. This was supported by the anti gp120 probed blot where no reactive antigen was detected in the sucrose gradient fractions. As described earlier, the Gag - Env protein exhibited many breakdown products, and this was also the case when cells from the co - infected culture were analyzed. Why this occurred when the Gag precursor alone was not extensively broken down is not known, however, it implicates the Env domain in promotion of the observed degradation. The possibility that the Env domain may affect the processing and localization of the fusion protein within the insect cell cannot be ruled out. Such an occurrence may, in part, explain the inability of the Gag - Env protein to self assemble, as well as being excluded from the

forming Gag particle. The direct effects of the size of the fused Env domain may have had on particle formation were consequently obscured.

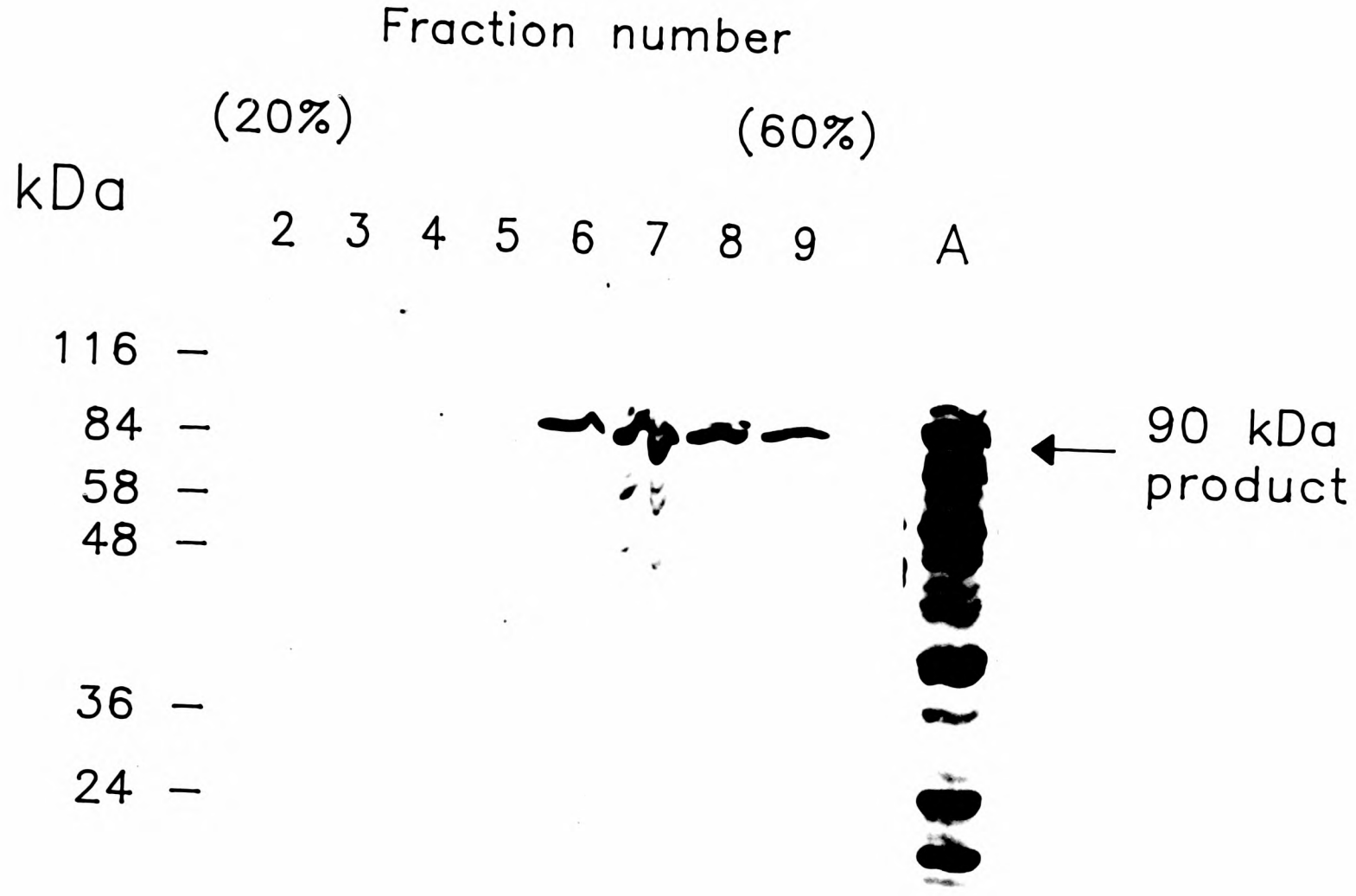
As Gag - Env fusions may represent a "worst case" construct for hybrid particles, an AcNPV recombinant virus expressing a Gag - Pol fusion protein was also examined. The construct kindly provided by Dr. Hughes of The Institute of Virology and Environmental Microbiology, included the entire *gag* and *pol* genes of HIV - 1 with two alterations. The first was a frameshift mutation near the authentic frameshift site (figure 6), which aligned both genes in the same reading frame. The second was a mutation in the region encoding the active site of the viral protease at the amino terminal end of the Pol domain that abolished the proteases activity. The latter alteration was necessary as the HIV - 1 encoded protease was found to be prematurely activated when expressed in this system (Madisen *et al.*, 1987), cleaving the Gag and Pol domains to their subunits and preventing the study of Gag driven precursor assembly. The mutant was included in this investigation to further explore the co - packaging of fusion proteins that include the Gag domain.

