Characterisation of a block to HIV-1 infection in rabbit cells as a model to study HIV-1 trafficking

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UMI Number: U593648

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Dissertation Publishing

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Abstract

This thesis focuses mainly on the analysis of the restriction to HIV-1 infection using rabbit cells as a model. Rabbit cells are poorly permissive to HIV-1 infection and the nature of this block is not well understood. This work shows that the restriction is independent of the cell receptor used by the virus for entry, as shown by infection of cells with HIV-1 pseudotyped with different types of envelopes and that it occurs mainly at the level of reverse transcription. It cannot be effectively saturated with high doses of virus or virus-like particles and has a recessive phenotype in human-rabbit heterokaryons. These results point to the existence of a factor required for HIV-1 infection that is absent in SIRC cells but can be complemented by human cells. The reverse transcription complexes extracted from human and rabbit cells have been analysed biochemically and found to have different densities but to be competent for reverse transcription in both cases in an in vitro endogenous assay. Cell fractionation of infected cells showed that HIV-1 is trafficked in a different way in human and rabbit cells and that correct intracellular trafficking is related to efficient reverse transcription and high infectivity in vivo. It is shown as well that viral DNA accumulates in rabbit cell nuclei only at a later stage of infection and fails to associate with chromatin, suggesting a further block prior to integration in SIRC cells. Finally, chimeric viruses are used to determine the viral components responsible for the block. Viral chimeras formed by HIV-1 and SIV or MLV are used to infect the human cell line HeLa and SIRC cells. It is found that HIV-1 capsid is the determinant of the block in SIRC cells. Our data point to the existence of cellular factors regulating the early stages of intracytoplasmic and possibly intranuclear HIV-1 trafficking.

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Part 1

Abbreviations

ADC	AIDS-defiining cancers
AGM	African green monkey
AIDS	Acquired immunodeficiency syndrome
AIP1	Actin-interacting protein 1
AP-1	Activating protein-1
ARD	AIDS-related dementia
AZT	Zidovudine
BAF	Barrier to auto integration factor
Bp	Base pairs
Brd4	Bromodomain-containing protein 4
CA	Capsid protein
CCR5	Chemokine receptor 5
CDK9	Cycline-dependent kinase 9
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
CNS	Central nervous system
CXCR-4	Cysteine/no cysteine/cysteine containing region receptor 4
COUP	Chicken ovalbumin upstream promoter
cPPT	Central polypurine tract
CRM1	Chromosome maintenance region 1 protein
CSA	Cyclosporine A
CypA	Cyclophilin A
DC	Dendritic cells
DIS	Dimer initiation signal
DNA	Deoxynucleic acid
ECF	Enhanced chemiluminescence
eGFP	Enhanced green fluorescent protein
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assays
Env	Envelope protein
Erk-1	Extracellular signal-regulated kinase 1
ERT	Endogenous reverse transcription
ESCRT	Endosomal sorting complex required for transport

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FACS	Fluorescence activated cell scanning
FDA	Food and Drug Administration
Fv1	Friend virus susceptibility factor 1
FDA	Food and drug administration
GL	Gag leader
GM-CSF	Granulocyte macrophage colony stimulating factor
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HFV	Human foamy virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HMGA1	High mobility group protein A1
hRIP	Human Rev-interacting protein
Hrs	Hepatocyte growth factor receptor substrate
HRV14	Human rhinovirus 14
HSV	Herpesvirus
HTLV-I	Human T lymphotropic virus type I
HTLV-II	Human T lymphotropic virus type II
IL-1	Interleukin type 1
IN	Integrase
INI1	Integrase interactor 1
L	Late domain
LAP-2	Lamina-associated protein 2
LEDGF/p75	Lens epithelium derived growth factor, p75
LP	Long progressors
LPS	Lipopolysaccharide
LTNP	Long term non-progressors
LTR	Long terminal repeat
Lv1	Lentivirus susceptibility factor 1
LysRS	Lysyl tRNA synthetase
MA	Matrix protein
MCMD	Minor cognitive motor disorder
MHC I	Major histocompatibility complex type I
MHC II	Major histocompatibility complex type II
MIP-1β	Macrophage inflammatory protein beta

MLV	Murine leukaemia virus
MMTV	Mouse mammary tumor virus
MoMLV	Moloney murine leukemia virus
M-PMV	Mason-Pfizer monkey virus
MRI	Modulator of retrovirus infection
MVB	Multivesicular bodies
mRNA	Messenger RNA
MTCT	Mother-to-child transmission
NAT	Nucleic amplification techniques
NC	Nucleocapsid protein
Nef	Negative factor
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NRE	Nuclear resonance element
NRTI	Nucleoside analogues reverse transcriptase inhibitors
NNRTI	Non-nucleoside analogues reverse transcriptase inhibitors
OMK	Owl monkey kidney cells
ORF	Open reading frame
PAK2	p21 (CDKN1A)-activated kinase 2
PBMC	Peripheral blood mononuclear cells
PBS	Primer binding site
PCR	Polymerase chain reaction
PI	Protease inhibitors
PIC	Preintegration complex
PML	Promyelocytic leukaemia
PPT	Polypurine tract
PR	Protease
P-TEFb	Positive transcription elongation factor b
Pts	*Pan troglodytes schweinfurthii*
Ptt	*Pan troglodytes troglodytes*
PVDF	Polyvinylidene difluoride
R	Repeat region
Ref	Resistance factor 1

Rev	Regulator of virion protein
RNA	Ribonucleic acid
RP	Rapid progressors
RRE	Rev responsive element
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RTC	Reverse transcription complex
RxRE	Rex responsive element
SFV	Semliki Forest virus
SIV	Simian immunodeficiency virus
SIVcpz	SIV from chimpanzee
SM	Sooty mangabey
SP	Spacer peptide
SS DNA	Strong-stop DNA
SSFV	Spleen focus-forming virus
STD	Sexually transmitted disease
SV40	Simian virus 40
TAR	Transactivation response
Tat	Transactivating protein
TCR/CD3	T cell receptor/CD3 complex
TNFα	Tumor necrosis factor alpha
tRNA	Transference RNA
TSG101	Tumor susceptibility gene 101
U3	Unique 3
U5	Unique 5
USF	Upstream stimulatory factor
V	Variable region
Vif	Viral infectivity factor
Vpr	Viral protein R
Vps	Vacuolar protein sorting
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus G-protein
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
ZAP	Zinc finger antiviral protein

Acknowledgements

I would like to thank my supervisor Ari Fassati for always being enthusiastic about my work and for his advice and patience during my PhD. Thank you for supporting me in all the things I have done in the last few years, both in the lab and elsewhere.

I would also like to thank Robin Weiss for his support throughout all the years I spent in the Wohl.

Thank you to all the people in the Wohl Virion Center, for being my friends, not only my colleagues and for everything that I learned from each and every one of you. Thank you Keith for your great patience, for going out of your way to help me out and for making me laugh. Thank you Aine for your interest professionally and also in my personal life. Thank you Greg for your support and useful discussions about my work. Thank you Anna and Luciano, for making the last years of my PhD a memorable time, for making me feel at home in the lab.

Thank you to my family that has always been beside me since I came to London, being as close as if I had never left. To my sister for sharing with me the good and bad moments of this PhD always believing I could do it. To my mother for her faith in me.

Thank you Darius, for being the best example of strength, hard work, and discipline for me to follow during our PhDs. Most of all, thank you for your infinite love and help in every step of the way. I am very lucky to share my life with you.

Dedicated to my son, a dream come true.

Chapter 1. Introduction

1. Overview

1.a The AIDS epidemic

Twenty-five years have passed since the report of some cases of *Pneumocystis carinii* pneumonia in homosexual men was published by the Mortality and Morbidity Weekly Report from the Centers for Disease Control in Atlanta, USA. This was followed a few months later, by the publication of a report in the New England Journal of Medicine of four homosexual patients with *Pneumocystis carinii* pneumonia and mucosal candidiasis. All these patients had fever, were infected with cytomegalovirus (CMV), presented lymphopenia and inversion of the T cell helper to T cytotoxic ratio which they proposed might be the result to repeated exposure to CMV (Gottlieb et al., 1981). In the same issue of the journal there was another report of eleven cases of homosexual patients, some of which were drug-abusers, with *Pneumocystis carinii* pneumonia and reduced total lymphocyte count, T-cell numbers and lymphocyte proliferation. The patients were studied between 1979 and 1981 and 8 of them died, with the remaining 3 left with no diagnosis despite intense study (Masur et al., 1981). This was followed by a series of reports on the newly described acquired immunodeficiency syndrome, linked to the homosexual community and characterized by a depressed cellular immune response, the presence of opportunistic infections and Kaposi's sarcoma (Durack, 1981; Follansbee et al., 1982; Gerstoft et al., 1982; Mildvan et al., 1982; Siegal et al., 1981). Because a decrease in the numbers of CD4+T cells was present in all of the patients, an agent that targeted these cells was a likely cause. The human T lymphotropic virus type 1 (HTLV-I) was a good candidate as not only had such a tropism, but also was transmitted by the same routes as the causing of AIDS was being transmitted according to the epidemiologic data: sexual, parenteral and materno-fetal routes. In May 1983, Luc Montagnier and his group published a paper on the isolation of a T-lymphotropic retrovirus that belonged to the human T cell leukemia viruses group but was very different to HTLV-I and HTLV-II. They isolated it from a 33 year old patient, homosexual, with cervical lymphadenopathy and asthenia. A biopsy of one of his cervical lymph nodes was

taken and cells from it were cultivated. Reverse transcriptase (RT) activity was detected in the supernatant after 15 days of culture, it could be propagated on normal lymphocytes and Montagnier and co-workers identified it as a retrovirus by its density and by electron microscopy. They distinguished it from HTLV-I and HTLV-II as anti-p19 and anti-p25 antibodies did not react with the patient's cells in culture but the patient's serum did recognize HTLV-I infected cells suggesting a common antigen between these two retroviruses. However, because this virus could not be propagated in permanent T-cell lines, its full characterization was not possible. They concluded that the retrovirus from this patient was a new human retrovirus that belonged to the HTLV family as it contained a similar p25 protein to that of HTLV-I, they later called it lymphadenopathy-associated virus or LAV. However, its role in the acquired immunodeficiency syndrome (AIDS) pathogenesis was still unclear (Barre-Sinoussi et al., 1983). In the same issue of Science, Robert Gallo and his group reported the identification of HTLV proviral DNA in two out of 33 patients with AIDS. They concluded that there might be an etiologic role for this retrovirus in AIDS but that their methods were not sensitive enough to detect it in all patients; they did not consider the possibility of infection by two different viruses, HTLV-I and HTLV-III (Gelmann et al., 1983). During 1984 and 1985, Gallo and his group described many different isolates of this new virus which they called HTLV-III (Gallo et al., 1984; Groopman et al., 1984; Shaw et al., 1984; Zagury et al., 1984), methods for continuous culture in T cell lines (Popovic et al., 1984) and characterized some of its main proteins (Sarngadharan et al., 1984; Schupbach et al., 1984), supporting its causative role in AIDS (Gallo and Montagnier, 2003; Hahn et al., 1984). All of the research led to the production of a blood test that became available for the human immunodeficiency virus (HIV) in blood-transfusion centers (Safai et al., 1984). During these years, the complete genome of HIV was published and its genes were defined (Ratner et al., 1985; Sanchez-Pescador et al., 1985; Starcich et al., 1986; Wain-Hobson et al., 1985b). Importantly, the virus variability was identified (Hahn et al., 1985; Wong-Staal et al., 1985) and its presence in semen (Zagury et al., 1984) and in the central nervous system (CNS) (Shaw et al., 1985) was determined. In 1986, both names, LAV and HTLV-III were dropped and the name HIV was adopted (Coffin et al., 1986). All these findings, along with the discovery of the virus receptor and co receptors, the CD4, CCR5 and CXCR4 molecules (Deng et al., 1996; Feng et al., 1996; Klatzmann et al., 1984; Weiss et

al., 1986) and the discovery of related simian retroviruses (Daniel et al., 1985) were among the most important advances done during those years (Gallo, 2002a; Gallo, 2002b; Gallo and Montagnier, 2003; Montagnier, 2002).

The identification of HIV made it possible to eliminate HIV contaminated blood from blood banks, to create prevention policies and the production of antiviral compounds, the first of which was zidovudine (AZT) (Mitsuya et al., 1985).

In the last twenty five years, great advances have been made in the field of AIDS research: the virus lifecycle is better understood, there are many different anti-virals that can extend the life of HIV-1 positive patients allowing them to lead almost normal lives. However, at the end of 2005, approximately 38.6 million people were living with HIV worldwide, about 4.1 million became newly infected and around 2.8 million people died because of AIDS according to the Report on the Global AIDS epidemic: Executive summary of UNAIDS in 2006. Africa remains as the centre of the pandemic and many countries in it show no evidence of decline in the incidence of AIDS, such as South Africa, Botswana, Namibia and Swaziland where up to 33% of adults are infected with HIV. During all these years, in the words of Kofi Annan, Secretary-General of the United Nations, AIDS has orphaned millions of children, exacerbated poverty and hunger and in some places, reversed human development altogether: what was first a mystery illness, now poses among the greatest threats to the world's progress in the 21st century.

The response to AIDS will require economical, political, social and scientific efforts to join for the next decades as the toll of the epidemic has not yet been felt in its entirety. For it to be successful, every aspect needs to be sustained in the long term: the development of new medicines and preventive technologies amongst them but without forgetting the fundamental drivers of this pandemic which are gender inequality, poverty and discrimination (Peter Piot, Executive Director of the Joint United Nations Programme on HIV/AIDS).

1.b Pathogenesis of AIDS

1.b.i Cellular tropism of HIV-1

Although multiple cell types have been found to be infected by HIV-1 *in vitro*, only CD4+ T lymphocytes and cells from the macrophage lineage are consistently infected *in vivo*. This cell tropism and its mode of transmission were the reason for HIV-1 to be called HTLV-III by some when it was first found. Both HTLV-I and HTLV-II target T lymphocytes and are transmitted from mother to child, through blood or sexual or contact, just as HIV-1. HTLV-I was isolated in 1980 from a patient with a cutaneous T-cell lymphoma (Poiesz et al., 1980). It was then described in a T-cell line from a patient with adult cell leukemia, endemic in Southwestern Japan (Hinuma et al., 1981). The second HTLV virus, HTLV-II, was isolated in 1982 from a patient with benign hairy cell leukemia (Kalyanaraman et al., 1982). However, although the mode of transmission of HTLV is similar to HIV, the clinical manifestations are different. While HIV-1 causes immunosuppresion, HTLV-I is associated with adult T-cell leukemia and other immune-mediated disorders including neurological disease such as HTLV-associated myelopathy/tropical spastic paraparesis (Uchiyama et al., 1977), HTLV-II is not so pathogenic and only in some infected individuals it has been associated with disease, like hairy cell leukemia (Feuer and Green 2005). This is reflection of the effect that these viruses have on their target cell: HIV-1 kills CD4+ T cells and HTLV-1 and HTLV-2 expand them by inducing their proliferation.

The main receptor for HIV-1, HIV-2 and all SIV is CD4, present on the surface of CD4+ T cells, cells of the monocyte/macrophage lineage and some other cells. Besides CD4, some chemokine receptors act as co receptors for HIV. The most important co receptors are CCR5 and CXCR4. The first is the receptor of MIP-1 α and β and RANTES and the second is the receptor of SDF-1 (Bleul et al., 1996; Alkhatib et al., 1996). All HIV-1 strains can use either of these co receptors to enter CD4+ cells and are called R5 or X4 viruses although some dual tropism strains have been described and are called R5X4 viruses (Collman et al., 2000; Yi et al., 1999). In most cases, the transmitted strains are R5 and these persist throughout infeciton. The viruses that use CXCR4 as a co receptor appear later

and are present in about 50% of patients (Clapham and McKnight, 2002). R5 viruses were formerly known as nonsyncitium-inducing or M-tropic and X4 viruses as syncitium-inducing or T-tropic (Tersmette et al., 1988; Gartner et al., 1986; Alkhatib et al., 1996). In the blood of HIV-1 infected individuals, the main cell population infected are the lymphocytes although there are infected macrophages as well. The emergence of X4 strains has been associated with the decline in CD4 cell counts (Penn et al., 1999) and the appearance of clinical symptoms (Koot et al., 1993; Richman and Bozzette, 1994). This can be attributed to higher cytopathicity (Fouchier et al., 1996), to the larger pool of cells that can be infected (Blaak et al., 2000; Grivel and Margolis, 1999; Ostrowski et al., 1999) or to the greater thymic depletion that X4 HIV-1 strains cause (Berkowitz et al., 1998; Berkowitz et al., 2000). Furthermore, this switch from R5 to X4 lies in the variable (V) region V2 of the gp120 protein. Additional amino acids in the V2 region have been observed in the circulating strains of individuals who have a rapid progression of the disease (RP) as compared with those of patients with a slow (long progressors, LP) or non-progressive disease (long term non-progressors, LTNP), pointing out a link between the V2 region, the switch to X4 tropism and the appearance of clinical symptoms (Masciotra et al., 2002). Other studies have found that it is the V3 region that determines these two phenotypes, but that the V1, V2 and V5 regions of gp120 of strains with dual tropism are also different from R5 and X4 strains (Ghaffari et al., 2005) and work in rhesus macaque models has shown that co receptor usage, determined by V3, is central in cell and tissue tropism and in CD4+ T cell depletion *in vivo* (Ghaffari et al., 2005).

Primary infection of HIV is associated with an acute retroviral syndrome associated with high-titer HIV-1 replication and a robust immunologic response (Clark et al., 1991; Daar et al., 1991). Forty to ninety percent of acutely HIV-1-infected patients present symptoms. The symptoms which can include fever, myalgia, rash, sore throat, arthritis, and lymphadenopathy resolve quickly (Daar, 1998) and the viraemia declines reaching a set point at around 6 months. The intensity of this syndrome and the viraemia levels at set point are predictive of the clinical course that the disease will have in each individual (Goedert et al., 1989; O'Brien et al., 1996; Pedersen et al., 1997). However, this syndrome is often undiagnosed or misdiagnosed because HIV-1 antibodies are usually not

detectable in these early stages of infection. It is still controversial if treatment during this primary infection is beneficial as it has been shown that it can diminish the establishment of cellular reservoirs of HIV-1 (Strain et al., 2005). The immune response to HIV infection has also been reported to be prognostic of disease progression independent of viraemia (Pantaleo et al., 1997). During the first 2-8 weeks post infection there is a significant decrease in the number of CD4+ T lymphocytes in the blood which can go back to normal when the patient enters the clinical latency stage, but seldomly goes back to the preinfection levels. During this acute phase there is a peak of viral replication in which viral proteins and infectious virus can be detected in blood and CNS fluid by quantification of CA p24 protein or viral RNA (Piatak et al., 1993). Specific cellular and humoral immune responses can be detected 3- weeks after infection which cause a fall in plasma viraemia and antigenemia and resolution of the clinical symptoms (Cooper, Imrie and Penny, 1987). The cell-mediated immune response consists mainly of cytotoxic T lymphocytes and eliminates virus-expressing cells (Koup et al., 1994). The humoral immune response consists of different HIV protein-specific antibodies that are trapped along with the virus in the reticulo-endothelial system as immune complexes (Heath et al., 1995; Schrager and Fauci, 1995). It is possible that both types of immune responses contribute to the transition from the acute to the chronic stage of HIV-1 infection.

It is during the initial stage that the lymphoid tissue and the CNS are infected. Along with the increase of viral titers there is an abrupt decline of CD4+ T cells in peripheral blood, in the lymphoid tissues and in the gastrointestinal tract (Brenchley et al., 2004; Guadalupe et al., 2003). In fact, most of the CD4+ T cell depletion occurs in the gastrointestinal tract breaking down the mucosal immunity (Mattapallil et al., 2005; Brenchley et al., 2004; Mehandru et al., 2004). This fall in CD4+ T cell counts could to be due to HIV-1 cytopathicity and its mechanism is still controversial, it has been reported to be caspase-independent (Bolton et al., 2002; Petit et al., 2002) by a necrotic mechanism (Lenardo et al., 2002) and the viral proteins Vif and Vpr have been reported to be responsible to induce the G(2) cell cycle arrest and death of CD4+T cells (Sakai et al., 2006; Somasundaran et al., 2002). However, there are also reports of a caspase 3 and interferon-dependent, apoptotic mechanism (Herbeuval et al., 2005) used by HIV-1 to deplete CD4+ T cells. On the other hand, HIV infects also bone marrow

progenitor cells, developing thymocytes and thymic stromal cells which could affect the ability of the immune system to regenerate itself (Dion et al., 2004; Hazra and Mackall, 2005). Chronic immune activation is also responsible for decreasing CD4+ T cell counts as proliferation and activation-induced cell death of memory T cells would exhaust naïve T cell pools (Grossman et al., 2002). In simian immunodeficiency virus (SIV) infection, recent studies reported that there is a massive infection of memory CD4+ T cells in mucosal tissues, lymph nodes and peripheral blood of macaques which causes their death within approximately 4 days; this occurs during the acute phase of infection, decreasing afterwards (Li et al., 2005; Mattapallil et al., 2005). Chronic immune activation is a better predictor of progression to AIDS than the viral load (Giorgi et al., 1999). As it was mentioned above, in the acute stage of HIV-1 infection, CD4+ T cell depletion and damage to mucosal immunity occurs primarily in the gastrointestinal tract and this causes chronic immune activation by increased translocation of luminal microbial products. A study measuring lipopolysaccharide (LPS) as an indicator of microbial translocation found that it was increased in HIV-1 infected individuals with progressive disease. Elevated LPS plasma levels correlated with immune activation and decreased with HAART treatment (Brenchley et al., 2006). Recent work also showed that, as opposed to what Nef protein from most primate lentiviruses and HIV-2 does, HIV-1 Nef protein in HIV-1 fails to downmodulate TCR-CD3 in infected T cells and to inhibit cell death. This function might have been lost during evolution in HIV-1's precursor and might be one of the causes for the large immune activation observed in HIV-1 infection (Schindler et al., 2006).

After the immune response to HIV appears, there is a long period with mild or no clinical manifestations but with a steady decline in the CD4+ T lymphocytes. Even in the absence of symptoms, a diagnosis of AIDS is done when the CD4+ T cell counts fall below 200 per microliter. Although the individual is asymptomatic (what is called clinical latency), there is active viral replication as demonstrated by the presence of HIV RNA in the lymph nodes, strong antibody responses for years and detection of virus from the blood of most infected individuals at all stages of infection (Piatak et al., 1993). However, the virus can establish latent infection at the cellular level, defined as a state of non-productive infection where no virion is released although, in the case of HIV-1, a small

amount of mRNA is produced (Hermankova et al., 2003). This mRNA however, does not lead to viral protein production as it mislocalized or prematurely terminated (Pomerantz et al., 1990; Malim and Cullen, 1991; Lassen et al., 2006). HIV-1 cellular latency is the result of the normal transition to memory cells that some CD4+ T cells suffer after encountering an antigen: but it is reversible as the cell can produce virus if exposed to the same antigen or iin the presence of cytokines. This has been detected *in vivo* in resting CD4+ T cells although with a low frequency (1 in 10^6 resting CD4+ cells) (Chun et al., 1995, 1997). These latently infected cells persist even when HIV-1 replication is inhibited by HAART (Finzi et al., 1997; Wong et al., 1997) constituting the main viral reservoir (Finzi et al., 1999; Siliciano et al., 2003). This reservoir is established during acute infection (Chun et al., 1998; Finzi et al., 1997).