When the Gag - Pol fusion protein was assessed for its ability to self assemble into particles using the previously described methods, an unexpected result was obtained, (figure 41), where the construct appeared to be particle assembly competent. A band of antigen reactive to the anti Gag antibody of approximately 90 kDa was observed in fractions 6, 7, 8 and 9 of the sucrose density gradient. This however was reconciled with previously published results on similar constructs (outlined in chapter one), as the fusion protein had a predicted molecular weight of 160 kDa, thus the smaller, self assembly competent, 90 kDa protein must represent a degradation product of the full

Figure 41.

Sucrose density gradient analysis of particle production of the Gag - Pol fusion protein

Western blot of fractions taken from a continuous sucrose density gradient loaded with culture supernatants from Sf cells infected with the HIV Gag - Pol expressing virus. The fractions were loaded onto the SDS/PAGE gel, from the top of the gradient (left side; 20% sucrose), to the bottom of the gradient (right side; 60% sucrose), and probed with monoclonal antibodies to the HIV Gag protein. Gag - Pol infected Sf cell extracts were included on the right side of the diagram (lane A). The 90 kDa Gag - Pol derived product has been indicated by an arrow. On the left side, the molecular weight markers have been indicated (kDa).


Fraction number
(20%)
(60%)
kDa
2 3 4 5 6 7 8 9 A
116 –
84 –
58 –
48 –
36 –
24 –
90 kDa
product

length precursor. Inspection of extracts from infected cells (figure 41; lane A), showed no detectable 160 kDa proteins, but an abundant species of approximately 90 kDa in size. Thus with the loss of 70 kDa of the fused Pol domain, the truncated Gag - Pol fusion precursor regained its self assembly proficiency. Fortuitously, this indicated that a protein of at least 40 kDa in size, can be fused to the Gag precursor before assembly competence is abolished.

The Gag - Pol and Gag p46 recombinant antigens were co - expressed in Sf cells. Cultures of insect cells (16 x 10^6 / 80 cm^2 flask), were co - infected with both recombinant viruses at an moi = 10 / recombinant, and harvested at 2 days post infection. The culture medium was recovered and analyzed for evidence of particle assembly. Duplicate western blots of the density gradient fractions were prepared, the first was probed with anti Gag antibody (three stage protocol), while the other with sheep anti HIV - 1 protease antibody (Holmes, 1991, Cat. No. ADP 413 / S 106), followed by anti sheep biotin conjugate (Sigma Cat. No. B - 7390), and finally ExtrAvidin alkaline phosphatase conjugate (Sigma Cat. No. E - 2636). The results (figure 42), revealed particulate antigen of both 90 and 46 kDa molecular weight in fractions 5, 6, 7, and 8 on the western blot, probed with anti Gag antibody (panel A), and antigen of 90 kDa only in the same fractions on the western blot probed with anti protease antibody (panel B). These observations confirmed that the protease domain is present only in the larger 90 kDa species indicating it is derived from the Gag - Pol fusion precursor and still includes the protease domain (the amino terminal 98 amino acids or 10 kDa of the Pol domain). Evidence for co - packaging was difficult to discern from this result, however there were some differences noted in the sedimentation characteristics of the Gag p46 and Gag - Pol constructs when expressed separately. The Gag p46 antigen, when expressed alone, was distributed through

Figure 42.

Sucrose density gradient analysis of particle production by co - expression of Gag p46 and Gag - Pol fusion proteins

Western blots of fractions taken from a continuous sucrose density gradient loaded with culture supernatants from Sf cells co - infected with the Gag p46 and the HIV Gag - Pol expressing viruses. The fractions were loaded onto the SDS/PAGE gel, from the top of the gradient (left side; 20% sucrose), to the bottom of the gradient (right side; 60% sucrose), and probed with monoclonal antibodies to HIV Gag protein (panel A), and polyclonal antibody to the HIV - 1 protease (panel B). Reactive antigen to the protease antibody (panel B), has been highlighted by an arrow to indicate its position. On the left side, the molecular weight markers are indicated (kDa).

A

Fraction number

(20%) (60%)

kDa 2 3 4 5 6 7 8 9 A B

116 –
84 –
58 –
48 –
36 –
24 –

B

kDa 2 3 4 5 6 7 8 9 A B

116 –
84 –
58 –
48 –
36 –
24 –

the density gradient fractions 4 to 7, reaching a peak in concentration in fractions 5 and 6 (figure 10), while Gag - Pol antigen migrated further through the gradient peaking in fractions 7 and 8 (figure 41), that suggested particles of a higher density. Both the 90 kDa and 46 kDa antigen, produced by co - expression of the constructs in the same cell, reached a peak in concentration in fractions 6 and 7, and furthermore there were equivalent amounts of both Gag p46 and Gag - Pol fusion antigen in each of the sucrose fractions, little in fraction 5, peaking in approximate equimolar amounts in fractions 6 and 7, then tailing off simultaneously in fraction 8. This observation could only be explained if hybrid particles with both the 46 kDa and 90 kDa antigen were being produced. Ideally however, formal demonstration of co - packaging would require the rescue of assembly deficient particles, that would allow more concise conclusions to be drawn from this work.

Incorporation of surface expressed integral membrane antigens into budding Gag particles

The question of whether the budding Gag particle could acquire surface expressed antigen was next considered. Initial evaluation involved determining whether a known abundantly expressed surface glycoprotein could be acquired by the particle. For this experiment, a recombinant baculovirus was used that expresses the rabies glycoprotein G (derived from the CVS strain), to high levels on the surface of the insect cell and for which a stock of monoclonal antibodies was available. The construction and analysis of this recombinant virus, termed AcNPV2, has been described in detail elsewhere (Prehaud *et al.*, 1989). Large cultures of Sf cells (50 x 10^6 cells / 175 cm^2 flask) were co - infected with Gag p46 (moi = 5), and AcNPV2 (moi = 5). Culture medium was recovered at 2