However, in an individual, the level of viral replication is constant with only a gradual increase during the course of infection probably as a result of equilibrium between the new infections and death of infected cells during this clinical latency phase (Coffin 1995). There are two explanations for this balance between production and clearance of infected cells: one is that the immune response controls the virus but cannot clear it completely. The second explanation is that HIV replication is limited by the number of target cells available that is, activated CD4+ lymphocytes (Klenerman et al., 1996). This balance eventually disappears and leads to high viral loads and a fall in CD4+ lymphocytes counts below 500 cells/μl that defines AIDS (normal range is 800-1000 cells/μl). The typical pattern of CD4+ T cell loss in an HIV-infected individual starts with a steep decline in the acute phase and is followed by an average loos of about 60 CD4+ T cells / μl every year (Lang et al., 1989). In Western countries, the average number of years between infection with HIV-1 and the appearance of clinical symptoms of immunodeficiency is ten years, according to studies done with patients in which the time of seroconversion is known (Hessol et al., 1994). The main opportunistic infections observed in patients whose CD4+ T cell counts fall below 200 cells/μl are *Pneumocystis carinii* pneumonia, disseminated *Mycobacterium avium* complex infection and CMV infection as well as AIDS-definining-cancers (ADC) such as Kaposi's sarcoma (Katz et al., 1994), cervical cancer or non-Hodgkin lymphoma. In a study done in the United States on 302 834 adults with AIDS, Hodgkin disease of the lymphocytic depletion and mixed cellularity subtypes

were found to be increased with immunosuppression (Frisch et al., 2001). Cancers such as penile, lung and lip cancer as well as testicular seminoma were also found increased in AIDS patients although heavy smoking or frequent exposure to human papillomavirus in these patients could be the reason for this finding (Mbulaiteye et al., 2003). Some studies have found a favorable effect of highly active antiretroviral therapy (HAART) on the incidence of ADC in patients with AIDS with the exception of cervical cancer that seems unaffected by the introduction of HAART (Clifford et al., 2005; Franceschi et al., 2003).

In some individuals, AIDS develops within months of acquiring HIV while others show no signs of disease progression after 12 or more years, the LTNP. In LTNP, although CD4+T cell counts and lymph-node architecture remain normal, viral replication persists (Pantaleo et al., 1995). The causes for this are still elusive, but a strong virus-specific CD8+ T cell response with some degree of viral attenuation have been reported in some of these patients (Cao et al., 1995; Musey et al., 1997). There are some reports of a defective Nef as a possible cause for non-progression in HIV-1 infected patients (Tobiume et al., 2002). Host factors like age or co infection with tuberculosis (Day et al., 2004; Whalen et al., 2000) or hepatitis B or C viruses (De Luca et al., 2002; Weis et al., 2006) may influence the severity of the disease although the effect of the latter remains controversial (Konopnicki et al., 2005; Sulkowski et al., 2002; Sullivan et al., 2006). Genetic variation in the host is also associated with non-progression. Some individual who are heterozygous for a 32-base deletion in the CCR5 deltion gene have a slower progression to disease. Homozygotes for this deletion are resistant to HIV-1 infection (Samson et al., 1996; Rappaport et al., 1997).

As mentioned before, macrophages are also infected and are one of the reservoirs for HIV-1 during the clinical latent phase (Saha et al., 2001; Zhu et al., 2002). In macrophages, HIV-1 seems to be able to maintain high level production of early proteins such as the negative factor (Nef), which allows immune evasion, but low production of structural proteins, such as p24 suggesting a latent infection (Zhu et al., 2002). This has important implications because infection of macrophages in the CNS constitutes one of the most challenging aspects of therapeutic intervention. Although there are a smaller

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number of infected macrophages in an HIV-1 positive patient compared to CD4 T lymphocytes, the dynamics of HIV-1 replication and macrophages' long half-life make them an important viral reservoir that is difficult to treat. They contribute to the recruitment and activation of CD4+ T cells by chemokines and viral proteins thus contributing to the pathogenesis of HIV infection. Most importantly, they are the main target of HIV-1 in the CNS and presumably this causes AIDS related dementia (see below). Although nucleoside analogues inhibitors of HIV reverse transcriptase (NRTI) are even more effective in macrophages than in lymphocytes (Aquaro et al., 1998), they have limited penetration in the CNS. On the other hand, protease inhibitors (PI) can inhibit virus production and release from chronically infected macrophages (Aquaro et al., 1998) but higher doses are required (Aquaro et al., 2002; Aquaro et al., 2006; Perno et al., 1994; Perno et al., 1998). Dendritic cells (DC) can also sustain HIV-1 infection (Patterson et al., 2001). Their susceptibility to infection depends on their stage of maturation (Dumont et al., 2004) and on their phenotype (Schmitt et al., 2006). There are reports of decreased HIV-1 infection in mature DC (Cavrois et al., 2006), of a block post integration, occurring at a transcriptional level (Bakri et al., 2001) and a block before reverse transcription (Granelli-Piperno et al., 1998). There are two different populations of DC: the plasmacytoid and the myeloid. Both express CD4, CCR5 and CXCR4 (Zenke and Hieronymus, 2006). Plasmacytoid DC can be infected by HIV-1 more efficiently than myeloid DC (Patterson et al., 2001) despite the fact that myeloid DC express DC-SIGN (van Vliet et al., 2006). DC-SIGN is a c-type lectin that binds HIV-1 but does not mediate its entry. It has been implicated in the transfer of HIV-1 from DC to T cells in lymph nodes, thus spreading viral infection (Arrighi et al., 2004; Geijtenbeek et al., 2000) and even in the vertical transmission of HIV because of the high levels of DC-SIGN RNA found in the placenta (Pedersen et al., 1997; Soilleux et al., 2001).

In other organs, like the liver, it is the resident macrophages (Kuppfer cells) that carry the virus. In the brain, which is isolated from the blood by the brain-blood barrier, the main cells infected are the microglia and perivascular macrophages (Dunfee et al., 2006). This colonization eventually manifests as dementia, present in approximately 30% of AIDS cases (Ghafouri et al., 2006).

1.c AIDS-related dementia

Dementia is a set of symptoms resulting from the damage or diseases of the brain. These symptoms depend on the specific region of the brain that is affected and can be cognitive, behavioural, affective, motor and psychiatric (Ghafouri et al., 2006).

In the United States, HIV-1 infection of the brain is the most important cause of dementia in young adults (McArthur et al., 1999). Before the introduction of HAART, about 20 to 30% of HIV-1 infected patients developed neurological symptoms such as impaired short-term memory, reduced concentration, leg weakness along with behavioural symptoms such as personality changes, social withdrawal and apathy, known as AIDS-associated dementia (ARD). With the introduction of HAART a milder form of CNS dysfunction known as minor cognitive motor disorder (MCMD) is observed in HIV-1 infected patients. This could be due to the positive effect of HAART in decreasing the viral load or in the longer life span of HIV-1 infected patients (Gonzalez-Scarano and Martin-Garcia, 2005). Thirty to sixty percent of HIV-1 positive patients are affected neurologically (Fischer-Smith and Rappaport, 2005) and some reports parallel its frequency to that of AIDS (Trujillo et al., 2005).

HIV-1 gets in the central nervous system in the early stages of infection and persists there for decades (An et al., 1999). Evidence for this is the occasional presence of meningitis or meningoencephalitis at the time of seroconversion, the presence of HIV antigen and of antibodies in cerebrospinal fluid during the presymptomatic phase (Goudsmit et al., 1986; Ho et al., 1985; Resnick et al., 1988) and the detection of HIV proviral DNA by PCR postmortem in brains of patients pre-AIDS. In the central nervous system, HIV-1 activates chemokine receptors, inflammatory mediators, glutamate receptor-mediated excitotoxicity and extra cellular matrix-degrading enzymes that disturb neuronal and glial function.

Although HAART has improved the control of viral infection in the periphery, the availability of an effective therapy for ARD is still controversial (Cysique et al., 2006; Giancola et al., 2006; Maschke et al., 2000; Sacktor et al., 2002). This

makes the immunologically protected CNS an important reservoir for HIV (Cashion et al., 1999; Lambotte et al., 2003). Virus in the CNS is protected at least in part because of the low penetration of anti-HIV drugs and this is evident as there is at least some degree of cognitive impairment even after treatment with HAART (Chao et al., 2004; Sacktor et al., 2002)

Immunohistochemistry and *in situ* hybridization studies have shown that perivascular macrophages are the main targets of HIV-1 in the brain. Multinucleated giant cells, a hallmark of HIV neuropathology, express CD14 and CD45, the cell-surface markers of macrophages (Gonzalez-Scarano and Martin-Garcia, 2005). Microglial cells seem to be best suited for maintenance of a viral reservoir as they are long lived, can produce virus in culture for several weeks and do not form virus-induced syncitia (Kramer-Hammerle et al., 2005). Astrocytes may also be infected even though they do not express CD4 receptor on their surface. However, they seem to have some block to HIV-1 infection restriction, particularly in paediatric patients (Saito et al., 1994; Tornatore et al., 1994). Although oligodendroglia and neurons are not the main target for HIV, the secondary damage to these cells probably is a major cause for the symptomatology of ARD.

The pathophysiology of ARD remains unknown but several mechanisms have been proposed. The direct injury hypothesis proposes that interaction of gp120 with co receptors can induce apoptosis through CXCR4-mediated signalling (Hesselgesser et al., 1998; Kaul and Lipton, 1999; Khan et al., 2003) However, most CNS isolates that have been described so far use CCR5. Also, gp120 needs to interact with CD4 before it interacts with the co receptor and finally, studies supporting this hypothesis use monomeric gp120 and not the trimeric form that is found in natural conditions so this mechanism of action still needs to be proven (Gonzalez-Scarano and Martin-Garcia, 2005). The release of viral particles and proteins, such as Tat and Vpr by infected macrophages and microglia is another pathologic mechanism that has been postulated as the source of nerve cell damage. Tat can directly injure neurons, alter tight junctions in the blood-brain barrier and up regulate inflammatory mediators in the brain. However, free Tat protein in body fluids has not been demonstrated so its role in neuropathogenesis is still uncertain. Vpr induces cell-cycle arrest and induces

apoptosis of human neuronal precursors and mature neurons through a caspase 8-dependent mechanism. It is uncertain if both Tat or Vpr can reach the necessary concentrations in the CNS as to cause the effects that have been observed in vitro, considering HIV-1 infection in the CNS is limited (Gonzalez-Scarano and Martin-Garcia, 2005). The third theory proposes the activation of uninfected microglia and macrophages by activated mononuclear cells from the blood with the release of proinflammatory cytokines such as interleukin type 1 (IL-1) and tumor necrosis factor alpha (TNF-α), glutamate receptor agonists and quinolinate to be the mechanism of injury in HIV-induced encephalopathy. These substances generate free radicals in neurons and the end result is damage and loss of neurons and myelin. These substances can also induce the migration of activated, HIV-1 specific T cells into the CNS (Gonzalez-Scarano and Martin-Garcia, 2005). An increased number of activated macrophages has been shown to correlate with dementia in HIV-1 infection (Glass et al., 1995).

The brains of patients with ARD are generally atrophic with reduced brain weight and ventricular dilatation. Histologically, the central white matter of the cerebral hemispheres and basal ganglia shows multinucleated giant cells (Budka, 1986; Sharer et al., 1985). This extensive white matter damage is designated HIV leucoencephalopathy and is still not clear if it is just a stage of ARD. Two common and prominent features are reactive astrocytosis and generalized microglial activation in both grey and white matter which can give rise to the formation of microglial nodules, the latter also seen in CMV and toxoplasma CNS infection (Budka et al., 1987) Similar changes have been observed in the neocortex and the spinal cord (Adle-Biassette et al., 1995; Budka et al., 1991; Everall et al., 1999; Shepherd et al., 1999)

Before HAART, CNS opportunistic infections and lymphomas were end stage conditions. Although they are now treatable, there are studies that have documented the neuropathological characteristics of HIV-induced neuropathology in cohorts from the pre to the post-HAART eras (Gray et al., 2003; Jellinger et al., 2000; Langford et al., 2003; Masliah et al., 2000; Morgello et al., 2002) and the reports show that HIV-induced encephalitis is almost as common in the post-HAART as it was in the pre-HAART era

1.d Clinical management

1.d.i Diagnosis and antiretroviral therapy

The diagnosis of HIV-1 infection is based on serological tests that detect antigens or specific antibodies. The availability of rapid HIV-1 antibody tests has been a great advance as they provide results in about 20 minutes and enable specimen collection and diagnosis at the same visit in clinical and non-clinical settings. They are interpreted visually (Greenwald et al., 2006). These tests are important tools for screening and surveillance and they can be done on plasma, serum, saliva or whole blood. Commercial enzyme-linked immunosorbent assays (ELISAs) have been developed since 1985 and have improved with the years (Brust et al., 2000; Thorstensson et al., 1998) allowing them to recognize a larger number of HIV-1 subtypes (Brust et al., 2000; Gurtler et al., 1994; Vanden Haesevelde et al., 1994). The newest assays detect both anti-HIV antibodies and HIV antigens (Saville et al., 2001; Weber et al., 2003; Yeom et al., 2006) so are useful in the acute and chronic phases of infection.

Current drugs cannot eradicate HIV-1 infection so lifelong treatment is usually needed. Of the 21 antiretroviral drugs approved by the US Food and Drug Administration (FDA), 20 target the viral RT or protease (PR): eight NRTI and three non-nucleoside (NNRTI) reverse transcriptase inhibitors inhibit viral replication post-entry but before integration. NRTI block RT by competing with the natural substrates thus being incorporated into viral DNA and terminating the synthesis of proviral DNA (Mitsuya and Broder, 1986). Currently FDA approved NRTI are zidovudine, didanosine, stavudine, lamivudine, abacavir, enofovir, emtricitabine, combivir, trizivir, truvada, epzicom and zalcitabine. Their most common side effects are lactic acidosis, severe hepatomegaly and steatosis (Temesgen et al., 2006). NNRTIs bind directly and non-competitively to the substrate binding site of RT disrupting it as they cause a conformational change that blocks the DNA polymerase activity of RT (Grob et al., 1992; Merluzzi et al., 1990). The currently FDA approved NNRTIs are nevirapine, delavirdine and efavirenz and their most most common side effects are rash, hepatotoxicity (Pollard et al., 1998), CNS side effects, dislipidemias (van Leth et al., 2004) and neural tube defects have been reported in the babies of women that have

received it during pregnancy (De Santis et al., 2002; Fundaro et al., 2002). PI prevent the maturation of virions resulting in production of structurally disorganized and non-infectious particles. The currently FDA approved PI are saquinavir, indinavir, ritonavir, nelfinavir, amprenavir, lopinavir/ritonavir, atazanavir, fosamprenavir, tipranavir, amprenavir, and darunavir. Efurtivide is the first and so far, the only fusion inhibitor approved by the FDA, and it binds to HIV-1 gp41 protein, blocking fusion with the cellular membrane (Temesgen et al., 2006). This drug is reserved for treatment of patients that have been treated extensively as it can be useful in cases of drug resistance; its most common side effects are erythema, induration, nodules or cysts or echymosis in injection sites (Lalezari et al., 2003; Lazzarin et al., 2003).

A combination of three or more drugs is usually necessary to decrease morbidity and mortality and to achieve near normal CD4+ and CD8+ T cell counts. The diversity of HIV-1 species or quasi-species in chronically HIV-1 infected patients increases the chances of resistance to appear so HAART becomes necessary; genetic recombination may contribute to multiple-drug resistance (Moutouh, Corbell and Richman, 1996). The combination of several antiretroviral agents which will suppress viral replication to low levels to prevent or at least delay the emergence of resistance. In industrialized countries, HAART has decreased morbidity and mortality in a striking way (Hogg et al., 1998; Mocroft et al., 1998; Palella et al., 1998). Plasma viraemia concentration, absolute CD4+ cell counts and clinical manifestations are the criteria used to decide when to start HAART (Simon et al., 2006). Patients with CD4+ counts lower than 200 per microliter or with AIDS-defining illnesses benefit from treatment. However, the optimal time to start HAART in asymptomatic patients with modest CD4+ T cells depletion (>350 per microliter) or viraemia (100 000 copies per mL) is not so clear (Mocroft and Lundgren, 2004). In the developing world, therapeutic success rates have been achieved despite restricted health infrastructures (Badri et al., 2004). Unfortunately, the cost of HAART and restrictive licensing policies in many countries leave an estimated 80% of HIV-1 infected people worldwide who need treatment without access to antiretroviral drugs (UNAIDS, 2006b).

1.e Transmission and prevention

1.e.i Sexual transmission

Heterosexual transmission is crucial in the spread of the epidemic in many parts of the world (Chan, 2005; UNAIDS, 2006a). Its prevention can be achieved by abstinence or sex between concordantly seronegative individuals. However, they are difficult to achieve so interventions should be directed towards preventing risk of transmission between seropositive and seronegative individuals, such as male and female condoms which have proven effective (de Vincenzi, 1994; Weller and Davis, 2002). Other prevention strategies include male circumcision, treatment of sexually transmitted diseases, microbicides and vaccines. Male circumcision has been reported to protect 60% of individuals in a South African trial and has been associated with a lower risk of HIV infection in other African countries (Auvert et al., 2005; Lavreys et al., 1999; Seed et al., 1995). The reason for this possibly being that the penile foreskin has Langerhan's cells expressing CD4 which can uptake virus and transport it to T cells (Soto-Ramirez et al., 1996). However, male circumcision as a prevention strategy still is under analysis. Post-exposure prophylaxis is recommended in certain situations such as sexual abuse or needle-stick injury but its efficacy and the optimum drug combination that should be used are yet not clear (Soto-Ramirez et al., 1996). On the other hand, the treatment of sexually transmitted diseases (STD) such as herpesvirus type 2, which might increase the risk of acquiring HIV-1 (Corey et al., 2004; Freeman et al., 2006; Grosskurth et al., 2000) is recommended. The role of other STDs is not clear.

As mentioned above, gender disparities are a major drive of the epidemic and make women vulnerable to acquiring HIV. Treatment of vaginal conditions such as bacterial vaginosis, vulvovaginal candidiasis and infection with *Trichomonas vaginalis* can reduce the risk of acquiring HIV-1 (Schwebke, 2005). Giving women access to methods that are under their control such as cervical caps, diaphragms and microbicides can empower them to protect themselves. Microbicides are applied topically and used to prevent rectal and vaginal transmission of HIV-1. Finally, vaccines would be the most efficient way to prevent HIV-1, however, despite the extensive research the development of a

vaccine is still elusive because of the safety concerns that a live-attenuated virus vaccine would have and the very high variability of the virus itself. Efforts to induce neutralizing humoral immunity with a recombinant monomeric envelope gp120 protein have been unsuccessful (Cohen, 2003; Francis et al., 2003; Pantophlet and Burton, 2006; Wyatt and Sodroski, 1998) and the focus more recently is to elicit cellular immunity. Most likely, a vaccine based in virus-like particles, that elicits both humoral and cellular responses, will prove to be the most effective, although the way to get it still is uncertain (Young et al., 2006).

1.e.ii Mother to child transmission

It has been many years since it was found that mother-to-child transmission (MTCT) of HIV-1 could be prevented in 67% of cases with a regimen of perinatal zidovudine (Connor et al., 1994). However, 750 000 children become infected worldwide with HIV through MTCT: without intervention the rate of MTCT is 15-20% and it doubles with prolonged breastfeeding (Newell et al., 1996). MTCT prevention focuses on antiretroviral prophylaxis during pregnancy, labour and early neonatal period along with elective Caesarean section and shortening or avoidance of breastfeeding (European Mode of Delivery Collaboration 1999; Newell, 2006). Maternal viral load is the key risk factor for vertical transmission of HIV so suppression of viral replication through drugs reduces it markedly (European Collaborative Study, 2005). On the other hand, Caesarean section and avoidance of breastfeeding are not feasible or safe in some settings but it has been demonstrated that peripartum nevirapine is an inexpensive and effective way of prevention of MTCT in low resource settings (Guay et al., 1999). The overall rate of MTCT of HIV has been reported to be reduced to around 2% in non-breastfeeding populations or 10% in breastfeeding populations, with the administration of antiretrovirals such as zidovudine or nevirapine to pregnant mothers and newborn babies (European Collaborative Study, 2005; Dabis et al., 2005; Lallemant et al., 2004; Leroy et al., 2002). There is concern as well about the possibility of adverse effects in the mother or the offspring with exposure to peripartum antiretrovirals: although there have been reports of increased premature delivery with the administration of protein inhibitors in early pregnancy (Thorne et al., 2004), there are no

reports of an increase in congenital malformations. However, mortality of HIV-infected mothers increases the chances of dying of their infected or uninfected children by 2-3 fold which emphasizes the fact that children of HIV-1 infected mothers have a higher mortality risk even if MTCT is prevented (Newell et al., 2004).

1.e.iii Transmission by blood and blood-derived products

The risk of acquiring HIV through a transfusion of infected blood or blood-derived products is nearly 100%. According to the World Health Organization, 5% to 10% of all HIV infections world-wide have been acquired in this way and there are up to 13 million units of blood supply that are not screened for HIV or any other infectious disease worldwide. The risk of HIV transmission via a blood unit is about 1 in 1000 in some African countries, 1 in 10 000 in some areas of Asia and 1 in 100 000 to 1 million in Europe and North America (Guertler, 2002). Keeping blood safe involves recruitment and selection of voluntary, non-remunerated donors from low risk populations, appropriate training of the staff involved in transfusions and screening of donated blood for HIV and other pathogens (WHO, 2006). Blood is screened via culture or enzyme immunoassay (EIA)-based antigen detection of HIV p24 antigen and anti-HIV antibodies. Their usefulness depends on HIV prevalence in the population and the duration of the window preceding the development of antibodies in the initial phase of infection (Allain, 2000). In the last decade nucleic amplification techniques (NAT) have been developed that allow identification of infectious units that are not identified by EIA methods. The most widely used of these NATs is polymerase chain reaction (PCR).

2. Retroviruses

2.a Overview of retroviruses

The *Retroviridae* are a family of enveloped ribonucleic acid (RNA) viruses defined by their ability to synthesize a double stranded deoxynucleic acid (DNA) molecule using the two molecules of RNA that compose their genome as template. This DNA copy of their genome integrates into the host's genome establishing a permanent infection. There are 7 major *genera* in the family of retroviruses: mammalian C-type retroviruses (of which the prototype would be murine leukemia virus [MLV]), avian sarcoma and leucosis viruses (of which the prototype would be the Rous Sarcoma Virus [RSV]), B-type retroviruses (of which the prototype would be the mouse mammary tumor virus [MMTV]), D-type retroviruses (of which the prototype would be the Mason-Pfizer monkey virus [M-PMV]), lentiviruses (of which the prototype is HIV-1), the HTLV-BLV group (the prototype is HTLV-1) and spumaviruses (the prototype is human foamy virus [HFV]). Retroviruses isolated from fish and flies (such as the Gypsy viruses) seem to be different to the already existing *genera* (Fodor and Vogt, 2002a; Fodor and Vogt, 2002b; Holzschu et al., 1997) and await to be classified. *Pol* sequence is the major criteria for assignment into the different *genera*, however, the shape of the core, its assembly in the cytoplasm or the plasma membrane and the presence or absence of accessory genes also count (Coffin, 1997). Viruses within a *genus* are identical in more than two thirds of the amino acid residues in the most conserved part of the reverse transcriptase. There are amino acid sequence motifs conserved among all retroviruses in the *gag, pro* and *pol* genes.

2.b Origins and diversity of HIV

Retroviruses have been isolated from sheep, goats, horses, cattle, cats and primates (Carlson et al., 2003; Dutta et al., 1970; He et al., 1992; Kawakami et al., 1967; Ortin et al., 2003; York and Querat, 2003). In humans, HIV-1 and HIV-2 are the only lentiviruses that have been found. HIV-related lentiviruses of nonhuman primates SIVs and have been isolated from several African monkeys (Chen et al., 1995; Peeters et al., 1992; Peeters et al., 1994; Sakai et al., 1992). There are five groups of primate lentiviruses. According to genetic sequencing, viruses within each group have around 60% aminoacid identity in the sequence of RT. These groups are: HIV-1/ SIVcpz, HIV-2 / SIVsmm, SIVmac, SIVagm, SIVmnd and SIVsyk (cpz, smm, mac, agm, mnd, syk stand for chimpanzee, sooty mangabey, macaque, African green monkey, mandrill and sykes monkeys respectively). SIVmac is used in all experiments shown in this thesis.