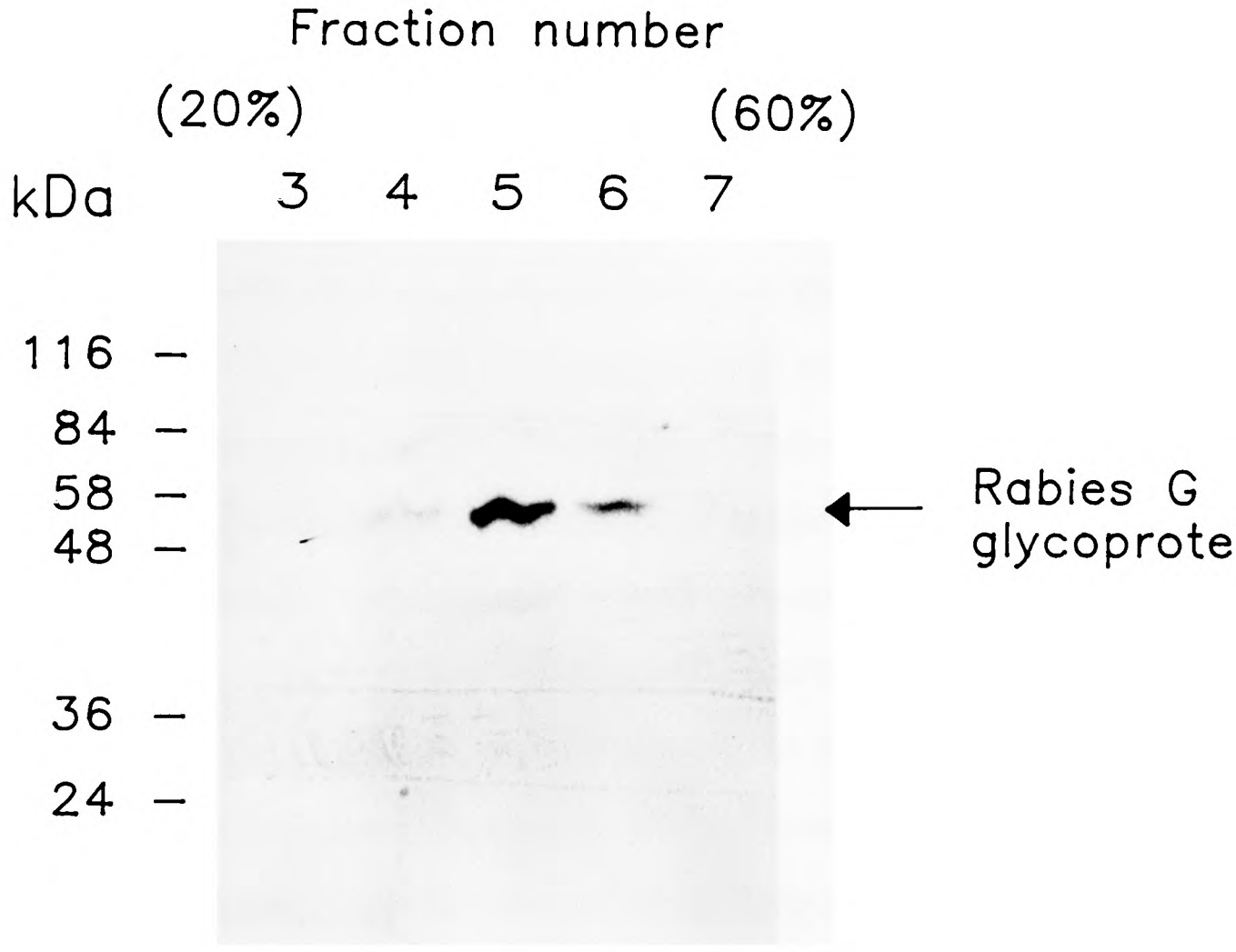
days post infection and analyzed for evidence of particle formation by sucrose density gradient formation, as previously described. Duplicate western blots of the SDS/PAGE separated sucrose gradient fractions were prepared, the first was probed with anti Gag antibodies as above (3 stage protocol), and the second with the monoclonal antibody 5D66 directed against the rabies G glycoprotein (Prehaud *et al.*, 1989), with the 2° and 3° stage antibodies being the same as for the first blot. The anti Gag probed blot revealed antigen of about 46 kDa molecular weight peaking in concentration in fractions 5 and 6 as has been seen previously (figure 10), while the blot probed with the anti rabies G antibody showed a band of approximately 63 kDa in size concentrated in fractions 5 and 6 (figure 43). The observed molecular weight correlated well with the expected molecular weight of the rabies glycoprotein, and its pattern of sedimentation, co - migrating with the particulate Gag antigen, implied that it had been acquired by the particle. Control infections where the rabies G glycoprotein was expressed in Sf cells alone, showed no evidence of extracellular antigen in the sucrose gradient fractions (data not shown).

In addition to the above findings, the ability of the Gag particle to acquire an immunodeficiency virus derived transmembrane glycoprotein when budding from the plasma membrane of the cell was investigated. For this experiment, the recombinant SIV gp42 expressing virus, constructed and analyzed in chapter three, was used. The above procedure was followed except the second of the duplicate western blots was probed with SIV positive monkey serum as described in chapter three. As above, the Gag p46 antigen was seen in the sucrose fractions, however no SIV specific antigen was detected in the gradient. A cross reaction was noted, however, where the SIV positive serum recognized the HIV Gag antigen, a result consistent with previously published work on the

Figure 43.

Sucrose density gradient analysis of particle production by co - expression of the Gag p46 and the rabies G glycoprotein

Western blot of fractions taken from a continuous sucrose density gradient loaded with culture supernatant from Sf cells co - infected with the Gag p46 and the rabies G glycoprotein expressing viruses. The fractions were loaded onto the SDS/PAGE gel, from the top of the gradient (left side; 20% sucrose), to the bottom of the gradient (right side; 60% sucrose), and probed with a monoclonal antibody to the rabies G glycoprotein (CVS strain). Reactive antigen localized to fractions 4, 5 and 6, has been indicated by an arrow. On the left side, the molecular weight markers are indicated (kDa).