HIV-1 is divided into three phylogenetical groups: M, the main group that is responsible for most cases of AIDS in the world, O, the "outlier" group, found primarily in West Africa, and N, "non-M" and "non-O", found mainly in Cameroon. M is further subdivided into clades or subtypes A to K, phylogenetically equidistant from each other (Sharp et al., 2001) (Figure 1). Chimpanzees (are divided into four subspecies, two of which *Pan troglodytes troglodytes* (Ptt) and *Pan troglodytes schweinfurthii* (Pts) harbor SIV from SIV chimpanzees. SIVcpz*Ptt* is the progenitor of HIV-1 and is the result of recombination between ancestors of different SIVs found still in red-capped mangabeys *and Cercopithecus* monkeys that inhabit West Central Africa. The virus was transmitted locally and then made its way south where the N epidemic was born from a second SIVcpz*Ptt* lineage (Keele et al., 2006). The M, N and O HIV-1 groups are interspersed between SIVcpz which indicates that they must have arisen from separate cross-species transmission events (Gao et al., 1999; Sharp et al., 2001; Sharp et al., 2000). HIV-1 M and N lie closer to SIVcpz from chimpanzees found in Cameroon, Equatorial Guinea, Gabon and Congo-Brazzaville but group O is probably the result of recombination among different lineages because its position in the phylogenetic tree depends on which region of its genome is analyzed, the 5' or the 3' end (Figure 1). The origin of HIV-1 group O remains obscure but recently an HIV-1 group O-like virus was identified

Figure 1. Geographical distribution of HIV subtypes worldwide. Estimates based on data from global HIV/AIDS surveillance; HIV-1 incidence in 2000 was estimated in geographical regions using a model designed by UNAIDS and the proportion of new infections with different subtypes was estimated by the WHO-UNAIDS Network for HIV Isolation and Characterization according to HIV molecular epidemiology studies from 1998 to 2000. Panel A shows the total incidence of HIV-1 subtypes according to *env* in 2000 and panel B the distribution of those new infections in the same year. Taken from Osmanov S et al. 2000. *JAIDS* 29: 184-190.

in wild gorillas rising the possibility that chimpanzees might have transmitted HIV-1 group O-like viruses to gorillas that then transmitted it to human or to gorillas and humans independently (Van Heuverswyn et al., 2006).

The most likely and simple explanation for the SIV's cross species jump that originated HIV is the direct exposure of human to animal secretions or blood through consumption of uncooked contaminated meat that occurs in "bushmeat" markets in many African places. However, there is a hypothesis that postulates a iatrogenic cause, and blames the contamination of oral polio vaccine when it was prepared in the kidneys of SIV infected chimpanzees (Sharp et al., 2001). Epidemiological and phylogenetical evidence weaken this last hypothesis but cannot point to a specific mechanism for the cross species jump. On the other hand, they can indicate SIVcpz from the common chimpanzee *P. troglodytes* as the source of HIV-1 (Gao et al., 1999), Western Equatorial Africa, this ape's habitat, as the place where this probably occurred and 1940 as the time around which the common ancestor of HIV-1 group M and N was already infecting humans (Sharp et al., 2001). The answer to the question of why the epidemic only started in the second half of the twentieth century is not yet clear but it is possible that medical interventions and cultural changes facilitated the rapid spread of the successfully adapted virus between humans (Chitnis et al., 2000).

HIV-1 evolves at a very high rate because of its high replication rate and the tolerance to changes of its envelope proteins (Coffin, Hughes and Varmus, 1997) that reflects in a great diversity between and within individuals. Recombination is another source of genetic diversity in primate lentiviruses. It contributes to the variation of the distinct viral quasispecies circulating within one infected individual and occurs primarily in gene segments generating diversity in sequences like *pol* and *env* that confer selective advantage (Charpentier et al., 2006). This is more pronounced when an individual is co infected with different viruses in an area where distinct subtypes circulate originating inter-subtype recombinants (Sharp et al., 2001).

2.c Genome Organization

The retroviral genomic RNA is dimeric (Stoltzfus and Snyder, 1975). This unique property of retroviruses was initially discovered in in the 1960's and this was determined by sedimentation analysis at first (Duesberg, 1968) but this property was later confirmed by electron microscopy studies on an endogenous baboon virus, a woolly monkey sarcoma virus and an endogenous feline virus about 10 years later (Kung et al., 1976). It was also shown that the two strands of RNA have a stable contact point close to the 5' end which was called the dimer initiation site (DIS) (Bender et al., 1978). The tertiary structure of HIV-1 DIS was analyzed by two-dimensional nuclear magnetic resonance (NMR) spectroscopy and found to be directed by a hairpin structure with a palindrome in what has been called a "kissing loop domain" (Mujeeb et al., 1998). The diploid genome allows the virus to switch from a damaged to an intact template. This is an advantage in itself but it also provides the opportunity to recombine which in HIV-1 is related to viral fitness and pathogenesis (Greatorex and Lever, 1998). There have been studies attempting to determine the significance of dimerization for HIV-1 infectivity. The DIS is in a ~109 nucleotide long region called psi (ψ). However, because there is another RNA structure called the packaging signal in ψ, it is difficult to separate the functions of these two regions and thus their overall effect on infectivity. However, many studies have shown a decrease in infectivity when the "kissing loop domain" is mutated (Berkhout and van Wamel, 1996; Clever and Parslow, 1997; Laughrea et al., 1997; Paillart et al., 1996). In HIV-1, ψ is found in the 5' leader region, in the splice donor of the viral RNA and contains helix-loop motifs with terminal purine-rich loops (Lever et al., 1989). There are additional sequences upstream of the splice donor site and in the Gag gene that are also involved in packaging (Aldovini and Young, 1990). The location of ψ ensures that is only present in unspliced mRNAs.

Retroviruses have an RNA genome that replicates via a DNA intermediate. Because of this, they must have a promoter that drives the expression of the genomic RNA that is located in the 5' end. For this reason, it is simpler to describe the retroviral genome organization in its DNA form, the provirus,

A

5'LTR   3'LTR

vif vpu
vpr   nef
tat
rev

pol   env
gag   PR   IN   SU   TM
                 RT p66   (gp120) (gp41)
         MA CA NC p6   p51
              p2 p1

                                    C4   fusion peptide N-helix C-helix membrane-spanning domain cytoplasmic tail

C1 V1 V2 C2   V3 C3 V4 V5 C5

B

nt 1   454/5 551/2 634 744 790/2

          U3   R U5 GL gag

nt -454   -105 -78 -1/+1 +60 +181
                      TAR tRNA
                 Basal
                Enhancer
      Modulatory
   -340   -184
      NRE

Figure 2. Schematic representation of HIV-1 genome. A) The structure of HIV-1 proviral DNA is shown above. *Adapted from Freed E. 2001. Somat Cell Mol Genet 16: 13-23.* B) Structure of the HIV-1 5'LTR and gag leader (GL) sequence; the U3 region contains the modulatory, enhancer and basal promoter elements. The negative regulatory element (NRE) is contained within the core modulatory promoter. The transactivating region (TAR) is within the repeat (R) region and the tRNA binding site is within the GL sequence. The numbering above starts from the first nucleotide of the provirus (HIV-1 clone HXB2 sequence) and the numbering below is relative to the transcription initiation site (nt +1). *Adapted from Pereira L et al. 2000. Nuc Ac Res 28: 663-8.*

because then the promoter, the RNA site and the polyadenylation site are positioned in the same way they are found in host cell chromosomes. At both ends of the retroviral proviral DNA are the long terminal repeats (LTRs). The two LTRs are identical repeats containing the unique 3 (U3), repeat (R) and unique 5 (U5) regions. These sequences are 300 to 1800 base pairs (bp) long in different retroviruses and are generated in their complete form during reverse transcription, when the 3' U3 region is copied to the 5' LTR. In HIV-1, the LTRs are 635 bp long and the U3 region of the LTR contains the viral promoter and enhancers, which are active in the 5' LTR (Jeeninga et al., 2000; Naghavi et al., 2001) and the R region contains the polyadenylation signal, which is active in the 3' LTR (Gee et al., 2006). The nature of the enhancer sequences in U3 plays an important role in pathogenesis as it is involved in controlling tissue specificity of the virus (Coffin, 1997). There are four functional regions involved in HIV-1 transcription regulation: the transactivation response (TAR) element within R and in the U3 the basal or core promoter, a core enhancer and a modulatory region. It has been proposed that the modulatory region has a negative regulatory element. Four Sp1 core promoter-binding sites and two NFκB core enhancer motifs have been reported to regulate HIV-1 transcription. Cellular proteins reported to interact with these regions include nuclear factor of activated T cells (NFAT), activating protein-1 (AP-1), upstream stimulatory factor (USF) the chicken ovalbumin upstream promoter (COUP) transcription factor. HIV-1 regulates its gene expression in different cell types under different stimuli through varied DNA-protein and protein-protein interactions involving these regions (Figure 2) (Pereira et al., 2000). Particularly interesting are the NFκB-binding sites. In most cells, NFκB is expressed in an inactive cytoplasmic form. Its activation in T cells can be a response to a number of stimuli such as mitogens, bacterial lipopolysaccharide, viral transactivators of cytokines and NFκB activation leads to the activation of genes coding for cytokines such as β-interferon, granulocyte macrophage colony stimulating factor (GM-CSF), IL-2, TNF-α and IL-6 and for surface receptors such as the major histocompatibility complex type I (MHC I). NFAT and AP-1 are also very important activators that interact with HIV-1 LTRs and along with NFκB are able to induce low level transcription of HIV-1 fully spliced ~2 kb messenger RNA (mRNA) (Cullen, 1991) coding for the transactivating (Tat), regulator of virion (Rev) and Nef proteins. Tat then is translated and establishes a feedback loop increasing HIV-1 gene

expression to very high levels and producing the other two HIV-1 mRNA species by binding to the TAR element. The TAR element is a 59 nucleotide RNA stem-loop structure located at the 5' end of all HIV-1 transcripts: its location and orientation are essential for its function. It possesses a three nucleotide bulge and a terminal loop to which Tat and cellular cofactors bind, respectively (Cullen, 1991).

Retroviruses have three common genes *gag*, *pol* and *env* (Chang et al., 1985; Ratner et al., 1985) (Figure 2). In HIV-1, the *pro* gene coding for protease and the *pol* gene are in the same (-1) reading frame as *gag* so the viral PR is translated at the same level as the RT and integrase (IN) proteins. The *env* gene is expressed from a subgenomic mRNA whose splice donor is upstream of *gag*. Within env is a 234 nucleotide RNA sequence called the Rev responsive element (RRE). Rev binds to the RRE and promotes the nuclear export of the ~9 kb and ~4 kb viral RNA transcripts so they cannot be spliced. The RRE possesses a 66 nucleotide stem-loop domain necessary and sufficient for Rev binding but the entire sequence is necessary for its full biological function, perhaps for stabilization of the Rev-binding RNA structure or to facilitate its exposure so that Rev can bind (Cullen, 1991) (Figure 2).

The *gag* gene codes for a polyprotein precursor called Pr55 that is cleaved by the viral protease into the matrix protein p17 (MA), capsid protein p24 (CA), nucleocapsid protein p7 (NC), p6, p2 and p1 (Henderson et al., 1992). These proteins participate in the process of uncoating, reverse transcription (Kiernan et al., 1998), assembly and maturation of the virion (Tang et al., 2001). The *pol* gene codes for the viral enzymes RT, IN and PR (di Marzo Veronese et al., 1986), all necessary for viral replication. Upstream from RT is the viral PR enzyme and downstream is the IN. The *pol* gene has no initiation codon so it is translated when there is a translation suppression of the stop codon of Gag or frameshifting mechanism, the result of which is the Gag-Pol polyprotein. This polyprotein is cleaved by the viral protease (Tarrago-Litvak et al., 1994). The RT has RNase H activity in a separate but contiguous domain of the protein and it degrades the viral RNA bound to single strand DNA that is used as a template during reverse transcription. HIV-1 IN is a multidomain enzyme that mediates viral DNA integration. The viral PR is essential for the processing of the primary

product of translation and virion maturation: it acts late in assembly and budding cleaving Gag and Gag-Pol which reflects in morphological changes and infectiousness of the viral particle. It is a homodimer (Coffin, 1997) . The *env* gene codes for the glycoproteins (gp) that compose the viral envelope: gp160, cleaved into gp41 and gp120, the transmembrane and surface domains respectively (Robey et al., 1985; Veronese et al., 1985).

HIV and SIV have six open reading frames (ORF) on top of *gag, pol* and *env* with additional genes involved in the regulation of viral replication and processing of RNA (Muesing et al., 1985; Ratner et al., 1985; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985a; Wain-Hobson et al., 1985b). They are: *tat. rev, nef. vpr, vif, and vpu* (in HIV-1) or *vpx* (in HIV-2 and SIVagm) accessory genes. *Tat* and *rev* are absolutely required for virus growth: Tat is an essential transactivator of the LTR and Rev ensures nuclear export of unspliced viral RNA and the switch from early to late phase in the viral gene expression pattern. The *tat* gene has two coding exons and encodes an 86 amino acid long protein. The rest of the proteins are called accessory as they can be dispensable for virus growth in some *in vitro* situations. All these genes are located downstream from *pol* and can overlap each other and over *env* and U3. All accessory genes are expressed from singly or multiply spliced RNA (Coffin, 1997). A more detailed description of the proteins they code for is given in section 2.d of Chapter 1.

2.d Viral proteins

Retroviral particles consist of a core surrounded by an envelope. The envelope (Env) consists of a host cell – derived lipid bilayer membrane and virus – encoded envelope glycoproteins, which mediate binding and fusion with the infected cell. In the infected cell, these glycoproteins are synthesized as a precursor, gp160 that is then cleaved by a cellular protease in the Golgi complex to result in gp41 and gp120 (San Jose et al., 1997; Willey et al., 1988) a process that is essential for HIV-1 replication. Both proteins assemble on the cell surface as trimers (Center et al., 2002). The gp41 glycoprotein has an ectodomain responsible for trimerization, a membrane-spanning domain and a long cytoplasmic tail of approximately 150 amino acids, important for Env fusogenicity (Earl and Moss, 1993; Gallaher, 1987; Miyauchi et al., 2005; Veronese et al., 1985; Wyss et al., 2005), surface expression and incorporation into virions (Freed and Martin, 1996; Freed et al., 1990). Gp120 is mainly responsible for binding to CD4 (Lasky et al., 1987) and is the main target for neutralizing antibodies (Wyatt and Sodroski, 1998). Gp120 is divided into five conserved and five variable domains: C1 to C5 and V1 to V5 respectively (Willey et al., 1986). The conserved regions of gp120 are covered by the variable regions, which shield them from neutralization by antibodies (Starcich et al., 1986; Wyatt et al., 1995). Gp120 has an inner and outer domains and a bridging sheet: in the unliganded protein, the conserved regions are in the inner domain and almost devoid of glycans while the outer domain contains a large quantity of glycans which lower their immunogenicity (Wyatt et al., 1998). The CD4-binding site is formed by all three domains in the unliganded form of the protein but once ligated to CD4, most of gp120 sites of contact lie within the outer domain. The CCR5-contacting site has been mapped to a conserved region between the bridging sheet and outer domain (Rizzuto and Sodroski, 2000) (Figure 3).
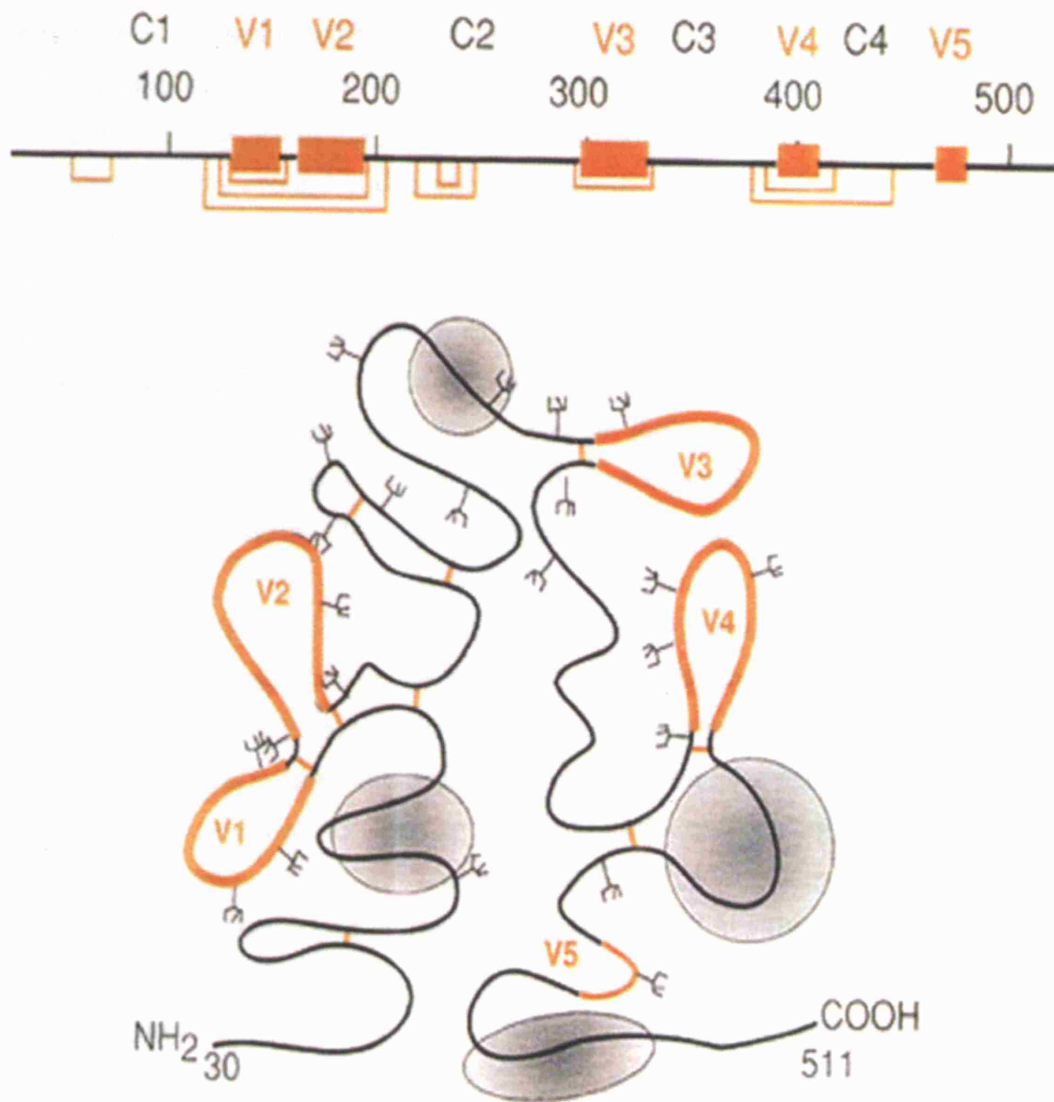
Figure 3. Structure and domains of HIV-1 protein gp120. In the top panel, a representation of gp120 amino acid sequence, the variable domains are shown as colored rectangles and the disulfide bonds between conserved cysteine residues are shown as the colored lines. The bottom panel shows HIV-1 gp120 folded with the disulfide bonds in color and side chains of N-linked oligosaccharides as the branched structures in black. Grey colored regions are domains involved in CD4 binding. *From Coffin, Hughes and Varmus. 1996. Retroviruses.*

The Gag polyprotein is the precursor of the proteins that form the internal structure of all retroviruses. Expression of *gag* on its own can lead to assembly of virus-like particles that can bud from the cellular membrane. It comprises about three quarters of the total proteins of a virus. The average immature HIV particle contains around 5000 copies of Gag (Briggs et al., 2004). Gag proteins from all retroviruses are organized in the following way: (NH2)-MA-CA-NC-(COOH) (Coffin, 1997). HIV-1 Gag is synthesized as a 55 kDa polyprotein precursor (Pr55 Gag) that during maturation is proteolytically processed by the viral PR transforming the virion structure into p17 MA, p24 CA, p7 NC, p6, p2 and p1. MA remains in the interface between the membrane and the core and CA condenses to form a conical shaped core containing the viral nuclei acid. The NC remains within the core with the viral RT and IN proteins.

The MA protein is involved in targeting of Gag to the membrane (Ono et al., 2000). This is possible through the exposure of its myristyl group, induced by binding to a cellular factor that regulates the localization of cellular proteins to the plasma membrane: phosphatidylinositol 4,5 bisphosphate (Saad et al., 2006). MA is also involved in Env protein incorporation into virions during virus assembly apparently through a cell factor, TIP47, that binds both Env and the MA domain of Gag (Lopez-Verges et al., 2006; Yu et al., 1992).

The CA protein, also called p24, is the largest of the Gag proteins: it is about 200-270 aminoacids in size. In HIV-1, CA is ~ 230 bp. The N-terminal region of CA is involved in virion maturation and incorporation of cyclophilin A (CypA). The C-terminal domain of CA is involved in multimerization allowing Gag-Gag interactions that dictate the size of the capsid and morphology and contains the major homology region that is the only part of CA that is highly conserved among retroviruses (Ako-Adjei et al., 2005; Chu et al., 2006; Ganser-Pornillos et al., 2004). Mutations in an alpha-helix structure located C-terminal to the major homology region cause defects in Gag multimerization that reflects in a marked decrease of viral particle production (Chu et al., 2006). Moreover, viruses with mutations in the C-terminal domain of CA show decreased virus particle production because of defects in Gag binding to the membrane and multimerization in addition to defects in CA folding (Joshi et al., 2006). The viral core changes from an hexameric lattice structure forming a spherical shell in

immature particles (Briggs et al., 2004) to a conical shape after proteolytic maturation (von Schwedler et al., 1998).The C-terminal region of CA also is responsible for the incorporation of Gag-Pol precursors into the assembling virion (von Schwedler et al., 1998). CA is involved in early steps after viral entry to the host cell. Proper uncoating of the virus is necessary for efficient nuclear entry, provirus integration (Dismuke and Aiken, 2006) and reverse transcription (Forshey et al., 2002).

The viral nucleocapsid is formed by NC proteins and nucleic acids. The NC protein is a small basic protein of about 60 to 90 amino acid residues long (in HIV-1 it is ~55 bp) that has zinc-finger motifs found in many cellular DNA binding proteins and participate in reverse transcription, protection of newly synthesized viral DNA and integration (Buckman et al., 2003; McGrath et al., 2003; Thomas et al., 2006). In the virions, NC proteins are in close association with the viral genomic RNA which they help encapsidating into the virion during assembly (Berkowitz et al., 1995). The NC protein promotes the annealing of the primer tRNA to the primer binding site (PBS) and in this way it facilitates reverse transcription. It also facilitates the formation of dimeric RNA by promoting the pairing of sequences at the DIS maybe by recognizing the viral encapsidation signal (Coffin, 1997).

The spacer peptide (SP) is also derived from Gag and it is 14 amino acids long and located in the carboxyl terminus of CA. Its deletion from the HIV-1 genome results in problems of budding and a decrease in infectivity (Coffin, 1997). Another polypeptide, p6, also has a role in budding defined as the interaction between viral proteins and the cellular machinery to assemble the virion), as shown by mutants in this region that can bud but cannot be released from the plasma membrane, so remain attached to the plasma membrane (Gottlinger et al., 1991). It is 60 amino acids long and is located downstream from NC partially overlapping the *pro* reading frame. Gag domains required during the late stages of the viral assembly are called late (L) domains and in HIV-1 the L domain is within p6. It contains a P(T/S)AP motif close to its N-terminus that is required for viral particle release along with a functional viral PR. P-X-X-P motifs (where P is a proline) are involved in protein-protein interactions (Huang et al., 1995).

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The *pol* gene codes for the viral enzymes PR, RT and IN; *pol* is located downstream from *gag* so these enzymes are synthesized as part of the 160 Kda Gag-Pol polyprotein. The viral PR is an aspartic-type protease, whose structure is a symmetric homodimer and each monomer is formed by the following structures in duplicate: a hairpin, a wide loop containing two conserved catalytic Asp residues, an alpha-helix and a second hairpin. This structural arrangement is found in the proteases of all retroviruses. In the loop there is a structure known as a "flap" because it changes its orientation when the enzyme binds to its ligand (Dunn et al., 2002). HIV-1 PR has a major role in the maturation of viral particles as it first cleaves itself from the Gag-Pol polyprotein precursor and then processes the viral RT and IN enzymes as well as the Gag protein into mature proteins that can form infectious virions.

The RT protein is a heterodimer that consists of one 66 kDa subunit that contains the polymerase and RNase H domain and a 51 kDa subunit containing only a polymerase domain. Both polymerase and RNase H domains reside together only separated by 18 nucleotides as was shown by a 3D structure study of HIV-1 RT in complex with its dsDNA template (Jacobo-Molina et al., 1993). These 18 nucleotide distance separation between the two domains may be important for correct spacing of both the tRNALys primer, that binds to a 18 bp region of the PBS sequence and the PPT, 19 bp long (Tarrago-Litvak et al., 1994). It contains highly conserved amino acid sequences that have been used to construct phylogenetic trees and it is a heterodimer in solution of p55 and p66. RT lacks proofreading activity: this facilitates mutation and recombination of the viral genome.

The viral IN is a 32 kDa enzyme that carries out the integration of the proviral DNA in a two-step reaction: 3' processing which involves the removal of 2 nucleotides from each 3' end of the proviral DNA and DNA strand transfer that involves two transesterification reactions and the integration of the ends of the provirus in the host cell DNA. HIV-1 IN consists of three domains, all required for integration, and it functions as a multimer, most likely as a tetramer (Dyda et al., 1994). Its catalytic site is in the core domain and contains two Asp and one Glu catalytic residues (Engelman and Craigie, 1992). It has an N-terminal zinc-

binding domain, the core domain with the catalytic activity and the C-terminal domain that can bind DNA non-specifically.

As mentioned earlier, besides the *gag, pol* and *env* genes, HIV-1 has six other ORFs: *tat, rev, nef, vif, vpr,* and *vpu.* The viral protein R (Vpr) is a basic protein of 96 amino acids (14 kDa) present not only in HIV-1 but also in HIV-2 and SIV. It is recruited into virions by p6 and it is one of the few accessory proteins that is incorporated in significant amounts into the virion. It has been found even in the extra cellular space. Vpr is nucleophylic, remains associated with the reverse transcription complex and may have a role in nuclear targeting of the preintegration complex (PIC) mainly in macrophages. Vpr arrests cells in the G2 phase of the cell cycle presumably to favour viral replication. It has also been implicated in cytotoxicity of infected and bystander cells, by apoptotic or necrotic mechanisms (Le Rouzic and Benichou, 2005; Moon and Yang, 2006).

The viral protein U (Vpu) protein is found in HIV-1 but not in other primate or nonprimate lentiviruses. It is a small integral membrane protein of 81 amino acids (16 kDa) (Strebel et al., 1988) that downregulates the levels of CD4 by interfering with its transport, stability and expression on the cell surface. It does this in conjunction with gp160 that forms complexes with CD4 in the endoplasmic reticulum where Vpu induces its degradation liberating gp160 (Willey et al., 1992). It also facilitates the budding of the virion from the plasma membrane, a function that involves a different domain of the protein. It is not clear yet if it does this by promoting the transport of Gag precursors to the cell membrane or by increasing the N-terminal domain of MA for the plasma membrane (Bour and Strebel, 2003).

The Vif protein is a basic 23 kDa phosphoprotein that is required for HIV-1 replication in cells like lymphocytes and macrophages called nonpermissive but is dispensable in other cells called permissive, examples of which are non-haematopoietic cells lines like HeLa-CD4, 293T and COS7 (Rose et al., 2004). Vif facilitates degradation and prevents incorporation of the cellular protein APOBEC3G present in nonpermissive cell lines into the nascent virions. Incorporation of APOBEC3G into virions results in hyper mutation of reverse transcripts by cytidine deamination and additional blocks to reverse transcription

(Kao et al., 2003; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Yu et al., 2003) although there also are reports about the suppression of translation of APOBEC3G by Vif (Stopak et al., 2003). Vif also has been reported to contribute to the arrest of infected cells in G(2) phase of the cell cycle (Wang et al., 2006). More details on the effect of the APOBEC protein family on HIV-1 replication are given in section 2.f of Chapter 1.

The viral protein Tat is an 86 to 102 amino acid protein that activates transcription by binding to the TAR RNA: a stem-loop structure at the 5' end of the viral transcript. In its absence, the HIV LTR produces short, incomplete transcripts. Tat contains multiple functional domains with different but complementary functions (Ulich et al., 1999): a strong transcriptional activation domain that consists of a cysteine-rich region and a hydrophobic core motif, with an arginine-rich RNA binding motif that is responsible for the specific binding to the bulge region of the TAR RNA structure (Jones, 1997). The cysteine-rich domain contains 6 to 7 highly conserved cysteines and is essential for transactivation along with the core domain (Siddappa et al., 2006). Thus, Tat acts as an adaptor protein that makes a host cell transcription factor Cyclin T1 viral-specific (Bieniasz et al., 1999). Tat is expressed early in the viral life cycle and is critical for viral infectivity and pathogenesis. It can be even be secreted in the extra cellular medium and activate transcription of the latent LTR in neighbouring cells (Frankel and Pabo, 1988) and also stimulates the production of cytokines in host cells (Siddappa et al., 2006).

Nef is a 27-34 kDa myristoylated protein (in HIV-1 it is 27 kDa) that is only present in primate lentiviruses and it has a major role in the development of viraemia in HIV-1 infection. Its best-known effect is down regulation of CD4 receptor and MHC I molecules on the cell surface. Nef binds to a dileucine motif in CD4 cytoplasmic domain acting as a lysosomal targeting signal (Renkema and Saksela, 2000). Down regulation of MHC I depends on a different domain of Nef, pointing to a different mechanism. The importance of these effects of Nef could be that down regulation of CD4 might prevent super infection and thus enhance HIV replication or that high levels of CD4 may interfere with virion production and release, as CD4 interferes with Vpu activity (Renkema and Saksela, 2000). On the other hand, MHC I down regulation might reduce the

amount of molecules incorporated into nascent virions, making them and their producer cells less susceptible to recognition by cytotoxic T cells (Collins et al., 1998). Nef also contributes to the pathogenesis of AIDS by altering gene expression of infected cells: a potent allele of SIVmac239 Nef has been reported to induce acute diarrhoea, rash and gastrointestinal lymphoid proliferation in macaque monkeys (Du et al., 1995). Nef can also modify the intra and extra cellular environment presumably to promote viral replication and spread. There are reports of Nef's paracrine and autocrine effects that exemplify this: Nef in macrophages has been reported to induce them to produce macrophage inflammatory protein 1 beta (MIP-1β), a chemotactic chemokine for T cells as well as a T cell stimulating factor, all of which would facilitate HIV-1 spread between cells (Swingler et al., 1999). Similarly, SIV and HIV Nef has shown to induce IL-2 production in IL-2 –dependent immortalized T cell clones derived from rhesus monkey (Swingler et al., 1999). Finally, Nef interacts with several cellular proteins involved in signalling pathways such as tyrosine kinases, p21 (CDKN1A)-activated kinase 2 (PAK2), the extracellular signal-regulated kinase 1 (Erk-1) and others that are probably involved in the effect that Nef has in the infected cell to promote the viral life cycle (Renkema and Saksela, 2000). In a recent report, SIV Nef from most primates was found to down regulate the T cell receptor / CD3 complex (TCR-CD3) as well as CD4 and MHC II. This was associated with normal CD4 T cell counts in their natural hosts as it reduced T cell activation and activation-induced cell death. The authors found that HIV-1 Nef from M, N and O groups enhances T cell activation in agreement with previous reports and interestingly, the only SIV Nefs are not found to down regulate TCR-CD3 are precisely those that are most closely related to SIVcpz and thus to HIV-1. The viruses containing these last Nef proteins also contain Vpu, which might compensate somehow the absence of this activity of Nef. The authors conclude that the ability to down regulate T cell activation was lost recently during evolution in a subset of SIV that included SIVcpz predisposing it to greater virulence when it made the jump to humans and gave rise to HIV-1 (Schindler et al., 2006).

Rev is an essential viral protein in HIV-1 life cycle. It interacts with the RRE and allows the export of unspliced (intron-containing viral mRNAs) viral RNA from the nucleus into the cytoplasm (Zapp and Green, 1989). This mechanism of

nuclear export is present in all lentiviruses and in HTLV-I and HTLV-II where the equivalent protein is called Rex and its target in the mRNA is called the Rex responsive element (RxRE) (Pollard and Malim, 1998). Rev is a 116 amino acid (~18 kDa) protein and the mRNA coding for it is expressed in the early stages of infection. It contains an amino-terminal domain with an arginine-rich sequence that acts as a nuclear localization signal (NLS) and an RNA-binding domain (Bohnlein et al., 1991; Hammerschmid et al., 1994). This domain is flanked by sequences that are necessary for multimerization (Madore et al., 1994; Malim and Cullen, 1991). Rev has a second domain, the carboxy-terminal domain that contains the nuclear export signal (NES) and is called the effector domain. Rev functions as a multimer: around eight or more Rev proteins bind to a single RRE while the effector domain might interact with cellular proteins that facilitate the export of the RNA and Revs' complex (Malim et al., 1991). More details about the cellular proteins that interact with Rev and the characteristics of the viral RNAs exported by Rev are given in section 2.e of Chapter 1.

2.e The Viral Life cycle
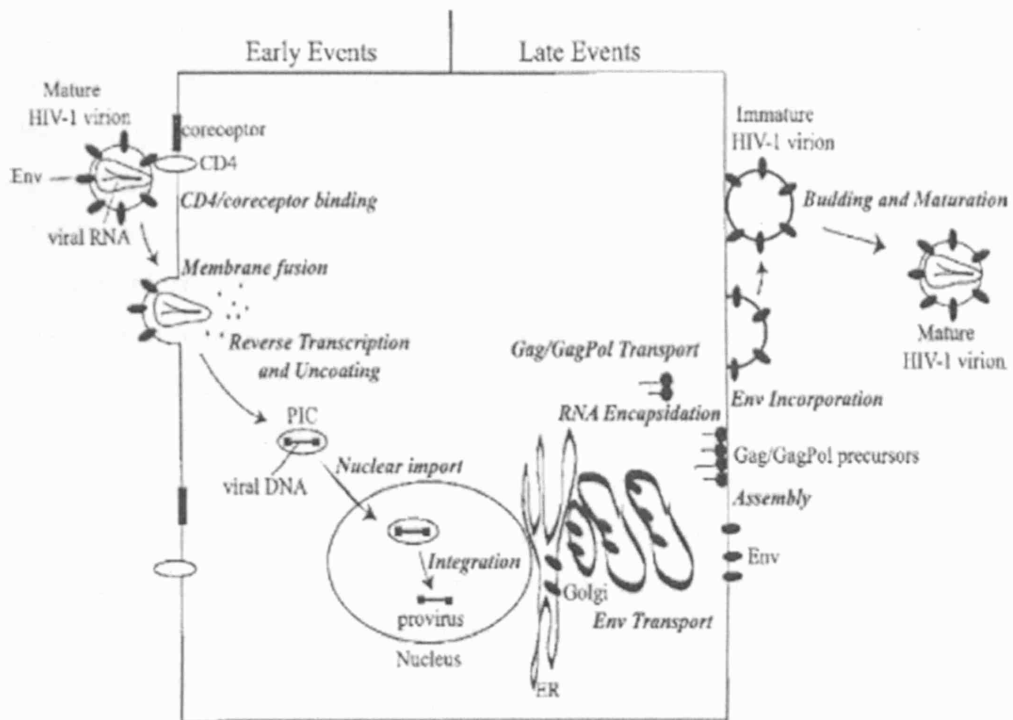
Figure 4. HIV-1 lifecycle. A schematic representation of the early and late events in HIV-1 lifecycle is shown above. From Freed E. 2001. *Somat Cell Mol Genet 16: 13-23.*

2.e.i Entry

The HIV-1 life cycle (Figure 4) begins with the interaction of the viral envelope surface glycoprotein with a specific cellular receptor and co-receptors and requires fusion of its membrane with the plasma membrane, a process mediated by the viral glycoproteins. After binding of the viral protein gp120 with its cellular receptor CD4 and interaction with its co-receptor CCR5 or CXCR4 (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996; Maddon et al., 1986), a conformational change occurs in the viral glycoprotein with subsequent exposure of a fusion domain in the viral envelope transmembrane domain (Hart et al., 1991; Sattentau and Moore, 1991; Sattentau et al., 1993). The V3 domain of gp120 is responsible for the recognition and utilization of the different co-receptors (Choe et al., 1996). Fusion of the viral envelope with the cell membrane then occurs in a pH-independent manner (Berson et al., 1996; McDougal et al., 1991) and a six-helix bundled gp41 ectodomain core structure that consists of three N helices with three anti-parallel C helices is formed concomitantly (Caffrey et al., 1998; Chan et al., 1997). A similar structure has been described for other enveloped viruses such as influenza virus and Ebola virus (Weissenhorn et al., 1999). The formation of this structure induces the fusion of viral and cellular membranes and the formation of a pore between both membranes (Markosyan et al., 2003). This ultimately leads to the release of the core into the cytoplasm (Figure 5).

2.e.ii Uncoating, reverse transcription and translocation to the nucleus

After viral entry, the viral genome is uncoated and reverse transcription is initiated (Figure 5). Little is known about the process of uncoating: after the delivery into the cytoplasm, the HIV-1 core undergoes a partial disassembly that leads to the formation of the reverse transcription complex (RTC). RTC composition varies between viruses: Moloney murine leukemia virus (MoMLV) RTC contains the viral genome, CA, IN and RT proteins. Sedimentation velocity analysis of MoMLV RTCs show that a gradual shedding of the CA is likely to occur during reverse transcription (Fassati and Goff, 1999).

On the other hand, HIV-1 RTCs are composed of the viral genome, IN and Vpr proteins mainly; most of the CA, MA and RT proteins dissociate early in infection (Fassati and Goff, 2001; Nermut and Fassati, 2003). HIV-1 RTCs associate quickly with the host cytoskeleton possibly through the interaction of the viral MA protein and the host's actin network (Bukrinskaya et al., 1998; McDonald et al., 2002). Some viral and cellular proteins influence uncoating and reverse transcription of retroviruses. Examples of this are cyclophilin A (CypA) and TRIM5α. The cellular proline isomerase Cyp A binds the HIV-1 capsid primarily in the target cell (Hatziioannou et al., 2005). CypA promotes HIV-1 infection in human cells as demonstrated in various experiments in which disruption of the CA-CypA interaction inhibited HIV-1 infection (Braaten and Luban, 2001; Thali et al., 1994; Towers et al., 2003). Cyp A binds specifically a proline residue in HIV-1 CA (Franke et al., 1994) and is involved in the correct disassembly of the viral core as has been shown by studies reporting the need for Cyp A before the initiation of reverse transcription (Braaten et al., 1996a; Braaten et al., 1996b). CypA also modulates restriction in some non-human primates like owl monkeys (Keckesova et al., 2006; Towers et al., 2003).

To initiate negative strand DNA synthesis (strong stop DNA), the viral RT uses a partially denatured cellular transfer RNA (tRNA): tRNA(Lys3) that binds to the primer binding site (PBS). Lysyl t-RNA synthetase (LysRS), a cellular enzyme that facilitates the selective packaging of tRNA (Lys3), is also packaged into HIV-1 virions by interacting with Gag (Kovaleski et al., 2006). Annealing of tRNA (Lys3) to the viral RNA requires RNA rearrangements and the disruption of the 3D structure of the tRNA to form a stable initiation complex that can be recognized by the viral RT. The 5' half of the tRNA (Lys3) binds the interface between the two subunits of the viral RT, which specifically interacts with the tRNA (Lys3) anticodon loop: this is necessary for the efficient negative strand synthesis of viral DNA (Arts et al., 1998; Mishima and Steitz, 1995). The annealing is mediated by the viral nucleocapsid but it also depends in the sequences flanking the PBS (Chan and Musier-Forsyth, 1997; Rong et al., 2001). HIV-1 has developed a strong preference for usage of tRNA (Lys3) as opposed to other tRNAs (Han et al., 2004).

HIV
membrane
HIV fusion
protein
gp120
CD4
chemokine
receptor

Extracellular
space

Cytosol

CD4 attachment   chemokine receptor binding   membrane insertion   fusion
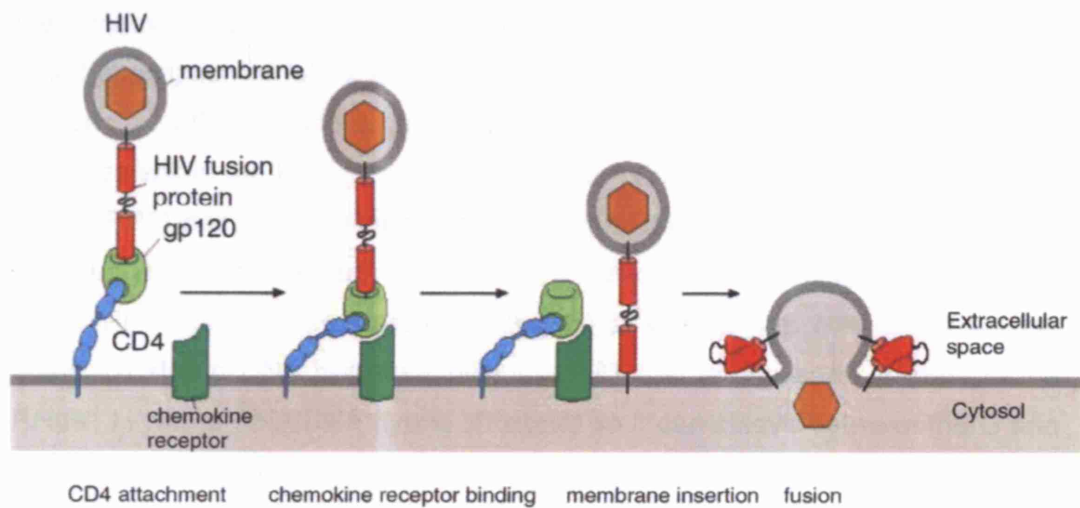
Figure 5. HIV-1 entry to the host cell. Panel shows first the binding of HIV gp120 with the CD4 molecule on the cell surface, it then shows binding with the co receptor (chemokine receptor that would be CCR5 or CXCR4); this interaction releases the fusion peptide (gp41) that was previously buried, to insert in the membrane and this ultimately leads to anchoring of gp41 to both membranes as a transient integral membrane forming then six helix bundles that liberates energy used to pull both membranes together with release of the core into the cytoplasm.(From Alberts et al, 2002; original adapted from a drawing by Wayne Hendrickson).

While RT synthesizes a DNA molecule using the RNA genome as a template, the RNaseH portion of RT degrades the RNA/DNA hybrid: the cleavage sites being selected according to the nucleotide sequence, permissible distance and accessibility (Schultz et al., 2006). The result of this first stage of reverse transcription is the negative strand strong stop DNA (SS DNA). It consists of the tRNA and a DNA copy of the 5' R and U regions. The homology between the 5' and 3' R regions allow a jump of the SS DNA and RT from the 5' to the 3' end of the genome, known as the first strand transfer. Reverse transcription continues until transcription of full length (-) DNA is complete. Before the synthesis of the full length (-) DNA strand is complete, the synthesis of the (+) strand starts from two sites within the HIV-1 genome: the 3' polypurine tract (PPT) and the central PPT (cPPT) sequences (Rausch and Le Grice, 2004): they are identical purine-rich sequences embedded within the viral genome. The precise initiation from the 3' PPT is critical. Processing of the (+) strand primers consists of several steps: (-) strand DNA synthesis over the PPT that is necessary to provide the RNase H with a RNA/DNA hybrid substrate so it can cleave between the G and A at the 3' end of the PPT. Data from structural studies suggest that this is possible by specific interactions between the abnormal structure in the PPT-U3 junction (i.e. bases display a geometry that deviates from the standard Watson-Crick) and residues in the HIV-RT p66 subunit (Rausch and Le Grice, 2004). After cleavage, (+) strand DNA synthesis and primer degradation takes place (Julias et al., 2002; McWilliams et al., 2006) (Figure 6). The additional PPT stretch in the *pol* gene also primes (+) DNA synthesis and this results in the formation of a 90 nucleotides triple stranded DNA "flap" at the center of the genome after completion of reverse transcription. This additional PPT is important for the synthesis of viral DNA and for efficient HIV replication (Charneau et al., 1992).

Movement towards the nucleus can not rely on simple passive diffusion because of pre-integration complexes' (PIC) relatively large size and because the cytoplasm is very viscous (Sodeik, 2000). This has led viruses to evolve mechanisms to hijack the cellular transport system to move within infected cells: the microtubule cytoskeleton is convenient as it radiates from the centrosome to the periphery, the minus end at the microtubule organizing centre and the plus end at the periphery for polarized movement (Sodeik, 2000). Examples of this

strategy are vaccinia virus, which has been shown to use the microtubules for its intracellular movement and the actin cytoskeleton to spread from cell to cell (Ploubidou et al., 2000; Rietdorf et al., 2001). Studies with herpes simplex virus 1 showed that dynein colocalizes with incoming capsids and that the administration of drugs that depolymerise the microtubules delays retrograde transport and viral protein synthesis (Kristensson et al., 1986; Sodeik et al., 1997; Topp et al., 1994). Adenovirus has been shown to use dynein and microtubules for its retrograde transport to the nucleus (Suomalainen et al., 1999). The shedding of the envelope or exit from the endosome is a requisite for movement of herpesvirus (HSV) and adenovirus towards the nucleus which points to the capsid as responsible for recruiting the transport machinery (Sodeik, 2000). Foamy viruses also use the microtubule network to translocate to the nucleus as demonstrated by studies with nocodazole (Saib et al., 1997b). Foamy viruses have been reported to concentrate around the microtubule organizing center after entering the cell and after their gag protein has interacted with dynein light chain 8 (Petit et al., 2003). The rabies virus and the African swine fever virus have also been shown to use the microtubule network to move within the cells (Alonso et al., 2001; Raux et al., 2000) and a similar role for the dynein light chain 8 has been reported with rabies virus (Poisson et al., 2001; Jacob et al., 2000). The dynein light chain 8 could be a bridge between the actin cytoskeleton and the microtubule network utilized by different viruses (Harrison and King., 2000).

In the case of HIV-1, it has been shown that an intact actin cytoskeleton is necessary for efficient reverse transcription (Bukrinskaya et al., 1998).
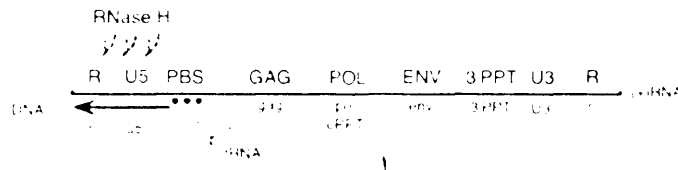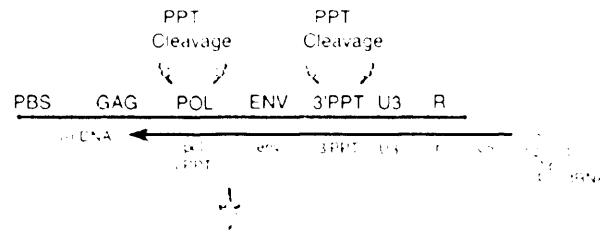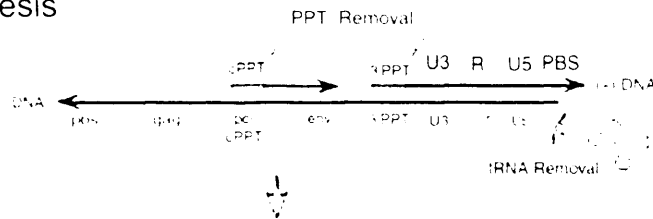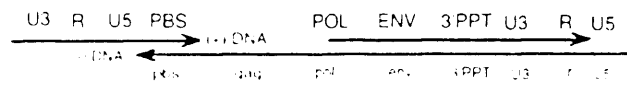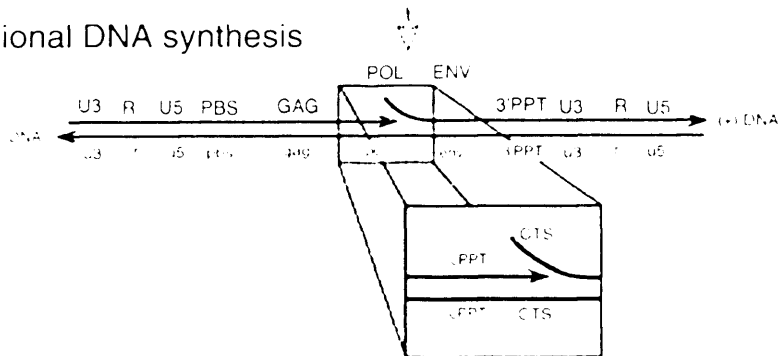
Figure 6. HIV-1 Reverse transcription A representation of HIV-1 reverse transcription is shown, with the most important steps indicated ((-) and (+) strand synthesis and transfer and bidirectional synthesis. The (-) DNA reverse transcription initiates from the PBS with the tRNA as a primer, after the (-) strand transfer, and once DNA has been synthesized over the 3'PPT and cPPT there is substrate for cleavage by either the RNase H or some other cellular enzyme, that creates the (+) strand primers. The synthesis of the (+) DNA carries on, the PPT primers are removed and the (+) DNA strand transfer occurs with bidirectional synthesis of both strands occurring: this creates a central DNA flap of 90 nt: this is removed by an endonuclease. The (+) strand synthesis is stopped by the central termination sequence (CTS). PBS: primer binding site, PPT: polypurine tract. From Rausch J and Le Grice S. 2004. *Int J Biochem Cell Biol 36*: 1752-66.