Fraction number
(20%)
(60%)
kDa
3
4
5
6
7
116
84
58
48
36
24
Rabies G
glycoprotein

serological cross reactivity of the immunodeficiency viruses (Schneider and Hunsman, 1988). Having established that the SIV gp42 TM glycoprotein was membrane bound (chapter three), the inability of the budding Gag particle to acquire it may be due either to localization to internal membranes or its presence only in levels below that of detection on the surface of the cell. Immunofluorescence experiments to verify this, using the SIV positive serum and an anti monkey FITC conjugate (Nordic Immunology Cat. No. 26 - 1180), yielded inconclusive results. Previously, it was shown that the HIV - 1 gp120 glycoprotein, expressed in insect cells, was efficiently secreted into the culture medium (Morikawa,Y *et al.*, 1990; Wells and Compans, 1990). This suggested that the HIV - 1 Env signal sequence was able to direct the protein through the appropriate secretory pathway and to the plasma membrane of the insect cell. It was possible, therefore, that the signal sequence would promote more efficient plasma membrane targeting of SIV gp42 if fused to the amino terminus of the glycoprotein. The resulting construct, SIV gp42^{SIG}, prepared in chapter three, was used in the following experiment on the basis that the protein may be expressed on the plasma membrane more efficiently than the construct lacking the signal sequence, and thus may be more likely to be acquired by the particle in detectable quantities. A repeat of the above experiment was done, where the SIV gp42^{SIG} expressing virus was substituted for the SIV gp42 construct. However, while similar cross reactivity to the HIV Gag protein was noted, there was no evidence of the TM glycoprotein present in the sucrose gradient fractions. It was concluded from this that, in contrast to the result obtained using rabies G glycoprotein, the Gag particles were not able to acquire detectable levels of the SIV TM glycoprotein in this expression system.

Discussion

In this chapter two methods of loading the Gag particle with additional antigens were investigated. Fusion of a foreign protein onto the carboxy terminus of the Gag precursor was carried out initially, using a portion of the HIV - 1 Env SU glycoprotein, that encoded several antigenic epitopes. However this HIV Gag - Env construct was unable to self assemble into particles. Attempts to rescue assembly by co - expression with Gag precursor only molecules (self assembly competent), were unsuccessful. The fusion protein was found to be highly susceptible to degradation, and consequently high levels of expression were difficult to obtain. The possibility, that the presence of a region of the Env precursor, or signals contained therein, having an influence on expression and transport of the protein, was raised. Further experiments with the Gag - Pol fusion precursor revealed that at least 40 kDa of protein could be fused to the carboxy terminus of the Gag protein and self assembly competence maintained. Co - expression experiments with this construct and the Gag p46 recombinant, gave an indication that co - packaging of the recombinant proteins expressed by baculovirus in insect cells, was possible.

Co - packaging of Gag fusion proteins has been tested using the ß - galactosidase protein fused to the Gag precursor of MoMuLV, and expressed in mammalian cells using the SV40 promoter. The authors found that the fusion protein, when co - expressed with wild type Gag precursor, was incorporated into particles that were made up of both the wild type Gag, and the Gag - ß - galactosidase fusion proteins (Jones,TA *et al.*, 1990). Along similar lines, a technique dubbed "retro - secretion" has been reported, where a protein of interest was fused to the carboxy terminus of the Gag precursor of RSV, and expressed in mammalian

cells as has been described in the general introduction. In the test case, the fusion protein, *iso* - 1 - cytochrome *c* derived from the yeast *Saccharomyces cerevisiae*, was isolated by recovery of the extracellular Gag particles by centrifugation. This exploited the assembly mechanisms of the retroviral Gag precursor to simplify purification of recombinant antigens rather than using the particle as an immunogenic carrier. Furthermore, it was suggested that engineering a known protease cleavage site into the junction between the Gag and fusion domains would further facilitate subsequent purification of the fused recombinant antigen (Wills, 1989; Weldon *et al.*, 1990).

The second method of loading the Gag particle by the expression of an integral membrane protein on the surface of the cell such that it is acquired during budding, was investigated. Initially, the rabies G glycoprotein was used as it was known to be expressed efficiently on the surface of the insect cell using the recombinant baculovirus system. The results have shown that particles acquired the G protein when budding from the co - infected insect cell. A similar experiment was done using the SIV gp42 TM glycoprotein in place of the rabies G protein, however there was no detectable amounts of the SIV antigen on the Gag particle. Examining the possibility that the protein may have been localized to internal membranes and thus not available for incorporation, the SIV gp42^{SIG} protein was used, as it included a signal sequence fused to its amino terminus, that was known to direct the HIV - 1 SU protein through the secretory pathway and into the insect cell culture medium. However the Gag particles in this experiment also remained free of any detectable SIV TM glycoprotein.

The observation that the rabies G glycoprotein could be acquired by the virus like particle, but not the SIV gp42 TM glycoprotein, might be explained in part

by the following hypothesis. First, the SIV gp42 glycoprotein, even if fused to a signal sequence, may still not be efficiently directed to the plasma membrane. Verification of surface expression of the glycoprotein was attempted by immunofluorescence, but yielded inconclusive results, probably because of cross - reaction of the SIV positive serum with Sf cell proteins. A stock of high titre antibodies with a high level of specificity suitable for immunofluorescence studies to the SIV TM protein was not available during the project. A future experiment might include the fusion of a signal sequence derived from either a surface expressed Sf cellular protein or an AcNPV derived glycoprotein, which may confer upon the recombinant protein, a more efficient plasma membrane localization signal. Conversely, the SIV gp42^{SIG} protein may have been expressed efficiently on the surface of the cell, but was excluded from the forming virion. However, the mechanism involved in this proposed exclusion remains obscure, although an interaction between the long cytoplasmic tail of the SIV TM protein and the HIV - 1 Gag precursors assembly mechanism cannot be ruled out. An exclusion interaction of this nature, would be unprecedented, and in the natural host mammalian cell, where the glycoprotein is acquired by the emerging virion (Haffar *et al.*, 1990a), the interaction would have to be either absent or overcome. Although unlikely, given the likelihood of common ancestry, a difference between the SIV and HIV TM glycoproteins and the nature with which they may associate with their respective Gag precursors, that may account for this proposed exclusion interaction, should not be overlooked.

In summary, these experiments demonstrate, that it was possible to use the Gag particle to carry foreign antigens, with incorporation either by fusion to the Gag precursor or by acquisition of a surface expressed glycoprotein at the budding stage. It was apparent, however, that dependant upon the nature of the foreign

protein some difficulties may arise, in particular excessive degradation or inappropriate intracellular targeting may impede production of the particulate carrier. There are two further considerations to be taken into account when developing the retroviral Gag particle as a carrier of immunodominant epitopes. The first of these, specific for production in the AcNPV / Sf cell expression system, concerns contamination by AcNPV virus particles of a preparation destined for an immunization regime. The Gag particles produced by expression of the Gag p55 construct co - migrate in the sucrose density gradient with the AcNPV virus particles, and persistent attempts to remove the AcNPV, were not able to achieve homogeneity (D. Gheysen, personal communication). Production in an alternative protein expression system maybe one method for alleviation of this problem. The second consideration involves the hazard of inadvertent introduction of foreign nucleic acid, packaged within the virus particle, into the host by vaccination. The use of the Gag p42 construct described in chapter one, may overcome both of these problems. Having a lighter density, may facilitate purification of the Gag particle to homogeneity, while the absence of the Cys - His motifs, known to bind nucleic acid, would greatly reduce the ability of the particle to encapsidate nucleic acid, alleviating the problem of subsequent introduction into the vaccinee. While the experiments in this chapter have been conducted using the Gag p46 construct as a model, it is likely that similar results would be obtained using the Gag p42 construct, which is both self assembly competent and buds from the plasma membrane of the cell.

Successful development of immunodominant antigen loaded Gag particles may constitute a safe and easily produced vaccine candidate capable of eliciting a broad range immune response against viral proteins. However, it remains to be

determined which epitopes or combination of epitopes, derived from the immunodeficiency virus, should be incorporated into an immunization regime, for creation of a protective immune response.

General Discussion

The baculovirus expression system has been used throughout this project to produce recombinant proteins under the control of baculovirus derived promoters in *Spodoptera frugiperda* insect cells. Molecular cloning techniques coupled with the ability to introduce specific mutations into the DNA coding sequence allowed detailed analysis of the roles of various domains within functional proteins of HIV and SIV. Initially the project was directed at understanding the mechanism of assembly of the Gag precursor protein derived from HIV - 1, by deletion mutagenesis. The protein was progressively truncated from the carboxy terminus and each mutant assessed for its ability to self assemble into particles. This was followed by an analysis of the role of the SIV TM glycoprotein cytoplasmic tail, and finally some practical uses of the HIV Gag particle were explored, where the assembly of immature core like particles bearing co - expressed foreign antigens was demonstrated.

Delineation of the carboxy terminal boundary of an HIV - 1 Gag assembly domain was established. The observations made in chapter one by electron microscopy of the insect cells expressing the mutant Gag proteins also lead to the proposal of separate oligomerization and circularization domains. The Gag protein was still observed to form long oligomeric structures under the surface of the plasma membrane in particle assembly deficient mutants. When this construct included the next 17 amino acid residues, ATIMMQRGNFRNQRKMV in single letter amino acid code, at its carboxy terminus, the precursor was found to circularize efficiently into discrete particles.