HIV-1 NC has been shown to interact with the actin cytoskeleton (Wilk, Gowen and Fuller, 1999) and HIV-1 mutants in NC did not co-fractionate with the cytoskeleton as opposed to wild type virus (Liu et al.,1999). During its trafficking through the cytoplasm, the RTC goes through a process of maturation. Although this maturation is not yet understood, it involves completion of reverse transcription and allows viral DNA to translocate into the nucleus and integrate in the host's chromatin more efficiently (Iordanskiy et al., 2006). Activation of the actin polymerization nucleator Arp2/3 has been involved in transport of HIV-1 towards the microtubules by studies using siRNA or inhibitors of this protein in which HIV, SIV and vaccinia virus infection was reduced. Interestingly, infection by viruses pseudotyped with VSV-G could not be inhibited (Komano et al., 2004). The migration of GFP-tagged HIV-1 along the microtubule network has been observed (McDonald et al., 2002) but it is unclear if this is the only cytoskeletal pathway used by HIV-1 to travel within the cytoplasm. HIV-1 IN has been shown to interact with microtubule-associated proteins like STU2p, a major component of the yeast spindle pole body in a two-hybrid system (de Soultrait et al., 2002). Rev has been reported to interact with microtubules destabilizing them (Watts et al., 2000) although the meaning of this is still not fully understood. There are some interesting reports on the trafficking of HIV-1 in polarized trophoblasts of the placenta. In these cells, HIV-1 enters by endocytosis and its trafficking involves different endocytic organelles. This process is coordinated by small GTPases of the Rab family, is independent of the viral proteins gp120 and gp41 and unlike what happens in CD4+ lymphocytes, it is essential for HIV infection to take place (Vidricaire and Tremblay, 2005).

2.e.iii Nuclear import

The retroviral life-cycle requires integration of the viral DNA into the host cell genome to form the provirus. Completion of reverse transcription with the generation of full length viral DNA produces the PIC. MLV and HIV PIC are large structures that contain viral and cellular proteins. MLV PICs have been reported to contain CA and IN while the PICs of HIV-1 are composed of the double stranded linear DNA plus the viral proteins MA, RT, IN and Vpr (Bowerman et al., 1989; Farnet and Haseltine, 1991; Fassati and Goff 1999; Li et al., 2000; Fassati and Goff 2001; McDonald et al. 2002; Nermut and Fassati 2003; Miller et

al., 1997). In addition to the viral proteins, PICs contain cellular proteins such as the barrier-to-autointegration factor (BAF), high-mobility group proteins (HMGs), Ku, lamina-associated polypeptide 2α (LAP2α) and lens-epithelium-derived growthn factor (LEDGF/p75) (Farnet and Bushman, 1997; Li et al., 2000; Lee and Craigie, 1998; Suzuki and Craigie, 2002; Lin and Engelman, 2003; Suzuki et al., 2004). The retroviral PIC is larger than the nuclear pore so it cannot rely on passive diffusion for traslocation into the nucleus. HIV-1 PICs have a Stokes diameter of at least 56 nm but the functional diameter of the nuclear pore is 39 nm maximum (Pante and Kann, 2002): this points to the fact that HIV must have developed a complex strategy to go across nuclear pores.

Murine oncoretroviral PICs on the other hand cannot enter the nucleus with an intact membrane so they have to wait for the dissolution of it when the cell is in mitosis: studies in which cells were arrested in at G1-S by starvation or addition of chemicals have shown that MLV RTC would finish reverse transcription but would not integrate (Roe et al., 1993). Lentiviruses PICs can access the nucleus directly (Lewis and Emerman, 1994; Weinberg et al., 1991). As a consequence, oncoretroviruses can replicate only in dividing cells but lentiviruses such as HIV-1 can replicate in non-dividing cells like macrophages (Weinberg et al., 1991).

The nuclear pore complex is a large protein structure that spans the nuclear membrane. Nuclear pores are channel-like structures constituted by approximately 30 different proteins called nucleoporins, with a diameter of 120 nm and a depth of 180 nm constituted by two rings joined by a central framework and with eight 10nm perforations. Nuclear pores allow the diffusion of molecules smaller than 9 nm across the nuclear membrane (Paine et al., 1975) and facilitate transport of molecules larger than 9 nm but up to 39 nm (Lim R et al., 2004; Mattaj and Englmeier, 1998). In fact, even small proteins or RNAs require active transport to cross the nuclear pores, even if their size theoretically would allow them to cross by passive diffusion (Breeuwer and Goldfarb, 1990; Zasloff et al., 1983). Nuclear pores respond to cellular stimuli: the presence of Ca2+ and the cell's state of differentiation and proliferation modulate the rate of nuclear import and the exclusion limit (Feldnerr and Akin, 1990; Stoffler et al, 1999).

Nuclear import is an active process that is driven by nuclear localizing signals (NLS) in proteins and requires interaction between the NLS with the nucleocytoplasmic shuttling receptors, known as importins, that belong to the karyopherin family (Peters, 2006). The first evidence of the existence of a nuclear transport signal was reported in 1982, in nucleoplasmin, a nuclear protein of *Xenoups laevis* oocytes (Dingwall et al., 1982). The first NLS identified was that of the SV40 large-T antigen which is the prototype NLS (Kalderon et al., 1984; Lanford and Butel, 1984; Robbins et al., 1991). NLS are short stretches of amino acids that bind to adaptors like importin beta or importin 7. The whole process is regulated by the small GTPase Ran (Pemberton and Paschal, 2005): an importin binds to its cargo (NLS) in the cytoplasm in the presence of RanGDP and it releases it in the nucleus as its affinity for the NLS decreases in the presence of RanGTP (Görlich et al., 1996).

As mentioned before, the capsid of many viruses, including HIV-1 is too big to cross the nuclear pore, thus an intact capsid may itself act as a negative element for nuclear import. There is evidence pointing to the capsid as a dominant negative regulator of nuclear import of retroviruses. Chimeric HIV-1 containing MLV CA, MA and p12 is unable to infect non-dividing cells (Yamashita and Emerman, 2004). It is possible that in these chimeric viruses MLV CA fails to dissociate from the RTC, making it too bulky to go across nuclear pores. Alternatively, excess CA on the HIV-1 RTC may bury NLS necessary for RTC nuclear trafficking. This model would explain why MLV infects dividing cells only.

Additionally, a general problem is that viral nucleic acids must enter the nucleus against a steep density gradient since the concentration of nucleic acids in the nucleus is very high and the central channel of the nuclear pore complex is hydrophobic while nucleic acids are charged, hydrophilic molecules (Fassati, 2006). To surpass all these obstacles, different viruses have evolved different strategies: adenoviruses dock their capsids in the cytoplasmic side of the nuclear pore (Morgan et al, 1969) where they uncoat and expose their genetic material to the nuclear (Greber et al., 1997). Herpes simplex virus docks its capsid at the nuclear envelope and then ejects its genome into the nuclear pore (Ojala et al., 2000). The strategy of HIV-1 seems to be the shedding of the capsid early in infection and the presence of NLS in many of the proteins present in the PIC.

Failure of HIV-1 to shed the capsid early in infection has been linked to inability to enter the nucleus and integrate (Dismuke and Aiken, 2006).

HIV-1 IN, MA, Vpr, all of which remain associated with HIV-1 PIC, and the viral cPPT element have been proposed to have a role in the nuclear import of HIV-1. In fact, the first NLS described in HIV-1 was in the N-terminal region of MA and mutations in two Lys residues in it blocked HIV-1 replication in terminally differentiated macrophages but not in proliferating cells (Bukrinsky et al., 1993). However, this matter remains controversial (Haffar et al., 2000; Popov et al., 1998) as there are reports showing that viruses with a mutant MA or even without most of MA can still infect dividing and non-dividing cells (Fouchier et al., 1997; Reil et al., 1998). However, there is some agreement in the fact that mutations in the N-terminal domain of MA modestly affect HIV-1 infectivity in both dividing and non dividing cells. The N and C terminal regions of the karyophilic protein Vpr also contain transferable NLS. They function in a Ran-independent way with a minimal need for energy (Jenkins et al., 1998), perhaps by direct interaction with nucleoporins. It has been therefore proposed that Vpr is responsible for docking of the PIC to the nuclear envelope (Popov et al., 1998). It has also been reported that Vpr disrupt the nuclear membrane which raises the possibility that Vpr can alter nuclear permeability to allow PIC to enter the nucleus (de Noronha et al., 2001). However, Vpr is dispensable for virus replication although it can increase viral infectivity and ability to propagate in certain cell types (Fouchier et al., 1998; Vodicka et al., 1998). The viral protein IN harbours non-classical NLS in its C terminal and central catalytic domain. IN can bind to and be imported by importin alpha, importin beta, importin 7 and transportin in a Ran-dependent way (Ao et al., 2005; Gallay et al., 1997; Tsurutani et al., 2000; Fassati et al., 2003). IN also interacts with the cellular protein lens epithelium derived growth factor, p75 (LEDGF/p75) (Cherepanov et al., 2003) that in turn interacts with chromatin and this interaction promotes integration and perhaps nuclear localization of IN (Fassati, 2006).

The central DNA flap might also play a role in nuclear import (Charneau et al., 1994; Zennou et al., 2000). The importance of the DNA flap in stimulating HIV-1 nuclear import has been confirmed in several studies using HIV-1 vectors (Ao et al., 2004; Manganini et al., 2002; van Maele et al., 2003; Zennou et al., 2001).

However the phenotype is less obvious when infectious HIV-1 clones such as LAI, YU-2, HXB2 and NL4-3 mutated in the cPPT element are used in spreading assays (Dvorin et al., 2002; Limon et al., 2002). More recently, a more substantial defect in nuclear import has also been observed in several infectious HIV-1 molecular clones mutated in the cPPT element (Arhel et al., 2006). The cPPT flap could be inducing the viral DNA to adopt a conformation that facilitates its translocation through the nuclear pore complex (Nisole and Saib, 2004).

Importin 7 has been shown to stimulate the nuclear import of HIV-1 PIC as depletion of this cellular protein by siRNA decreases HIV-1 infection although only by a few fold (Fassati et al., 2003). However, one report (Zielske and Stevenson, 2005) has failed to see a similar phenotype in imp7 knocked-down cells. This could be explained by the fact that sometimes even small amounts of residual cellular factors can still support near-normal levels of infection (Llano et al., 2006). More recently stable knock down of imp7 by shRNA has been confirmed to reduce HIV-1 infection (Fassati et al, personal communication). Imp7 may be implicated in chaperoning hydrophilic and charged viral nucleic acids across nuclear pores and may have a more general role in helping nuclear import of nucleic acids in general. Recently, tRNAs have been implicated in HIV-1 nuclear import, through the interaction of their T arm with cellular factors and their anticodon loop with the viral complex. These tRNAs have been found to be incorporated into and recovered from viral particles and could be transported into the nucleus on their own, in an energy and temperature-dependent fashion providing the first demonstration of retrograde nuclear import of tRNAs (Zaitseva et al., 2006).

2.e.iv Integration

The result of reverse transcription is a double stranded DNA copy of the viral genome containing the LTRs at each end: this constitutes the PIC (Fassati and Goff, 2001). Integration into the host cell genome involves two chemical reactions: 3' processing and the strand transfer steps. The 3' processing involves the removal of a pGT dinucleotide at the 3' end of both LTRs, adjacent to a conserved CA dinucleotide. During the strand transfer step the viral IN facilitates a concerted nucleophilic attack on phosphodiester bridges on both

sides of the major groove in the target DNA by the 3'-hydroxyl groups in the viral DNA (Van Maele et al., 2006). The 3' processing occurs in the cytoplasm within the PIC and the strand transfer inside the nucleus. The sites of the strand transfer for HIV-1 are 4-5 bp apart in the cell's chromosomal DNA which results in the duplication of 4-5 bp at each side of the integrated provirus: a hallmark of retroviral integration (Van Maele et al., 2006) (Figure 8). The integration process finishes with the repair of the gaps in the cell's DNA by host cell polymerases (Yoder and Bushman, 2000) (Figure 7).

Although the key protein necessary for integration is the viral IN enzyme, cellular factors also assist in the efficient and successful integration of the proviral DNA like HMGAI, BAF and the transcriptional co activator LEDGF/p75.

BAF is an 89 amino acid long cellular protein with a role in nuclear structure organization along with the lamina-associated protein 2 (LAP2), a protein that associates with the nuclear lamina (Zheng et al., 2000). BAF was first shown to be present in MoMLV PICs and to prevent auto integration (Lee and Craigie, 1994) but was later shown to restore the integration activity of salt-stripped HIV-1 PICs (Chen and Engelman, 1998). BAF is thought to contribute to virus assembly, to prevent self-integration and to promote correct conformation of HIV-1 PIC (Van Maele et al., 2006).

On the other hand, the nonhistone chromosomal high mobility group A1 (HMGA1) protein, has also been shown to restore the integration activity of purified and salt-stripped PICs *in vitro* (Beitzel and Bushman, 2003; Chen and Engelman, 1998; Farnet and Bushman, 1997) although HMGA1 does not seem to be important for integration in vivo (Beitzel and Bushman, 2003).

LEGDF/p75 stimulates *in vitro and in vivo* PIC integration activity and directs PICs to highly transcribed chromatin regions susceptible to LEGDF/p75 regulation (Cherepanov et al., 2005; Ciuffi et al., 2005; Van Maele et al., 2006). LEDGF/p75 is a 530 amino acids long (76 kDa) protein. It has a protein-protein and a protein-DNA interaction domain as well as a NLS. It also has an IN-binding domain and is mainly found in the nucleus, bound to chromosomes. There are studies showing that radical knock down of LEDGF/p75 by siRNA significantly
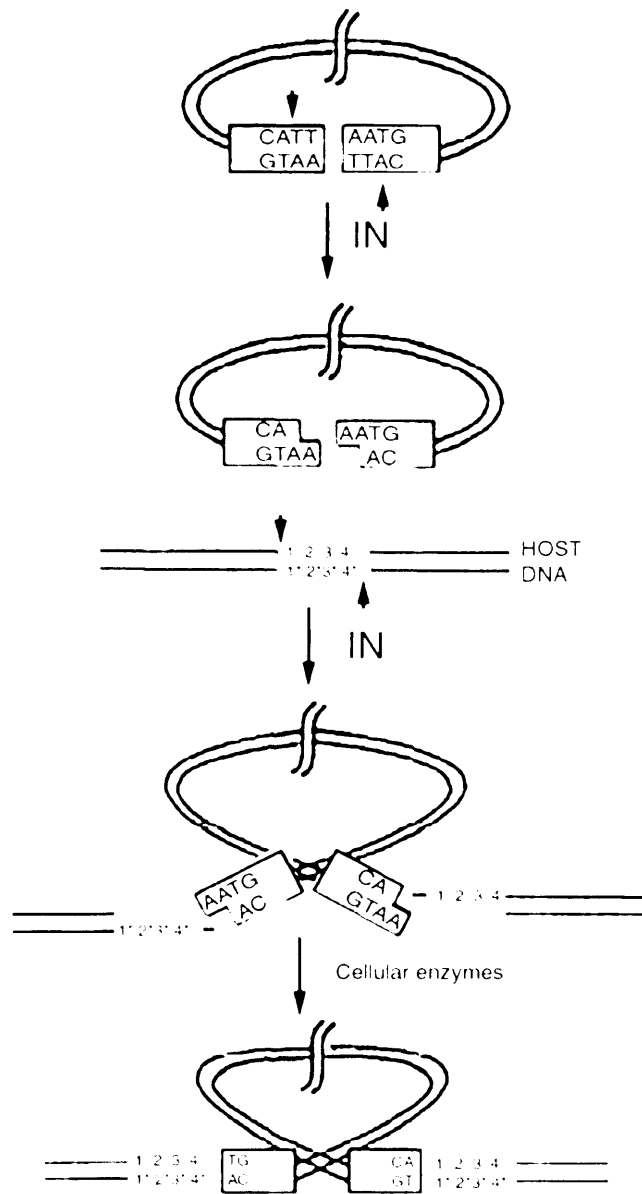
Fig 7. HIV-1 Integration. HIV-1 integration requires viral and cellular enzymes. The first step involves 3' processing of the viral DNA by the viral integrase (IN) which removes two nucleotides from each 3' strand, these attack the target DNA with a spacing of 4-5 nucleotides. The viral provirus integrates into the host DNA and cellular enzymes repair the gaps leaving 4-5 sequence duplication at either side of the provirus :the hallmark of retroviral integration. *From Goff S. 1992. Ann Rev Gen 26: 527-44.*

inhibit HIV-1 integration (Llano et al., 2006; Van Maele et al., 2006). Similarly, mutant viruses that cannot interact with LEDGF/p75 cannot integrate although they can enter the nucleus and these observations have been confirmed in nuclear import assays (Emiliani et al., 2005). The main mechanism used by LEDGF/p75 to facilitate integration is tethering of viral IN to the host DNA (Llano et al., 2006; Van Maele et al., 2006)

Finally, the integrase interactor 1 (INI1) protein, a 385 amino acid cellular protein can also bind the viral IN and interact with various cellular proteins. It has been reported to be important for HIV-1 virion production and has been found present in HIV-1 virions (Van Maele et al., 2006). Its role in HIV-1 replication is still unclear.

The sites of integration of HIV-1 PICs in the human genome are mainly within transcription units, maybe because of increased exposure and accessibility of actively transcribed regions or maybe because of interactions between the PIC and specific transcription factors, like LEDGF/p75 (Schroder et al., 2002). However, additional mechanisms may contribute to the selection of the integration site as MLV vectors have been shown to integrate selectively near or at 5' ends of genes (Wu et al., 2003) and ALV's integration is actually disfavored by high levels of transcription (Weidhaas et al., 2000).

2.e.v Transcription and nuclear export

Once integrated, the provirus is transcribed by the cellular RNA polymerase from its promoter and enhancers located in the 5' LTR and mRNAs are polyadenylated, spliced and exported from the nucleus. At the 5' end of all nuclear and cytoplasmic HIV-1 spliced and unspliced RNAs is the transactivation-responsive region (TAR), the Tat-responsive region of the HIV LTR. This is a folded stem-bulge-loop structure that binds Tat as well as cellular proteins. Tat has been shown to recruit the human cyclin T1 component of the positive transcription elongation factor b (P-TEFb) to the TAR element. Cyclin T1 binds cyclin-dependent kinase 9 (CDK9) also a component of P- forming the Tat-Cyclin T1-CDK9 complex This complex causes hyperphosphorylation of the C-terminal domain of the RNA polymerase II increasing its processivity and can

also recruit other cellular proteins and mediate efficient transcriptional elongation. TAR can also bind other cellular proteins that can increase or decrease HIV-1 translation. Mutations in TAR RNA have demonstrated its importance as they can greatly reduce HIV-1 trans-activation, translation and viral production (Bannwarth and Gatignol, 2005).

HIV-1 transcription generates a primary 9 Kb RNA that can follow different fates. It can be multiply-spliced to produce several short RNAs coding for Env and the regulatory proteins Tat, Rev and Nef, Vif, Vpr, Vpr. Tat transactivates the HIV-1 LTR by binding of the TAR element in the LTR and recruitment of cellular proteins enhancing transcriptional elongation by recruiting transcription elongation factors such as P-TEFb (composed of cyclin T1 and CDK9 and the bromodomain-containing protein 4 [Brd4]) which phosphorylate RNA polymerase II increasing its processivity. Other reports support the idea that Tat's main mechanism of action is at the transcription complex assembly, increasing the stability of the transcription complex (Brady and Kashanchi, 2005).

On the other hand, when Rev accumulates, it promotes the nuclear export of the two main mRNA species of 9 Kb and 4 Kb, the latter coding for Env. Rev does this by binding the RRE (Malim et al., 1989) (Figure 8) and recruiting the cellular protein Crm1, member of the karyopherin or importin/exportin nucleocytoplasmic transport factors (Fornerod et al., 1997; Fukuda et al., 1997; Yi et al., 2002). Crm1 binds to a leucine-rich motif in Rev that works as a nuclear export signal (Malim et al., 1991; Malim et al., 1990; Wen et al., 1995). This binding requires two cellular cofactors: the GTP-bound form of Ran and the Ran-binding protein RanBP3 (Fornerod et al., 1997; Lindsay et al., 2001). Rev is also aided in the nuclear export of viral RNA by host proteins such as actin and RNA helicases (Hofmann et al., 2001; Kjems and Askjaer, 2000; Yedavalli et al., 2004).

Once mRNA is in the cytosol, other cellular proteins such as Sam68 or the human Rev-interacting protein (hRIP) have been reported to facilitate trafficking to the sites of translation or assembly (Yu et al., 2005). The hRIP protein is very specific for RRE-containing RNAs, does not bind cellular mRNA (Sanchez-Velar et al., 2004), is located in the perinuclear region where the cellular machinery for vesicular trafficking is located (Doria et al., 1999) and contains a zinc-finger

domain with sequence similarity to a domain found in the family of ArfGAPs proteins that are involved in vesicular trafficking (Randazzo and Hirsch, 2004). The complete 9 Kb RNA is substrate for translation to produce structural proteins and enzymes and also for encapsidation in the new viral particles.

Gag and *pol* genes are expressed through an unspliced mRNA that goes from the 5' to the 3' LTR. Translation of this mRNA generally terminates after the *gag* open reading frame (ORF) but ~5% continues to the *pol* ORF producing a *gag-pol* polyprotein precursor. This occurs by either a termination suppression event or frameshifting between the *gag* and *pol* genes, depending on the retrovirus. The most common organization is that one found in HIV-1 in which *pro* and *pol* are in the same reading frame but (-1) relative to *gag* so frameshifting is necessary for translation of *pro* and pol (Jacks et al., 1988). In MLV and its relatives, *gag, pro* and *pol* are in the same reading frame which indicates *pro* and *pol* are translated by a read-through mechanism of the *gag* termination codon (Feng et al., 1989). HIV-1 Env protein is expressed from a spliced mRNA whose donor site is located between the 3' end of the 5' LTR and the 5' end of the *env* gene.

2.e.vi Particle formation, budding and maturation

To get to the plasma membrane, the viral proteins associate with the host cell's secretory pathway (Figure 5). HIV Gag has been shown to associate with actin *in vivo* and *in vitro* (Liu et al., 1999; Rey et al., 1996) and HIV virions have been shown to contain actin and actin-binding proteins such as erzin and moesin, found associated with the plasma membrane and more importantly, treatment of HIV-1 infected cells with cytochalasin D which disrupts the actin cytoskeleton reduces the release of HIV-1 by 40%. The viral NC p7 protein has been reported to bind F-actin directly, probably playing a role in assembly, in cellular fractionation experiments in which NCp7 and Gag co-purified with the cellular cytoskeleton except when Gag lacked the NC and p6; this was also true in co-sedimentation experiments (Liu et al., 1999; Wilk et al., 1999).