However, the particles were larger and less uniform in morphology than the particles produced by expression of the full length precursor. This implied, that although the region downstream of the amino terminal boundary of the proximal Cys - His motif was not required for particle assembly, it could be involved in condensation and stabilization of the particle. Further work in this area using deletion mutagenesis may allow the complete delineation of Gag assembly domain boundaries, which in turn would lead to a more comprehensive understanding of the mechanism by which the precursor associates with itself to promote assembly.

There are regions within the Gag protein that have roles which are distinct from virion assembly. One of these regions, that included most of the nucleocapsid protein, was shown to play an important part in the binding of RNA that contained the *cis* - acting encapsidation signal. It was found that the binding of this particular RNA was dependant upon the presence of at least one Cys - His motif, while the presence of two motifs increased the efficiency of binding dramatically. Analysis of the particle self assembly proficiency of these RNA binding deficient mutants revealed that the presence of the RNA binding regions were unnecessary for assembly of immature core like particles.

The presence of other domains within the Gag protein that could function during the early stages of infection have also been suggested. Within the capsid region p24, that makes up the central conical core structure of the virus particle, it is possible that determinants are exposed following cleavage of the region from the precursor. These determinants would, in this model, play a role in events following fusion of the virion with the plasma membrane early in the replication cycle, including the promotion of uncoating, reverse transcription and

translocation of the genome to the host cell nucleus. However such roles for these putative domains have yet to be established. This model, if supported by experimental data, would help also to explain why the virion, in its immature state, is non - infectious, as these determinants would remain buried within the Gag precursor.

The evaluation of phenotypic changes, as a result of genetic alteration, can give rise to useful experimental data on proteins that function independently of other factors. However, careful choice of protein expression systems should be made when wishing to analyze protein functions that involve an interaction with other factors. The baculovirus / insect cell expression system was highly efficient in production of large quantities of recombinant Gag protein, and was well suited for the study of HIV - 1 Gag precursor assembly, as the protein interacts with itself to form particles. However, it was noted that phenotypic effects, resulting from genetic mutation of domains that are suspected to interact with additional factors, may differ between insect cells and those of the natural host. This was particularly apparent, when a report outlined the restriction of particle release from mammalian cells expressing a mutant where the carboxy terminal p6 domain of the Gag precursor was deleted (Gottlinger *et al.*, 1991). In insect cells, there was no such retention observed at the plasma membrane, when the p6 domain was removed. Additionally, in the cited experiment, all other HIV coding sequences were present in the mammalian cell, thus reconciliation of these observations would lead to the proposal that the p6 domain of the Gag precursor has some interaction with other virally derived factors, or with factors present in mammalian cells, but absent in the Sf insect cells that mediate particle retention. The high levels of expression of the protein in insect cells, may also account for these discrepancies where a putative retention mechanism could be

overridden or swamped by the amount of protein. It is noteworthy that no equivalent domains to the p6 region have been found in Gag proteins from other members of the retroviridae. An open reading frame, located in the equivalent position, in the Mason - Pfizer monkey virus *gag* gene has been documented, however, when this domain was deleted, no effect was observed on either particle assembly or release (Wills and Craven, 1991). In another study, Klimkait and colleagues found that if the *vpu* gene was deleted from the HIV - 1 genome, particles were observed to assemble, but release from the cell surface was significantly reduced (Klimkait *et al.*, 1990). While this may be circumstantial evidence, the possibility was raised for an interaction between the p6 domain and the Vpu protein as they are both involved in the bud release mechanism. In the light of this example, where functions of proteins that may include interaction with host cellular or viral derived proteins are investigated, then analysis within the natural system would be recommended.

In chapter three, an investigation into the role of the cytoplasmic tail of the SIV transmembrane protein was undertaken. Previous reports have established that the presence of the SIV TM protein cytoplasmic tail conferred a growth disadvantage for certain strains of SIV, when propagated in certain human cell lines (Hirsch,VM *et al.*, 1989a; Kodama *et al.*, 1989, 1990). This investigation examined the role of the cytoplasmic tail in the location and glycosylation of the molecule. A full length TM glycoprotein was expressed, characterized and compared to a mutant, where the cytoplasmic tail of the integral membrane protein had been deleted. The data suggested that the presence of the cytoplasmic tail allowed glycosylation of protein and its association with the lipid membrane. It was found that removal of the tail, lead to the production of a non glycosylated protein that was largely soluble and displayed little or no association

with the membrane fraction of the cell, despite retaining the putative hydrophobic membrane spanning region. The role of the tail was discussed in the light of data gained from experiments on the related HIV - 1 transmembrane glycoprotein, together with the significance it may have in intracellular processing and trafficking.