Early Phase (no or low Rev)
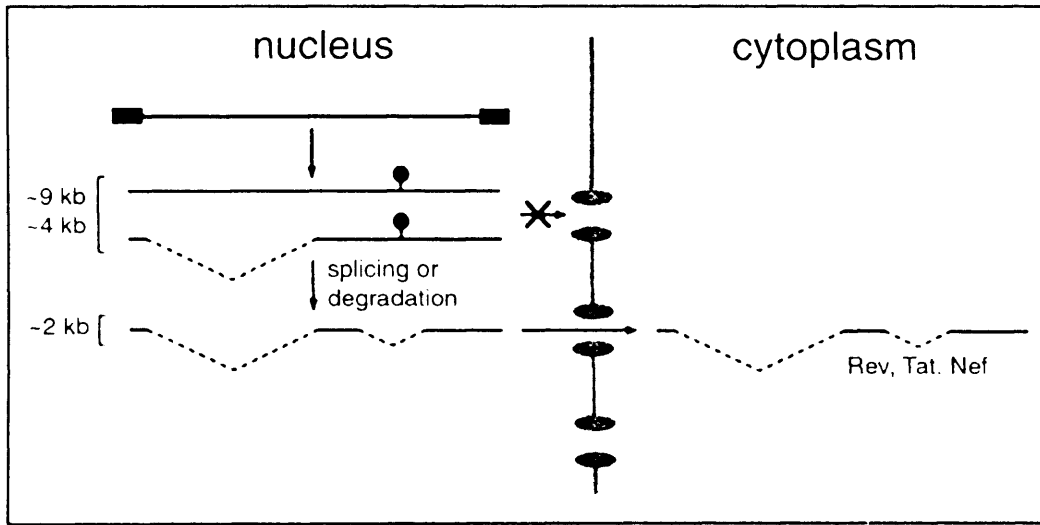
Late phase (high Rev)
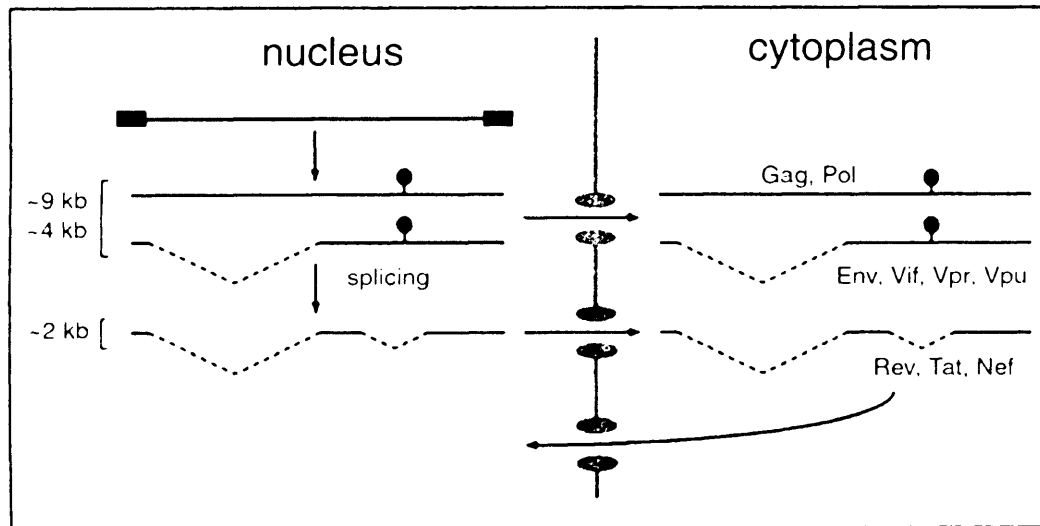
Figure 8. HIV-1 mRNA expression and splicing Full-length RNA (~9kb), partially spliced (~4 kb) and fully spliced (~2 kb) mRNAs are expressed in the nucleus in the early phase of mRNA transcription (while Rev levels are absent or low). The ~9kb and ~4kb species cannot be exported to the cytoplasm and are spliced or degraded but the ~2kb transcripts are exported and translated to produce Rev, Tat and Nef proteins. When Rev accumulates, it activates the nuclear export of ~9kb and ~4kb transcripts and all viral proteins are produced. Broken lines indicate introns, ball and stick indicates the RRE. From Pollard V and Malim M. 1998. *Ann Rev Microb 52*: 491-532.

Some full-length transcripts of the viral genome are packaged into the virion, a process mediated by the packaging signal or Ψ. The packaging signal is located at the 5' end of the genome within an intron, which ensures only unspliced full-length viral transcripts are incorporated. Recognition of the RNA is mediated by the NC portions of Gag. There is a balance between Gag's translation and RNA packaging to ensure that there are enough Gag proteins before the genome is encapsidated and this is regulated by Gag itself: translation is stimulated at low concentrations and inhibited at high concentrations of viral mRNA (Anderson and Lever, 2006).

The 5' untranslated leader sequence of the retroviral genome contains *cis* acting sites that have a role in RNA encapsidation (Lever et al., 1989) and efficient *gag* translation (Miele et al., 1996) besides dimerization as mentioned above. The packaging signal is located at the 5' end of the genome and it is an RNA structure that consists of three to four stem-loops (Harrison et al., 1998) termed SL-1 to SL-4 that form a cloverleaf secondary structure. The interaction between the packaging signal and the Gag polyprotein during encapsidation seems to rely on a conserved secondary structure of the viral RNA as there is little nucleotide sequence conservation across all retroviruses (Baudin et al., 1993; Harrison and Lever, 1992). The stem-loop 1 contains the DIS (Skripkin et al., 1994) and two highly-conserved internal loops called loop A and loop B (Greatorex et al., 2002). The tertiary structure of loop A resembles that one of the RRE and can bind Rev specifically (Gallego et al., 2003). In a recent study done with the infectious clone HIV_{HXB2}, this interaction has been found to be involved in nuclear export of viral genomic RNA and to some extent with RNA packaging into new virions (Greatorex et al., 2006). Mutations in this loop were reflected in a severely decreased viral replication despite a normal production of viral proteins. Virions with a mutant loop A showed dimeric genomic RNA, albeit more unstable than the wild type, suggesting non-dimerization of the genome was not the cause for the effects observed. The authors suggest that the interaction of Rev with loopA might influence the RNA trafficking that then affects its encapsidation.

During viral assembly, MA targets gag to the cellular membrane, CA establishes protein-protein interactions and NC interacts with the viral RNA genome recruiting it into the nascent virions. HIV-1 localize to lipid rafts to assemble and

bud from the cell as indicated by the enrichment of raft-associated molecules in virus particles, the finding of HIV-1 proteins in characteristically detergent-resistant membrane fractions and the inhibition of virus production with depletion of cellular cholesterol (Ono and Freed, 2001).

Other regions of Gag, like p6, recruit cellular proteins that aid in the process of viral assembly and budding. Gag proteins have short sequence motifs that when mutated, arrest the viral replication at late stages and for this reason, they have been called late domains (L domains) (Parent et al., 1995). They have been identified in many different viruses, including retroviruses, rhabdoviruses, filoviruses, arenaviruses and paramyxoviruses, amongst others (Morita and Sundquist, 2004). Different L domains recruit different cellular factors that are necessary for virus release. The mechanism of viral particle release promoted by retroviral L domains is not yet understood but recruitment of a cellular ubiquitin ligase by the L domain in p6 to the budding site has been proposed (Strack et al., 2000). Ubiquitination of lysine residues near HIV-1 L domain could be important for the interaction of Gag with cellular proteins and has been reported to be important for viral budding (Gottwein et al., 2006). The first L domain identified was p6 in HIV-1 Gag that recruits the cellular protein encoded by the tumor susceptibility gene 101 (TSG101) whose normal function is to sort proteins into multivesicular bodies (MVB). TSG101 has been shown to interact with the L domain in the p6 region of Gag through its N terminal region and it has been proposed that the ubiquitination of Gag resultant from this interaction is necessary for viral particle release (Goff et al., 2003; VerPlank et al., 2001). HIV-1 L domain in p6 belongs to the P(T/S)AP group. Another kind of late domain present in HIV-1 p6, a YPLTSL motif related to the YP(X)nL L domain group recruits the cellular actin interacting protein 1 (AIP1) that also normally helps in the process of cellular MVB vesicle formation (Morita and Sundquist, 2004). Normally in the cell, molecules that traffic from the plasma membrane or Golgi network to be degraded in the endosomal pathway do it through MVB. There are about 17 proteins identified so far that are involved in the formation of MVB and they are known as class E vacuolar protein sorting (Vps) proteins. They include the hepatocyte growth factor receptor substrate (Hrs) and the endosomal sorting complex required for transport (ESCRT) I to III complexes: TSG101 is part of some of these complexes. Ubiquitination of the proteins to be

degraded is generally needed as a sorting signal and three of the Class E proteins have ubiquitination activity. Mutations in the surface of ubiquitin block MVB sorting and HIV-1 budding and release although it has not yet been shown that Gag proteins are the target for ubiquitination (Morita and Sundquist, 2004).

When Gag molecules are targeted to the membrane, they form semispherical structures, the membrane then curves and pinches off releasing the newly formed virion. This is achieved by the concerted action of viral and cellular proteins and possibly lipids. AIP1, mentioned above, can interact with endophilins, cellular proteins that can curve membranes toward themselves and help regenerate the membrane during endocytosis (Morita and Sundquist, 2004).

Retroviruses can also bud intracellularly into endosomal membranes and exit via sites of cell-cell contact termed virological synapses. A virological synapse is a site of cell-cell contact where viral particles and receptors concentrate to allow viral entry and infection from an infected to an uninfected cell. Infection with cell-associated virus is more efficient than with cell-free virus (Carr et al., 1999; Jolly et al., 2004). The virological synapse is similar to the immunological synapse in that both are specialized contacts between a lymphocyte and some other cell, both contain protein microdomains and involve polarization of the cell cytoskeleton. They differ in that only the immunological synapse is triggered by T-cell recognition of an antigen while the virological one is formed as a consequence of viral infection and cell-cell contact (Nejmeddine et al., 2005). Virions budding intracellularly do it via the MVB and they are released from the cell when the endosomal compartment fuses with the plasma membrane (Morita and Sundquist, 2004). HIV-1 and SIV envelope proteins have trafficking signals that can direct the sites of virus budding that is necessary in polarized cells and in sites of cell-to-cell contact, for example, between infected and uninfected T cells or between dendritic cells and T cells, important for viral spread (Jolly et al., 2004; McDonald et al., 2003). An intact cytoskeleton and membrane lipid rafts are required for recruitment of Gag and Env proteins and for the formation of the virological synapse (Jolly, Mitar and Sattentau, 2007; Jolly and Sattentau, 2007). Virological synapses have a high concentration of adhesion molecules and show polarization of the microtubules network towards it (Morita and Sundquist, 2004; Nejmeddine et al., 2005). This has been observed most importantly in

Immature virion

- SU
- TM
- Gag precursor
- Vpr
- RNA
- Gag-Pol precursor
- HLA II DR
- HLA I
- Lipid
- Cyclophilin

Trimer

Mature virion

- Cyclophilin
- SU
- TM
- CA
- MA
- NC
- p6
- RT
- PR
- RNA
- HLA II DR
- HLA I
- Vpr
- IN
- Lipid

Figure 9. Virion maturation. The top panel on the left shows an immature HIV-1 virion. The Gag and Gag-Pol proteins are in different colors. The SU (surface) and TM (transmembrane) domains of the Env protein are shown protruding from the membrane as well as the HLA class I and II that have been incorporated into virions from the cell surface. The top panel on the right shows an EM photograph of an immature virion budding from the cell surface. The bottom panel on the left shows a diagram of a mature virion with the characteristic conical-shape core. The bottom panel on the right shows an electron microscopy photograph of a mature virion. From Coffin, Hughes and Varmus. 1996. *Retroviruses. CSHL Press.*

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macrophages chronically infected with HIV-1 but there are reports of this happening in other cell types such as activated Langerhans cells that can facilitate trans-infection of target cells (Fahrback et al., 2007).

During and after virus budding from the cell membrane, further processing of Gag and Gag-Pol by the viral PR takes place. Retroviruses are immature particles after assembly and require proteolytic maturation by the viral PR. This involves cleavage of the Gag polyprotein into MA, CA, NC and p6 to become infectious particles with a mature conical capsid (Briggs et al., 2003). Some of this processing occurs in the producer cell (Kaplan and Swanstrom, 1991) but most happens in the virion after budding. After cleavage, the CA protein dimerizes via its carboxyl-terminal domain which contains the major homology region, important not only for maturation but also for capsid assembly and infectivity (Gamble et al., 1997) (Figure 9).

2.f Cellular factors that interfere with viral replication

Many mammals have developed strategies to limit or restrict retroviral replication, some of which have been identified in recent years although their mechanism of action is still not very well understood in several cases (Figure 10). Restriction factors that block or inhibit retroviral replication can act at distinct stages of the life cycle and some have only been found in some species while the presence of some others is conserved among different species.

In the 1960s genetic studies revealed that the phenotypes of resistance to leukemia caused by the Friend murine leukemia virus had single locus patterns of inheritance and were dominant. The genes responsible for this phenotype were called Friend virus susceptibility factor 1 (Fv1) and 4 (Fv4) (Lilly and Pincus, 1973). Fv4 was identified in Japanese wild mice as a defective provirus lacking *gag* and most of *pol* but whose *env* protein was expressed on the cell surface (Kai et al., 1976; Odaka et al., 1981; Suzuki, 1975). Its expression conferred resistance and its mechanism of action was by interference with the ecotropic receptor in the target cell (Ikeda and Sugimura, 1989; Kai et al., 1986). Transgenic mice expressing FV4 were resistant to infection by Friend murine leukemia virus confirming that this gene conferred the block (Limjoco et al., 1993). This *env* gene had mutations that when introduced in MLV caused a block in its replication but could interfere with infection by wild type virus. This suggested the idea that a defective viral protein could protect from infection by a related pathogen (Goff, 2004).

On the other hand, Fv1 was found to decrease the incidence of leukemias in mice infected with restricted MLV strains (Lilly, 1967; Pincus et al., 1971; Pincus et al., 1975). Fv1 was shown to have two different alleles: $Fv1^n$ (present in NIH/swiss mice) and $Fv1^b$ (present in Balb/c mice). N-tropic strains of MLV can infect cells from Fv1n/n but cannot infect cells derived from Fv1b/b mice, and B-tropic strains of MLV does the opposite (Best et al., 1996; Pincus et al., 1971; Towers et al., 2002). There is a third class of MLV strain that is N and B-tropic, being able to resist restriction by both alleles. Fv1 blocks MLV infection after viral reverse transcription but before integration (Jolicoeur and Rassart, 1980; Pryciak and Varmus, 1992) and N- and B-tropism is determined by the residue 110 in the

CA protein (Kozak and Chakraborti, 1996). MLV infection has a 'two hit' kinetic when titrated in restrictive cells as preexposure with restricted virus or viral particles can overcome the block. Fv1 was later on found to be derived from the *gag* gene of endogenous retroviruses HERV-L and murine MuERV-L (Benit et al., 1997; Best et al., 1996). Its mechanism of action remains obscure. In humans, no ortologue of Fv1 was found but human cells also restrict N-MLV infection although they do it before reverse transcription. The restriction factor responsible for this phenotype was first called resistance factor 1 (Ref1) (Towers et al., 2000). It was also observed that the equine infectious anemia virus (EIAV), a very distantly related virus was also restricted in human cells and interestingly, it could abrogate infection by EIAV and by N-tropic MLV (Towers et al., 2002). It was also observed that lentiviral infection could be subject to restriction in a similar way in non-human primates: the restriction was saturable and was targeted to the viral capsid as shown by experiments done with chimeric SIV containing HIV-1 CA-p2 that was restricted as HIV-1 (Dorfman and Gottlinger, 1996). The block was again before reverse transcription (Hofmann et al., 1999; Towers et al., 2000). Due to these similarities, the presumed restriction factor in non-human primate cells was called lentivirus susceptibility factor 1 (Lv1) (Besnier et al., 2002; Cowan et al., 2002; Munk et al., 2002; Owens et al., 2003). Furthermore, it was observed that Lv1 and Ref1 could be abrogated with HIV-1 or with viral-like particles without RT activity (Besnier et al., 2003; Cowan et al., 2002; Kootstra et al., 2003). It was also observed that Lv1 and Ref1 restriction of infection of a given virus could be abrogated by a given virus could be abrogated by a different virus as long as both were restricted even and that they could inhibit multiple retroviruses with little sequence homology, leading to the idea that they could actually be species-specific ortologues of the same factor (Besnier et al., 2002; Cowan et al., 2002; Munk et al., 2002; Owens et al., 2003; Hatziioannou T et al., 2003).

The gene responsible for Lv1 activities was identified and is now known as TRIM5α. It was identified by screening a cDNA library derived from rhesus monkey (Stremlau et al., 2004) and confirmed by RNAi to knockdown the expression of TRIM5α. TRIM5α belongs to a large family of proteins characterized by an N-terminal domain that contains a RING, B-box and coiled-coil motifs and a C-terminal domain that in TRIM5α contains a B30.2 or SPRY

domain. The RING domain is a cysteine-rich zinc binding sequence of about 40-60 residues involved in protein-protein interactions. Some RING domains have ubiquitine E3 ligase activity (Joazeiro et al., 2000). The B box domains are zinc finger motifs of about 40 residues that have been found on several proteins important for development. The coiled –coil region is a structure formed by intertwining of multiple alpha helices, it is typical of myosin and probably responsible for the homo and heterodimerization of the TRIM proteins (Reymond et al., 2001). There are about 50 different TRIM proteins in mammalian genomes and they each localize in cellular compartments forming small structures that have been seen by immunostaining (Reymond et al., 2001). While in humans TRIM5α cannot restrict HIV, it is responsible for the Ref1 phenotype (Keckesova et al., 2004; Perron et al., 2004; Stremlau et al., 2004). In the African green monkey, TRIM5-α can restrict at least 4 different retroviruses and as mentioned before, in the owl monkey, the TRIM locus suffered an insertion of a CypA pseudogene resulting in a fusion protein: TRIM-CypA. As has already been pointed out, the discovery of this fusion protein suggested that the C terminal domain of TRIM5α is responsible for the ability to bind capsid (Bieniasz, 2004). Although it is clear that TRIM5α and TRIM-CypA bind the retroviral capsid, this has not been possible to prove with Fv1. The mechanisms of action of TRIM5α and Fv1 are not completely clear: they could be preventing interaction with some other host factor, or could be delaying or accelerating the capsid disassembly (Stremlau et al., 2004; Stremlau et al., 2006) or could be interfering with the correct trafficking of the virus (Schwartz et al., 1998). On the other hand, these factors could be recruiting additional cellular factors that lead to degradation of the virion: one possibility that has been suggested is ubiquitination, since some splice variants of TRIM5 have ubiquitinating capacity and proteosome inhibitors can rescue the reverse transcription defects mediated by TRIM5α (Wu et al., 2006). It has been reported, however, that the restriction mediated by TRIM-CypA is independent of the ubiquitin/proteasome system, irreversible and occurs almost immediately after the virus enters the cell (Perez-Caballero et al., 2005).

Exogenous and endogenous rhesus monkey TRIM5α (rhTRIM5α) has recently been reported to restrict HIV-1 in two steps: reverse transcription and nuclear entry. The first block can be alleviated with the addition of proteosome inhibitors

that leads to accumulation of reverse transcription products but the block to nuclear entry remains, as shown by the lack of 2LTR circles in the nucleus (Wu et al., 2006). This suggests that rhTRIM5α interacts with the viral CA altering its normal trafficking, which can then lead to RTC degradation.

Lv1/Trim5α activity against different retroviruses such as NMLV, HIV-2 and HIV-1, has also been found in cattle. However, the sequence homology between the bovine Lv1 and human TRIM5α is not extensive enough to be considered a TRIM5α ortholog. Interestingly, bovine Lv1 inhibits reverse transcription of HIV-1 and NMLV but not of HIV-2 (Ylinen et al., 2006).

Another important antiretroviral activity is provided by the APOBEC family of cytidine deaminases (Sheehy et al., 2002). The discovery of the restricting activities of this family came from studies showing that some cells, called permissive, could support HIV-1 infection with Vif-deleted strains while in cells called restrictive, Vif was necessary for efficient HIV-1 replication (Gabuzda et al., 1992). The restrictive phenotype was dominant in heterokaryons of permissive and non-permissive cells and the block was determined by the producer cell, rather than the target cell (Simon et al., 1998) (Madani and Kabat, 1998). APOBEC3G was then identified to be responsible for this phenotype and it has since been shown to mediate cytidine deamination in its main target: the (-) strand viral DNA (Yu Q et al., 2004). APOBEC3G is incorporated into nascent HIV-1 virions and cytidine deamination occurs during reverse-transcription in the target cell. This mutated viral DNA can suffer either of two fates: it is degraded after the uracil DNA-glycosylase enzyme removes the uracil residues making it a target for specific endonucleases, or it becomes hypermutated as the C to U substitutions are replicated to A residues on the (+) strand viral DNA. Hypermutation can lead to the production of stop codons when tryptophan codons (TGG) are encountered (Bieniasz, 2004) as APOBEC3G has particular preference for GG dinucleotides (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang H et al., 2003). Other members of the family also have antiviral activity, such as APOBEC3F although, perhaps because of its dinucleotide preference (GA) its effect is less lethal than that of APOBEC3G (Bieniasz, 2004; Zheng et al., 2004). As it has already been mentioned, HIV-1 has developed a way of defending itself from the deleterious effects of

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APOBEC3G: the Vif protein. Vif has been shown to counteract APOBEC3G in different ways: it may impair APOBEC3G translation, or it may bind to APOBEC3G targeting it for proteosomal degradation by binding at the same time the Cul5-elongin B-elonginC-Rbx1 ubiquitin ligase complex (Mariani et al., 2003; Sheehy et al., 2003; Stopak et al., 2003; Yu et al., 2003). There have been many gene duplications in the APOBEC locus in primates (Jarmuz et al., 2002). Even in the presence of Vif, G to A substitutions are relatively frequent in HIV-1 suggesting that Vif's counteraction is incomplete and as a result, the lentiviral genome is adenosine-rich

The existence of a still unidentified factor that restricts HIV-1 particle assembly and release unless overcome by Vpu has also been suggested (Varthakavi et al., 2003). Vpu is required for virus release in some cells but not in others and fusion between these two kinds of cells results in the production of heterokaryons in which Vpu is required for viral production (Geraghty et al, 1994; Gottlinger et al., 1993; Sakai et al., 1995). However, the putative factor has not been found and its mechanism of action is unknown. Vpu has been found to counteract some mechanism that stops assembled HIV-1 from being released from the surface of HeLa cells. This leads to endocytosis by a Rab5a and clathrin-dependent mechanism and sequestration inside endosomes of the viral particle (Neil et al., 2006).

In rat cells, a protein called Zinc finger antiviral protein (ZAP) inhibits viral gene expression posttranscriptionally by eliminating cytoplasmic viral mRNA of MLV and various Alphaviruses (Bick et al., 2003; Gao et al., 2002). A library of rat cDNA was introduced into the Rat2 cell line that lacks thymidine kinase, an enzyme that phosphorylates trifluorothymidine making it toxic. To identify dominant virus resistance genes, the transfected cells were grown in trifluorothymidine-containing medium and infected repeatedly with MuLV vectors containing the thymidine kinase gene. Uninfected cells were recovered and in one of them, the cDNA coding for ZAP was identified (Gao et al., 2002). ZAP contains a cluster of four CCCH-type zinc fingers typical of RNA-binding proteins, an example of which is a protein called tristetraprolin that targets RNAs rich in AU for destruction (Carballo et al., 1998). ZAP was found to interact with a protein complex responsible for mRNA turnover in mammalian cells (Mitchell et

al., 1997). A stretch of 653 nucleotides in the 3'LTR of MLV was identified as the target for ZAP activity (Guo et al., 2004). The antiviral activity of ZAP includes resistance to Sindbis, Semliki Forest, Ross River, Venezuelan equine encephalitis viruses and alphaviruses (Bick et al., 2002).

Absence or variation of host factors can also determine viral tropism (Bieniasz and Cullen, 2000; Yap et al., 2005). For example, murine cells possess a cyclin T1 (mCyclin T1) that has a tyrosine instead of a cysteine at residue 261. This inhibits its recruitment to TAR by HIV-1 Tat although it can bind the activation domain of HIV-1 Tat (Bieniasz et al., 1998). This cysteine residue has been reported to be essential specifically for interaction of human cyclin T1 (hCyclin T1) with Tat and the tyrosine residue in mCyclin T1 causes it to form only weak zinc-independent bond with Tat (Garber et al., 1998) which decreases its binding to TAR, even though hCyclin T1 has been found to have separate domains responsible for Tat and for TAR binding. However, rodent cells expressing a permissive cyclin T1 protein that supports Tat function have been shown to support reverse transcription, integration and early gene expression like human cells but show late defects which result in very low yields of infectious virus. These defects have a recessive phenotype in rodent and human heterokaryons pointing to possible factors necessary for the late stages of HIV-1 life cycle that are absent in rodent cells (Bieniasz and Cullen, 2000; Mariani et al., 2001).
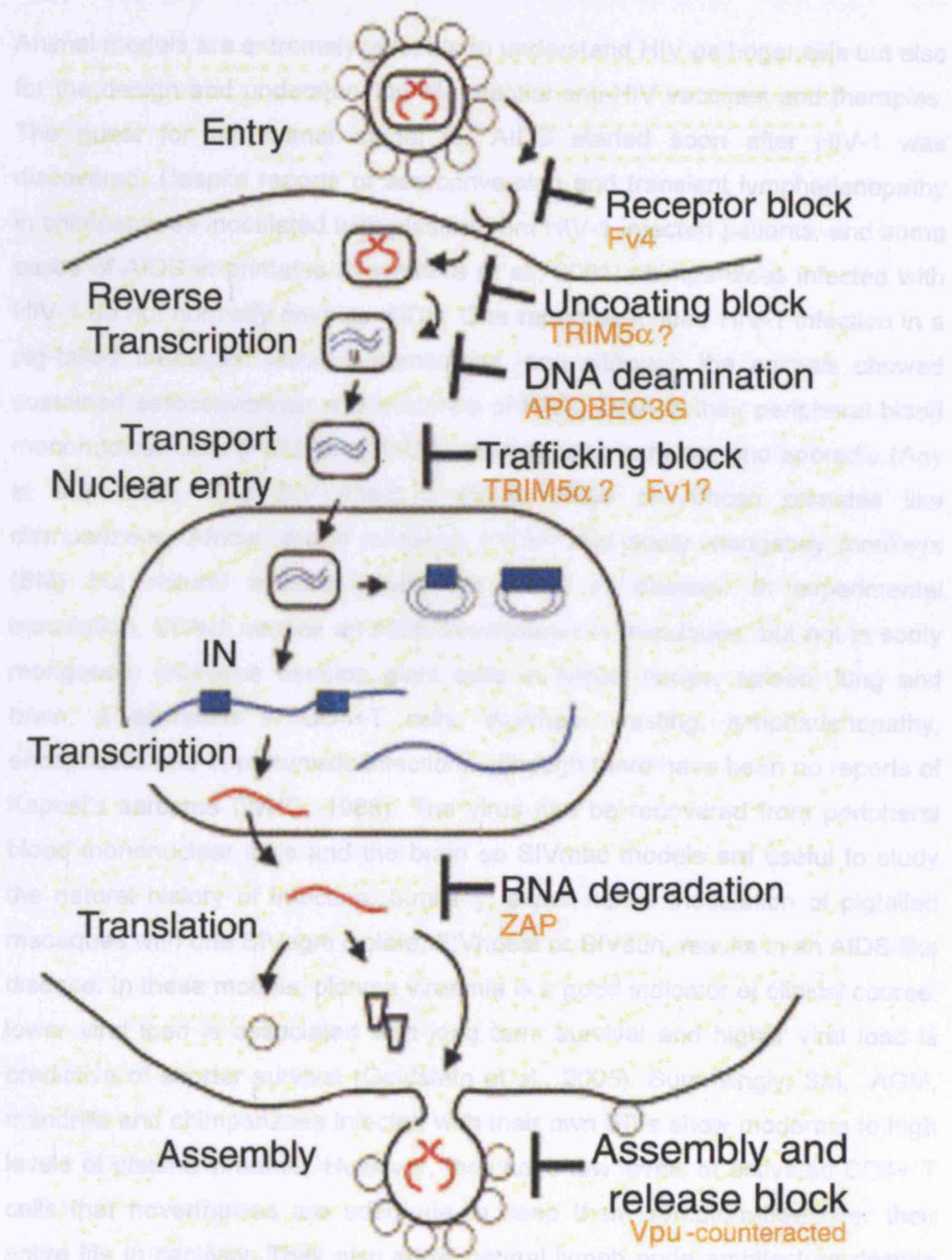
Figure 10. Blocks to retroviral replication. Identified blocks in different steps in the replication of retroviruses are shown. The name of the restriction factors is shown in colored letters. (?) Dubious. Adapted from Goff S. 2004. *Molecular Cell* 16: 849-59.

2.g Animal models for HIV infection

Animal models are extremely valuable to understand HIV pathogenesis but also for the design and understanding of potential anti-HIV vaccines and therapies. The quest for an animal model for AIDS started soon after HIV-1 was discovered. Despite reports of seroconversion and transient lymphadenopathy in chimpanzees inoculated with plasma from HIV-1 infected patients, and some cases of AIDS in primates (Novembre et al., 2001) chimpanzees infected with HIV-1 do not normally develop AIDS. One report described HIV-1 infection in a pig-tailed macaque: *Macaca nemestrina*, and although the animals showed sustained seroconversion and evidence of HIV-1 DNA in their peripheral blood mononuclear cells (PBMC) by PCR, infection was transient and sporadic (Agy et al., 1992). The SIV infect a broad range of African primates like chimpanzees, African green monkeys (AGM) and sooty mangabey monkeys (SM) but natural infection does not result in disease. In experimental inoculation, SIVsm causes an AIDS-like disease in macaques, but not in sooty mangabey: macaque develop giant cells in lymph nodes, spleen, lung and brain, a decrease in CD4+T cells, diarrhea, wasting, lymphadenopathy, encephalitis and opportunistic infections although there have been no reports of Kaposi's sarcoma (WHO, 1988). The virus can be recovered from peripheral blood mononuclear cells and the brain so SIVmac models are useful to study the natural history of infection. Similarly, experimental inoculation of pigtailed macaques with one SIVagm isolate, SIVhoest or SIVsun, results in an AIDS-like disease. In these models, plasma viraemia is a good indicator of clinical course: lower viral load is associated with long term survival and higher viral load is predictive of shorter survival (Goldstein et al., 2005). Surprisingly, SM, AGM, mandrills and chimpanzees infected with their own SIVs show moderate to high levels of plasma viraemia. However, they have low levels of activated CD4+ T cells that nevertheless are adequate to keep them symptom-free over their entire life in captivity. They also show normal lymph node architecture despite an antiviral cellular and humoral immune response. It is possible that SM and AGM can maintain a long-term balance between T cell renewal and loss and thus do not develop immunodeficiency (Hirsch, 2004).

On the other hand, the feline immunodeficiency virus also infects CD4+ T cells and produces respiratory, oral and gastrointestinal pathology with lymphadenopathy and opportunistic infections in infected cats and bovine immunodeficiency virus causes a generalized lymphadenopathy similar to that observed in AIDS.

Attempts to make an HIV-1 small animal model have been made with mouse and rats and there are reports of some limited success (Keppler et al., 2002; Potash et al., 2005) but none of the models so far can support robust viral replication in the context of an intact immune system or in the natural conditions of infection. Mouse cells do not support HIV infection because of several blocks in different steps of the viral replication: entry, transcription, nuclear import (Tsurutani et al., 2006), RNA splicing, polyprotein processing, assembly and release. Some rat cells have a block at the level of entry (Keppler et al., 2001) and reports of limited activity of CycT1 in these cells also exist. In some studies, mouse lymphocytes transgenic for human CD4 and human CCR5 or human CD4 and human CXCR4 were engineered but little or no infection was detected (Browning et al., 1997; Sawada et al., 1998). Other authors expressed human Cyclin T1 in mouse cells improving transcriptional activity of HIV-1 but mouse cells were still unable to support the complete replication cycle (Bieniasz and Cullen, 2000; Mariani et al., 2000).

The transgenic mouse models hu-PBL-SCID and SCID-hu can aid studies of HIV pathogenesis and vaccine design (Lapenta et al., 2003; Miura et al., 2001) although they have limitations: they cannot be used for long-term studies as they lack multi-lineage human haematopoiesis and a functional immune system, they are only useful for simulating acute HIV infection (Vieillard et al., 1999). Other humanized mice, the RAG-hu models, can sustain long term multi-lineage human haematopoiesis and so can mount immune responses after infection with HIV. They are useful for longer term studies as they can mimic HIV-1 pathogenesis such as CD4 T cell depletion in blood and thymus (Berges et al., 2006). However, they can sustain viraemia for only around 30 days.

The rabbit has also been investigated as a potential animal model for HIV-1 infection, because it is easy and inexpensive to breed. Rabbits have been used

as animal models extensively, for example in the design of a human papillomavirus vaccine (Brandsma, 2005; Mejia et al., 2006), in the study of HTLV-1 clinical course (Akagi et al., 1985; Cockerell et al., 1990; Kindt et al., 2000; Simpson et al., 1996a; Simpson et al., 1996b; Taguchi et al., 1993; Zhao et al., 2005), to analyze the immunological response to human foamy virus (Saib et al., 1997a; Santillana-Hayat et al., 1993; Swack and Hsiung, 1975) and infection by bovine immunodeficiency virus (Onuma et al., 1990; Van Der Maaten and Whetstone, 1992). However, they have not been used as animal models for HIV-1 with much success. Human CD4-expressing transgenic rabbits (Dunn et al., 1995; Sell and Tseng, 1995) have been engineered but have not proved useful. There is still controversy on the utility of these transgenic animals for the study of HIV-1 replication (Leno et al., 1995; Snyder et al., 1995). Some authors report an immune response to HIV-1 proteins and viral nucleic acids in the PBMCs of intraperitoneally infected rabbits (Debiaggi et al., 1995; Reina et al., 1993), and others report infection of rabbits by intravenous inoculation of HIV-1 infected T cells (Cockerell et al., 1991) but no manifestations of disease could be detected in any of these animals. In fact, susceptibility of rabbit to HIV-1 infection is a controversial issue

What is clear from all these studies is the great interest that rabbits as animal models have arisen since the early days of the HIV-1 epidemic (Filice et al., 1988; Truckenmiller et al., 1989) and that, after years of efforts, they still cannot be used to this end. Interestingly, the need for some factor provided by human cells was reported in early attempts to infect CD4+ rabbit cells with HIV-1 (Yamamura et al., 1991).

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2.h Objectives and scope of this thesis

HIV-1 infection is significantly impaired in rabbit cells (Besnier et al., 2002; Hofmann et al., 1999), however the nature of this block has not been investigated. The work described in this thesis attempts to elucidate the nature of the block, its stage in the viral life cycle, its consequences for viral replication and the viral determinants of this block. Elucidating the nature of the block to HIV-1 infection in rabbit cells may lead to the discovery of new cellular factors important for HIV-1 replication and open the way to the development of the rabbit as a new small animal model of HIV-1 infection.

Chapter 2. Materials and Methods

1. Materials

1.a Buffers and Solutions

Reagent	Composition
Terrific Broth	47 g Terrific Broth in 1000 ml of distilled water, + 4 ml glycerol, autoclaved at 121°C for 15 minutes
PBS	137 mM NaCl, 3 mM KCl, 10 mM Na$_2$HPO4, 2 mM K$_2$HPO$_4$ (pH 7.4)
TAE	40 mM Tris-HCl pH 7.8, 20 mM sodium acetate, 1 mM EDTA
DNA loading buffer	40% Glycerol, orange G in 1XTAE or distilled water
Endogenous reverse transcription buffer	10 mM Tris-HCl [pH 8.1], 15 mM NaCl, 6 mM MgCl$_2$, 1 mM DTT, 2 mM each dATP, dCTP, dGTP, dTTP
Preparation of competent bacteria	
TfB1	30 mM CH$_3$COOK, 100 mM RbCl, 10 mM CaCl$_2$, 50 mM MnCl$_2$, 15% glycerol
TfB2	10 mM PIPES, 75 mM CaCl$_2$, 10 mM RbCl, 15% glycerol
SSC 20X	3 M NaCl, 0.3 M C$_6$H$_5$Na$_3$O$_7$, pH 7.0
Western blot	
Western blot loading buffer	2% SDS, 125mM TRIS pH 6.8, 20% Glycerol, 0.1% Bromophenol blue, 2 mM EDTA and 2 mg/ml PMSF in distilled water
Western blot running buffer	30 g TRIZMA + 144 g Glycine + 10 g SDS in 1 L distilled water
Western blot transfer buffer	30 g TRIZMA + 144 Glycine + 5% CH$_3$OH in 1 L distilled water

TBE 1X	0.9 M Tris, 450 mM H_3BO_3, 20 mM Na_2EDTA pH 8.0
Virus production	
50 mM Phosphate buffer pH 7.4	7.74 ml of 1M Na_2HPO_4 + 2.26 ml of 1M NaH_2PO_4 + 190 ml of distilled H_2O
25% sucrose	w/w solution prepared in 50 mM sodium phosphate buffer pH 7.4, 1.12 g/ml density
45% sucrose	w/w solution prepared in 50 mM sodium phosphate pH 7.4, 1.22 g/ml density
Fractionation studies	
Hypotonic buffer	10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl
Isotonic buffer	10 mM Tris HCl [pH 7.4], 160 mM KCl, 5 mM $MgCl_2$
High Salt buffer	0.5M KCl, 10mM TRIS HCl pH 7.4, 5mM $MgCl_2$
1% Triton buffer	160mM KCl, 10 mM TRIS HCl pH 7.4, 5 mM $MgCl_2$, 1% Triton
SDS buffer	20 mM Tris HCl pH 8, 0.4% SDS, 10 mM EDTA
HIRT Lysis buffer	0.6% SDS, 100 mM Tris HCl pH 7.5, 10 mM EDTA
Fusion assay	
Permeabilisa tion solution	0.2% Triton in PBS containing 20 µg/ml RNAse DNase-free (see 2.1.a) and 5 mM $MgCl_2$
Blocking solution	10% foetal calf serum (FCS) in PBS

1.b Commercial reagents

Reagent	Supplier	Composition
Leupeptin	Sigma	20 µg/ml final
Dithiothreitol (DTT)	Fermentas	1 mM final
Aprotinin	Sigma	20 µg/ml final
SYBR-Gold Nucleic Acid Gel Stain (S-11494)	Molecular Probes	1mM in DMSO; excitation/emission peaks (bound to nucleic acids): 495/537 nm.
MOPS Buffer	Invitrogen	20X concentrated, proprietary mix, contains SDS
LB Broth	Sigma	1% Bacto Tryptone, 0.5% Bacto Yeast, 0.5% NaCl
In situ immunocytochemistry for HIV infection		
X-gal	Novolabs	0.5 mg X-gal (5-bromo-4-chloro-3-indolyl-ᕓ-ᴅ-galactopyranoside) substrate / ml in PBS, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1 mM magnesium chloride
Antibiotics		
Ampicillin	Sigma	50 µg/ml final
Tetracyclin	Sigma	12.5 µg/ml final
Molecular Weight Markers		
GeneRuler™ DNA Ladder	Fermentas	0.1 µg/µl DNA from pUC, pBR322, λphage and yeast genome Fragments range: 1 Kb: 10000 to 250 bp; 100 bp: 3000 to 100 bp
BenchMarkTM PreStained Protein Ladder	Invitrogen, UK	Protein ladder in 50 mM Tris-HCl, pH 6.8; 5 mM EDTA; 10 mM DTT; 1% (w/v) SDS; 10% (w/v) glycerol
DNA/BsuRI (*HaeIII*) Marker 9	Fermentas	0.5 µg/µl of ΦX174 completely digested with BsuRI Fragments range: 1353 to 72 bp.
Enzymes		
Proteinase K	Sigma	10µg/ml final

Taq Polymerase in Storage buffer B	Promega	5 u/μl, buffer composition: 20 mM Tris-HCl (pH 8.0 at 25°C), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween® 20 and 0.5% Nonidet®-P40
SuperScript™ III RT	Invitrogen	200 U/μl
RNase OUT™	Invitrogen	40 U/μl
E.coli RNase H	Invitrogen	2 U/μl
DNase I	Sigma	70 U/ml final concentration
Qiaprep Miniprep Kit		
P1 Buffer (Resuspension	Qiagen	50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A
P2 Buffer (Lysis)	Qiagen	200 mM NaOH, 1% SDS (w/v)
N3 Buffer (Neutralization)	Qiagen	Proprietary Mix, contains guanidine hydrochloride and acetic acid
PB Buffer (Binding)	Qiagen	Proprietary Mix, contains guanidium chloride, propan-2-ol
PE Buffer (Wash)	Qiagen	Proprietary Mix, contains 70% ethanol
EB Buffer (Elution)	Qiagen	Proprietary Mix, contains 10mM Tris, pH 8.5
QIAamp ® DNA Mini Kit		
Buffer AL (Lysis)	Qiagen	Proprietary Mix, contains guanidine hydrochloride
Buffer AW1 (Wash)	Qiagen	Proprietary Mix, contains guanidine hydrochloride
Buffer AW2 (Wash)	Qiagen	Proprietary Mix
PCR		
1x PCR buffer	Promega	500mM KCl, 100mM Tris-HCl pH 9.0, 1% Triton X-100

Quantitect PCR Probe Kit		
2X QuantiTect Probe PCR Master Mix	Qiagen	Proprietary mix, contains Tris, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$,pH 8.7 (20°C)
GenEluteTM High Performance (HP) Plasmid Midiprep Kit		
Resuspension solution	Sigma	Proprietary mix
Lysis Solution	Sigma	Proprietary mix
Neutralization Solution	Sigma	Proprietary mix
Binding Solution	Sigma	Proprietary mix
Wash Solution (1 & 2)	Sigma	Proprietary mix
Elution Solution	Sigma	Proprietary mix
Column Preparation Solution	Sigma	Proprietary mix
HiSpeed™ Plasmid Maxi Kit		
Buffer P3 (Neutralization)	Qiagen	3 M Potassium acetate, pH 5.5.
Buffer QBT (Equilibration)	Qiagen	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100(v/v/)
Buffer QC (Wash)	Qiagen	1 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)
Buffer QF (Elution)	Qiagen	1.25 M NaCL; 50 mM Tris-Cl, pH 8.5; 15% isopropanol (v/v)
Fusion Assay		
Fugene-6	Roche	Proprietary mix, contains lipids and other components in 80% ethanol, sterile-filtered.
BODIPY 630/650 cell tracker	Molecular Probes	8-bromomethyl-4-,4-difluoro-3, 5-bis-(2-thienyl)-4-bora-3a, 4a-diaza-s-indacene,

		Molecular Formula: $C_{18}H_{12}BBrF_2N_2S_2$. Molecular Weight: 449.14
TOTO-3 iodide (642/660)	Molecular Probes	1 mM solution in DMSO, molecular weight: 1355, absorption and fluorescence emission: 642 nm and 660 nm, excitation light source: He-Ne 633 nm
Silver Stain Plus		
Fixative Enhancer	Biorad	50% v/v methanol, 10% v/v acetic acid, 10% v/v fixative enhancer concentrate (proprietary mix), 30% v/v distilled water
Silver Complex Solution	Biorad	Proprietary mix; contains NH_4NO_3 and $AgNO_3$
Reduction Moderator Solution	Biorad	Proprietary mix; contains tungstosilicic acid
Image Development Reagent	Biorad	Proprietary mix; contains formaldehyde
Development Accelerator Reagent	Biorad	Proprietary mix; contains sodium carbonate
Silver Stain SDS-PAGE Standards, Low Range	Biorad	Proteins: rabbit muscle phosphorylase b, bovine serum albumin, hen egg white ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, hen egg white lysozyme in 50% glycerol (w/v), 300 nM NaN3, 20 mM Tris, 4 mM EDTA. Range: 14.4 to 97.4 kDa
Western and Southern Blot		
Enhanced Chemiluminiscence (ECL)	Amersham	Solution A: Proprietary mix, Solution B: Proprietary mix

Enhanced Chemiluminiscence Plus (ECL)	Amersham	Solution A: Proprietary mix, Solution B: Proprietary mix
Rapid-hyb solution	Amersham	Proprietary mix
Lenti-RT™ activity kit		
RT Reaction Component	Cavidi	Proprietary mix
Reconstitution Buffer	Cavidi	Proprietary mix
Concentrated Washing Buffer	Cavidi	Proprietary mix
RT Product Tracer	Cavidi	Proprietary mix
AP Substrate Tablets	Cavidi	Proprietary mix
AP Substrate Buffer	Cavidi	Proprietary mix

1.c Cell Culture

Reagent	Supplier	Composition
DMEM (Dulbecco's Modified Enrichment Medium)	Invitrogen	Contains GlutaMAX^{TM}-I substituted on a molar equivalent bases for L-glutamine, 1000mg/L D-glucose and 110 mg/L sodium pyruvate
Minimum Essential Medium	Invitrogen	Contains Earle's salts and L-glutamine
Fetal Calf Serum	Invitrogen	Heat Inactivated
PBS+EDTA		137 mM NaCl, 3 mM KCl, 10 mM Na$_2$HPO4, 2 mM K$_2$HPO$_4$ (pH 7.4), 0.5 mM EDTA
Trypsin	Gibco/BRL, Life Technologies	0.25% Trypsin + 0.05mM EDTA
Optimem Medium	Invitrogen	Modification of Eagles Minimum Essential Medium; hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors supplementation, 1.1 mg/L. phenol red, buffered with HEPES and sodium bicarbonate (2.4 g/L)
Freezing solution (2x)		30% FCS, 20% DMSO in DMEM media

1.d Antibodies

Antibody	Supplier	Specificity and use
anti-SIVgagp55/p27 antibody (ARP 3061)	NIBSC	Used for virus detection in immunostaining
anti-human PML (PG-M3)	SantaCruz Biotechnology	Texas Red conjugated mouse monoclonal IgG1 antibody, used for nuclei labelling in fusion assay.
Alpha tubulin Monoclonal (MCA77G)	Serotec, UK	Purified IgG2a (rat) in phosphate buffered saline pH 7.4; immunogen: yeast tubulin; used for Western blot.
HIV-1 p24 Monoclonal (AG3.0)	NIH AIDS Research & Reference Reagent Program	IgG1 isotype (Balb/c splenocyte x SSP2/10 oma); immunogen: p24, p55 and p150 (gag-rom HIV-1, HIV-2 and SIV; used for Western
HIV-1 p24 Monoclonal (#24-2)	NIH AIDS Research & Reference Reagent Program	Balb/c mouse splenocyte x SP2/0 myeloma (non-EBV transformed); Immunogen: Hexahistidine, amino-terminal tagged HIV-1 (HXB-3 isolate) p24 Gag protein; used for Western blot.
HIV-1 IN (IN2 and IN4)	Dr.Michael Malim, Guy's, Kings and St. Thomas' School of Medicine, UK	Monoclonal antibodies (mouse); Used for Western blot.
SIVgagp55/p27 (ARP 3061)	Central Facility for AIDS Reagents	Monoclonal (BALB/C); Immunogen: SIV p55/p2, reacts with ADP714 peptides 16 and 17. Used for immunostaining of SIV.
anti-mouse IgG (PO 447)	Dako, Denmark	Polyclonal anti-mouse IgG (goat), horseradish peroxidase conjugated; secondary antibody used for Western blot. .
Goat Anti-mouse IgM+IgG+IgA (H+L) (1010-06)	Southern Biotechnology	Pooled goat antisera hyperimmunized with IgM, IgG and IgA paraproteins from mouse; Immunogen: heavy and light chains of mouse

	Associates, Inc.	IgM, IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, IgG$_3$ and IgA.