Some potential practical uses of the Gag particles were investigated in chapter four. It was demonstrated that co - expression of Gag only and Gag fusion precursors was possible, with the incorporation of both proteins into the immature core like particles. There was a limitation on the size of the fusion domain demonstrated by Shioda and colleagues, where the Gag - Pol fusion protein was found to be unable to self assemble (Shioda and Shibuta, 1990). The fused Pol domain in this case was approximately 120 kDa in molecular weight. Data gained from experiments in chapter four, revealed that a fusion domain of approximately 45 kDa still allowed the Gag fusion protein to maintain self assembly proficiency. Thus, it was concluded that a Gag fusion protein could be at least 90 kDa in total molecular weight, where the Gag portion makes up approximately 45 kDa, before its size may interfere with the assembly process. Larger self assembly proficient fusion proteins may be possible, however the maximum size has not yet been determined. Other constraints were also evident, when constructing assembly proficient Gag fusion proteins. In the case of the HIV Gag - Env construct, the fusion domain itself was found to affect the ability of the Gag domain to assemble into particles, despite the size of the fused domain being less than 45 kDa. The mechanism of this disruption, mediated by the fused domain, was thought to be either by promotion of degradation of the fusion protein or by interference with its intracellular processing and targeting. It was proposed that sequences may have been present within the fusion domain

that were susceptible to proteolytic cleavage, or interacted with intracellular processing and trafficking mechanisms. Additionally, the possibility that the fusion domain caused a destabilization of the native state of the Gag protein, coupled with a conformational change and disruption of the required assembly domains could not be ruled out. Furthermore, a conformational change may give rise to increased access of cellular proteases, promoting the degradation of the molecule.

Baculovirus expressed Gag particles were also shown to be able to acquire co - expressed glycoproteins that were targeted to the cell surface. The efficiently expressed rabies G glycoprotein was found to be present on Gag particles, that had budded from the plasma membrane of the insect cell. Demonstration of the acquisition of SIV derived glycoproteins was also attempted, but was unsuccessful. The experiments suggested that both the efficient and abundant expression of the glycoprotein at the plasma membrane of the cell was necessary for detectable levels of the glycoprotein to be acquired by the particles. The failure to demonstrate SIV glycoprotein acquisition was thought to be due to expression levels being below the threshold of detection with the available antiserum.

The future exploitation of this system may include the multiple expression of Gag and Gag fusion domains to produce particles that bud from the plasma membrane and acquire a cell surface targeted glycoprotein. With the use of dual and triple expression vectors, recombinant baculoviruses could be generated that produce approximately equimolar amounts of 2 or 3 recombinant proteins within a single insect cell. This would overcome the problems associated with co - expression by co - infection, where a cell may only become infected with a single

recombinant virus. These multiple expression systems, may, in the longer term, allow the co - expression of several different types of Gag precursor, together with several types of glycoprotein, further increasing the range of epitopes incorporated into the virus like particle. This would be particularly appropriate for the development of a vaccine candidate directed against infection by HIV, where a polyvalent immune response is desirable for protection against the numerous forms of the virus.

Making use of the Gag assembly proficient Gag p42 recombinant, described in chapter one, that has both the RNA binding Cys - His motifs deleted, would add an additional factor of safety. The particles would be unable to specifically encapsidate RNA, during the assembly process, thus the likelihood of introduction of foreign genetic material into the host would be greatly reduced.

In summary, this project has been primarily directed at elucidating the mechanism of particle assembly by the HIV Gag protein. The presence of separate functional domains for assembly and RNA encapsidation within the precursor has been shown. The possibility of additional domains within the precursor that promote uncoating, reverse transcription and nuclear localization, were discussed. Further phenotypic analysis of mutants, with internal deletions in the *gag* gene, may yield data on the location and roles of these proposed domains. Ultimately, a detailed understanding of the functions of the precursor, may eventually facilitate the design of novel "molecular monkey wrenches" that could be tossed into the system to thwart virion formation. Finally, some practical applications of the Gag particle as potential immunogens were investigated. Loading of the Gag particle with additional antigens was demonstrated, that may allow the design of a multiple epitope particulate

vaccine. The potential of this non - infectious particulate carrier in the development of a candidate vaccine against HIV infection was discussed.

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