2. Methods

2.a Molecular Biology

2.a.i Bacteria

2.a.i.1 Bacteria strains

The *Escherichia coli* strains used for propagation of all our plasmids were XL-1 blue (Invitrogen, UK) and HB101(New England Biolabs, USA), except the MLV-HIV capsid chimera plasmids, which were propagated in DH5-α (Invitrogen, UK) and the SIV-HIV RT chimera plasmids that were propagated in TOPO-10 (Invitrogen, UK).

2.a.ii Preparation of competent bacteria (heat shock)

10 ml cultures of HB101, XL1-blue, DH5-α or TOPO-10 (Invitrogen, UK) bacteria were grown overnight in LB broth (see 1.b). The following day, 2.75 ml of these cultures were used to inoculate 150 ml of LB broth and grown at 37°C with shaking until the OD 550 was 0.45 to 0.55. Cells were incubated on ice for 10 minutes and then centrifuged for 10 minutes at 3300 xg in a CR422 Centrifuge (Jouan, Thermo Electron, USA). The pellet was resuspended in 20 ml of TfB1 solution (see 1.a) and incubated on ice for 5 minutes. The cells were centrifuged as before and the bacterial pellet resuspended in 2 ml of TfB2 solution (see 1.a). Bacteria were incubated on ice for 10 minutes. Competent bacteria were frozen in 100 microliters aliquots and stored at –80°C.

2.a.iii Transformation of competent bacteria

One hundred µl aliquots of competent XL-1 blue, HB101, DH5-α, or TOPO-10 bacteria were thawed on ice and then mixed with 1 -10 ng of the plasmid DNA to be propagated. The cells and the DNA were incubated on ice for 30 minutes, heat-shocked at 42 °C for 45 seconds and then incubated on ice for 2 minutes. Bacteria were added to 1 ml of pre-warmed LB media and incubated at 37°C for 1 hr with shaking and centrifuged at 16060 xg for 30 seconds on a table micro centrifuge. The bacterial pellet was resuspended in 100 µl of LB media with no antibiotics, cells were diluted 1:10 and plated on agar plates containing the appropriate antibiotic (100 µg/ml ampicillin or 35 µg/ml tetracycline).

2.b Plasmid purification

2.b.i Minipreps

Plasmids were transformed into competent bacteria that were then grown overnight at 37 °C on agar plates. Single colonies were picked and grown in liquid culture with LB, at 37°C with shaking overnight. The next day, cells were pelleted by centrifugation at 3000 xg for 10 minutes 4°C and resuspended in buffer P1 (see 1.b.) transferred to a 1.5 Eppendorf tube and 250 μl of buffer P2 (see 1.b) were added inverting the tubes 6 times to mix. 350 μl of buffer N3 (see 1.b) were added and tubes were immediately inverted. Samples were centrifuged for 10 minutes at 17900 xg and supernatants applied to a QIAprep column (Qiagen, UK) which was centrifuged at 17900 xg for 1 minute. The column was washed once with 0.5 ml of buffer PB (see 1.b) and once with 0.75 ml buffer PE (see 1.b). DNA was eluted from the column using 50 μl of molecular biology grade water or EB buffer (see 1.b), letting the columns to stand for 1 minute and then centrifuging at 17900 xg for 1 minute.

2.b.ii Midipreps

Plasmids were transformed into competent bacteria that were then grown overnight at 37 °C on agar plates. Single colonies were picked and grown in liquid culture with LB (see 1.b) at 37°C with shaking until the OD at 600 nm was 1.1. This culture was then was diluted 1:500 in 250 ml of LB broth or Terrific Broth (see 1.a and 1.b) and incubated at 37°C for ~16 hours with shaking. Cells were harvested by centrifugation at 3000 xg for 10 minutes at 4 °C and the pellet was resuspended in 4 ml of Resuspension solution (see 1.b) and the tube was then vortexed, the mix was added 4 ml of Lysis solution (see 1.b) inverting the tube gently 6 times. Samples were allowed to sit for 5 minutes and then added 4 ml of Neutralization solution (see 1.b) gently inverting the tubes 6 times. Three ml of Binding solution (see 1.b) were added to the mix which was then immediately poured into the barrel of a filter syringe. This was allowed to sit for 5 minutes during which a binding column was prepared by addition of 4 ml of Column Preparation solution (see 1.b) that was left to pass through. The plunger of the syringe was inserted in and the cleared lysate was expelled into

the column. The column was centrifuged at 3000 xg in a swinging bucket for 2 minutes and eluate was discarded. The column was washed with 4 ml of Wash solution 1 (see 1.b) and centrifuged again for 2 minutes at 3000 xg. The column was washed again by the addition of 4 ml of Wash solution 2 (see 1.b) and centrifugation at 3000 xg for 5 minutes. DNA was eluted with ~1 ml of Elution solution (see 1.b) or molecular biology grade water to the column and centrifugation at 3000 xg for 5 minutes.

2.b.iii Maxipreps

Plasmids were transformed into competent bacteria that were then grown overnight at 37 °C with shaking. This culture was diluted 1:500 in 150 ml of LB broth or Terrific Broth (see 1.a and 1.b) and incubated at 37°C for ~16 hours with shaking. Cells were harvested by centrifugation at 3000 xg for 10 minutes at 4 °C and resuspended the pellet in 10 ml of buffer P1 from the HiSpeed^{TM} Plasmid Purification kit (see 1.b) followed by 10 ml of buffer P2 (see 1.b) after which tubes were gently inverted and incubated at room temperature for 5 minutes. After incubation, 10 ml of chilled buffer P3 (see 1.b) were added, inverting the tube immediately 6 times and pouring the mix into the barrel of the QIAfilter Cartridge. This was incubated at room temperature for 10 minutes and a HiSpeed Midi tip was equilibrated with 10 ml of buffer QBT (see 1.b) allowing the column to empty by gravity flow. The plunger was inserted into the QIAfilter Midi cartridge and the cell lysate was filtered into the equilibrated tip. The cleared lysate was allowed to enter the resin by gravity flow and the tip was washed with 60 ml of Buffer QC (see 1.b). DNA was eluted by ~10 ml of buffer QF (see 1.b) and precipitated with ethanol if further concentration was needed.

2.c.i Phenol-chloroform extraction and ethanol precipitation of nucleic acids

Nucleic acids (either DNA or RNA) were extracted treating samples with 1 volume of phenol, mixed and centrifuged at 17900 xg for 3 minutes. The aqueous phase (upper layer) was removed and mixed with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1), mixed and centrifuged at 17900 xg for 3 minutes. The aqueous phase (upper layer) was removed and mixed with 1 volume of chloroform, mixed and centrifuged at 17900 xg for 3 minutes. The aqueous phase (upper layer) was removed and adjusted to 300 mM sodium acetate ($C_2H_3NaO_2$), 2.5 volumes of 100% ethanol and 1 μl glycogen (5mg/ml), incubated at least 2 hours at -80 °C and centrifuged at 17900 xg for 10 minutes. The pellet was washed in 200 μl of 70% ethanol, centrifuged at 17900 xg for 5 minutes and air dried at room temperature. DNA or RNA pellets were resuspended in molecular biology grade water and kept at 4 °C for immediate use or stored at -20 °C.

2.c.ii Total DNA extraction from cells

Approximately 6 X 10^6 cells were trypsinized and washed with PBS and pelleted by centrifugation at 3000 xg for 5 minutes. Total DNA was extracted with the Qiamp® DNA Minikit (Qiagen, UK) as follows: the pellet was resuspended in PBS to a volume of 200 μl, 20 μl of Proteinase K (see 1.b) were added and 200 μl of buffer AL (see 1.b) while vortexing for 15 seconds. The tubes were incubated at 56 °C for 10 minutes and added 200 μl of 100% ethanol. After pulse-vortexing samples were applied to a QIAmp Spin Column (Qiagen, UK) and centrifuged at 6000 xg for 1 minute. The column was washed with 500 μl of Buffer AW1 (see 1.b), centrifuged at 6000 xg for 1 minute, washed again with 500 μl of Buffer AW2 (see 1.b) and centrifuged at 23900 xg for 3 minutes and placed in a clean tube to collect the DNA by eluting with 200 μl of molecular biology grade water and centrifuging at 6000 xg for 1 minute.

2.c.iii Electrophoresis of nucleic acids

DNA was fractioned according to size by electrophoresis using either agarose (Roche, Basel, Switzerland) or polyacrylamide (BioRad, USA) gels. Different concentrations of agarose were used according to the expected size of the fragment to analyze: 1% agarose for 10 to 0.5 kb DNA fragments, 1.5% agarose for 3 to 0.2 kb DNA fragments or 1% agarose plus 2% low melting agarose (NuSieve, Cambrex, USA) for 300-100 bp DNA fragments. Gels were made with 1% or 1.5% w/v agarose in 1X TAE buffer (see 1.a) and 0.1 µg/ml ethidium bromide and submerged in 1X TAE buffer in a horizontal electrophoresis tank Horizon 11-14 (Gibco, UK). DNA was prepared in DNA loading buffer (see 1.a) and 15 µl loaded into each well and electrophoresed for approximately 45 minutes at 80 to 100 V. DNA was visualized by illumination with short wave (254 nm) ultraviolet light, photographs were taken with a Kodak DC290 digital camera and analyzed with KODAK 1D Image Analysis Software (V 3.6).

Polyacrylamide gels were made with 7.5% AA-Bis polyacrylamide (BioRad, USA) in TBE (see 1.a) with 75 µl of 10% Ammonium Persulfate and 20 µl of N,N,N',N'-tetramethylethylene diamine (TEMED) to polymerize for a 5 ml volume. Gels were placed in a mini-vertical gel unit Hoefer SE-250 (Amersham, UK) with 0.5X TBE buffer. DNA was prepared in DNA loading buffer (see 1.a) and samples loaded into each well and electrophoresed for 45 minutes at 100 V. Gels were then submerged in a 1:10 000 solution of SybrGold (Molecular Probes, UK) in 1XTBE (see 1.a) for 45 minutes at room temperature and analysed in a STORM 860 phosphorimager (Molecular Dynamics, Uppsala, Sweden).

Depending on the size of the DNA to be analyzed, the molecular weight markers used were GeneRuler TM 1 kb DNA Ladder, GeneRuler TM 100 bp DNA Ladder Plus, or ΦX174 DNA/BsuRI (*HaeIII*) Marker 9 (see 1.b).

2.d Polymerase chain reaction

The PCR allows the amplification of specific DNA sequences. The reaction has an initial logarithmic increase of the target sequence followed by a plateau where there is no increment. The final amplified product can be visualised by ethidium bromide after agarose gel electrophoresis or Sybr Gold staining after polyacrylamide gel electrophoresis. All PCR reactions were performed in a final volume of 50 µl containing 1x PCR buffer ($MgCl_2$-free), 100 µM of each dNTP, 1 mM $MgCl_2$, 5 U of Taq polymerase (Promega, USA), and 30 pmol of each primer. Cycle parameters were as follows: 94°C for 3 min the first cycle; 94°C for 1 min, 55°C for 30 s, and 68°C for 1 min for 25-30 cycles; followed by one final extension cycle at 68°C for 10 min. PCR product were resolved on a 1.5% agarose gel and visualized by ethidium bromide. The primers' sequences are shown in Table 1.

2.e Real time PCR

Real time PCR allows very close monitoring of the amplification process at every cycle in real time. This is possible by the measurement of fluorescence signals that are proportional to the amount of the PCR product that is generated (Wilhelm and Pingoud, 2003). Sequence specific fluorescence probes (TaqMan) or fluorescence dyes that bind double stranded DNA can be used to generate the fluorescence signals. The DNA extracted from various experiments was analyzed by TaqMan PCR using the QuantiTect Probe PCR kit (Qiagen). Quantitative PCR reactions were carried out in 25 µl volume containing 2X Quantitect Probe Master Mix (see 1.b), 0.3pmol of each primer, 0.15 pmol of the probe (GFP and Late) and 208 ng of carrier DNA. For amplification of early products of reverse transcription conditions were slightly different: 200 nM of primers and probe (RU5) were used with 2X Quantitect Probe Master Mix and 208 ng of carrier DNA in a final volume of 50 µl. For primer sequences and probes please refer to supplementary Table 1. In all reactions, samples to be analyzed were standardized to 100 ng or 500 ng of DNA or a volume of 1 microliter. Cycle conditions were the following: step 1 was 95 °C for 10 minutes, step 2 was 95 °C for 15 seconds and 60 °C for 1 minute for 50 cycles.

All quantitative PCRs were performed in the ABI Prism® 7000 Sequence Detection System (SDS). This system consists of a thermal cycler and a laser directed via optical cable fibres to each of the 96 sample wells. The emission of the fluorescence signal from each sample is collected and the data is analysed automatically by SDS software to calculate the threshold cycle (Ct) value, melting curve and gene copy number. The Ct parameter is the cycle number at which the fluorescence passes a fixed threshold above the baseline. The threshold is calculated as ten times the standard deviation of the average baseline fluorescence signal measured between cycles 3-15. The fluorescence signal detected above the threshold representing the background is analysed to calculate the Ct value. After amplification, a melting curve in which the temperature is slowly increased from 60 °C to 95 °C is performed. To determine DNA copy number an external standard curve was constructed using plasmids containing the target sequences: pCNCG and pHR' were used in dilutions

series from 10 to 10^5 copies, to run reactions with the same conditions as above. All samples were run in triplicate.

2.f RT PCR

Nucleic acids were extracted from the different fractions of a cellular fractionation (see 2.h.vi) carried out in RNAse-free conditions by phenol-chloroform extraction and ethanol precipitation (see 2.c.i) and then resuspended in RNase-free water. Samples were digested with 10U/ml RNAse-free DNase I (see 1.b) at 37°C for 30 minutes and the DNAseI was inactivated by the addition of 2mM EDTA and incubation at 60^0C for 20 minutes. For first strand cDNA synthesis, RNA was mixed with 200 pmol Oligo dT_{20} probe, biotin-labelled (Invitrogen, UK) and 1mM dNTPs mix (Invitrogen, UK) in a total volume of 10 μ following the manufacturer's instructions. Samples were incubated at 65°C for 5 minutes, placed on ice for 1 minute and then adjusted to 10 mM $MgCl_2$, 2 mM DTT, 40 units of RNase OUT (see 1.b) and 200 units of Super Script III Reverse Transcriptase (see 1.b) in its own buffer. Samples were incubated at 50°C for 50 minutes followed by one minute at 85°C and treated with 2 U of *E.coli* RNAse H (see 1.b) at 37°C for 20 minutes. The reaction products were then used as template for PCR or TaqMan real time PCR (see 2.d and 2.e).

2.g Cell culture

2.g.i Cell lines

HeLa (human cervical carcinoma) (Gey et al. 1952), 293T (human embryonic kidney) (Graham et al., 1977), rabbit cell lines SIRC (rabbit cornea epithelial) (Leerhoy, 1965), EREp (rabbit embryonic skin epithelial) (American Type Culture Collection CRL-6498,(Pifat et al., 1992)) and CrFK (*Felis catus*, kidney cortex epithelial) (American Type Culture Collection CCL-94,(Crandell et al., 1973) cell lines were grown in Dulbecco modified Eagle's medium (DMEM, Invitrogen, UK) supplemented with heat-inactivated 10% foetal calf serum (FCS, Gibco/BRL, Life Technologies) in 5% CO_2/95% air at 37°C . Owl monkey cells (OMK) (adult owl monkey kidney) were also grown in 5% CO_2/95% air at 37°C in minimal essential medium (Invitrogen, UK) supplemented with 10% FCS, non-essential amino acids, penicillin 100 U/ml, streptomycin 100 µg/ml (Gibco/BRL) and glutamax (see 1.c).

2.g.ii Cell passaging

Cells were passaged every other day rinsing with PBS+EDTA (see 1.c) and then incubating with trypsin (see 1.c) at 37 °C for approximately 2 minutes, diluted in 10ml of their own media, centrifuged at 1000 *xg* for 3 minutes in a Sorvall tabletop centrifuge RT7, the pellet resuspended in media and replated in 1:4 dilutions or in specific densities as required.

2.g.iii Freezing and thawing cells

Cells were pelleted as described above, resuspended in DMEM (see 1.c) and an equal volume of freezing solution (see 1.c) was added. Cells were frozen at -70°C in Nunc cryovials inside in a polystyrene box to freeze slowly. The next day they were transferred to vapour phase nitrogen. When needed, cells were thawed at 37°C, immediately added to 10 ml of fresh media and centrifuged at 1000 *xg* for 3 minutes. The cell pellet was then resuspended in media and plated.

2.h Cell biology

2.h.i Virus production

2.h.i.1 Plasmids

pV1/HTLV, used in the fusion assays, is an HIV-1-based expression vector, pV1, in which the sequence of HTLV-I *env* gene has been inserted. It only has Tat, Rev, and Vpu HIV-1 genes and the *cis*-acting sequences necessary for their expression as well as a CMV promoter replacing the HIV-1 5' U3 region (Cowan et al., 2002).

Viral vectors

HIV-1 vectors were produced by co transfection of pMDG, pCMVΔ8.2 and either of two plasmids: pHR or pCSGW / pCSPW. These last two plasmids are the transducing vectors, which contain the cis-acting sequences required for packaging, reverse transcription and integration. pHR' contains HIV-1 sequences such as the two LTRs, the packaging signal, and the RRE; pCSGW is a self-inactivating vector that also contains non-HIV sequences such as the spleen focus-forming virus strain P long terminal repeat sequence (SFFV) and the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) in addition to the cPPT element (Bainbridge et al., 2001; Demaison et al., 2002; Naldini et al., 1996). pCSGW carried the enhanced green fluorescent protein (eGFP) reporter gene and pCSPW carried the phosphorybosil acetyl transferase reporter gene (Hobbs et al., 1998). The latter construct was a kind gift from Dr. Greg Towers. pCMVΔ8.2 derives from pCMVΔR9 but contains a stop codon in the *env* reading frame followed by a *Not I* site as well as a deletion of a large portion of the *env* coding sequence (Naldini et al., 1996). Plasmid pMDG encodes the vesicular stomatitis virus G-protein (VSV-G) envelope driven by the human cytomegalovirus immediate early promoter (hCMV). It also contains polyA site from the β-globin gene (Naldini et al., 1996; Zufferey et al., 1997). All these plasmids contain the simian virus 40 (SV40) origin of replication in the backbone.

The SIV packaging constructs were derived from SIVmac251 (GenBank accession number M19499). The Gag-Pol expressor used in the production of SIV vectors was pSIV3+. This plasmid has the human cytomegalovirus early-immediate promoter and enhancer region in place of the U3 region in the 5' LTR. It has the RRE, *tat* and *rev* genes and the SV40 polyadenylation sequence instead of the 3' LTR. In pSIV3+ the R, U5 and 5' half of the leader region have been removed (Negre et al., 2000).The SIV packaging vector used was pSIV-RMES4 which contains the eGFP gene under the control of the CMV promoter placed between the LTR and the leader sequence. It carries the RRE elements but no *gag* or *pol* sequences. In fact, only 15% of its sequence is derived from the parental SIVmac251 RNA genome (Mangeot et al., 2000).

For MLV vectors, the Gag-Pol expressor used was pC1G3N or phCMV-intron. The *gag-pol* sequences of pC1G3N are derived from pWN41, a clone of WN1802N MuLV virus of BALB/c (Boone et al., 1989). Its backbone is the pCI-neo (Promega) and it contains the SV40 polyadenylation sequences (Bock et al., 2000). The *gag-pol* sequences of phCMV-intron are derived from pHIT60 and are CMV-driven. This plasmid contains the SV40 origin of replication (*ori*) (Soneoka et al., 1995).

For the production of the integrase-mutant HIV-1 vector, pD64V gag-pol expressor was used along with the VSV-G and packaging vectors described above. pD64V is pHIV-Hygro, a vector with most of the env gene replaced by a sequence of a hygromycin resistance gene as a selectable marker (Leavitt et al., 1996; Leavitt et al., 1993) with a single D → V substitution at position 64 of integrase.

To make the RT-SHIV chimeric virus and its SIV control, we used pBRmac239 and pBRmacRT-SHIV, a kind gift from Klaus Überla (Bochum, Germany). Both plasmids are molecular clones of SIVmac239 provirus (Naidu et al., 1988) but pBRmacRT-SHIV has the RT sequence HIV-1 IIIB clone HXBc2 instead of SIV RT (Uberla et al., 1995). These plasmids were co-transfected with pMDG to make virus capable of infecting non-CD4 expressing cells in single rounds of infection.

To make the MLV-HIV *gag* chimeric viruses, we used HIV-based chimeras in which the MLV sequences replace precisely the HIV sequences and are derived from the infectious HIV provirus pLai and MLV provirus pAMS (Miller et al., 1985; Peden et al., 1991). They were used as Gag-Pol expressors along with pMDG and pCSGW to make virus. HIV-mMA (pME411-deltaEnv), an HIV-1 molecular clone, contains MLV MA sequences, MHIV-mMA12 (pBru3-deltaEnv-mMA12) contains MLV MA and p12 sequences and MHIV-mMA12CA (pBru3ori-deltaEnv-mMA12CA-luc2) contains HIV MA, p12 and CA sequences (Yamashita and Emerman, 2004).

2.h.i.2 Viral Production, purification and storage

Viral vectors were prepared by co-transfection of sub confluent 293T cells with three plasmids (Soneoka 1995, Zufferey et al, 1998). Cells were plated onto a 10 cm dish diameter and after overnight incubation transfected with a mixture of 18 µl Fugene-6 in 200 µl Optimem media (see 1.c) and 1 µg of a VSV-G expressor plasmid, 1.2 µg of the corresponding Gag-Pol expression vector and 1.5 µg of the retroviral vector This mix was incubated at room temperature for at least 15 minutes and then added drop wise to the cells. Twenty-four hours later, transfection media was replaced by fresh DMEM + 10% FCS. Virus-containing supernatant was collected at 48, 72 and 96 hours post-transfection.

The supernatant was filtered through a 0.45 µm filter and pH was adjusted with 1M HEPES pH 7.4 (10mM final). Viral stocks were incubated in the presence of 70 U/ml DNase I (see 1.a) with 5mM $MgCl_2$ for 1 hour at 37°C and purified through a two step 25-45% sucrose gradient (20ml viral supernatant was placed first in cold 37ml polypropylene tubes, followed by 5 ml of 25% sucrose and by 45% sucrose placed sequentially at the bottom of the tube) at the bottom of the tube by centrifugation at 68,726 *xg* at 4°C for 2 hours in a Sorvall Discovery Surespin Rotor. The sucrose solutions (w/w 25% and 45%) were prepared adjusting the final density of the solutions to 1.12 g/ml and 1.22 g/ml respectively. This was done by weighting 100 µl of the sucrose solutions in a Mettler Analytical balance AE260 Delta Range. After centrifugation, virus was collected by perforating the bottom of the tubes, discarding the first 3.5 ml and collecting the following 3 ml that have the density at which HIV-1 bands. The

virus was then aliquoted and stored at -70°C. Stable producer cells of HIV-1 pseudotyped with amphotropic MLV envelope, ISTAR and 100R26 virus, were kindly provided by Yasuhiro Ikeda (Ikeda et al., 2003). Supernatant from these cells was harvested, filtered through a 0.45 µm filter and kept frozen in aliquots at −70 °C.

2.h.ii Virus detection assays

2.h.ii.1 RT ELISA

Reverse transcription activity of viral stocks was measured with a commercial kit, the Lenti-RT™ Activity Assay (Cavidi Tech, Uppsala, Sweden) following the manufacturer's instructions. This assay measures the incorporation of Bromo-deoxyuridine triphosphate (d-UTP) into an immobilised primer/template that is catalysed by the viral RT. An anti-BrdUTP antibody conjugated to alkaline phosphatase is then added to the plate. The color change of the AP substrate is proportional to the amount of RT activity. The kit provides a polyA plate that must be incubated initially reaction mixture (RT Reaction Components reconstituted by the Reconstitution Buffer and distilled water, see 1.b) at 33°C for 60 minutes, the samples were then added in 1:5 and 1:10 dilutions as starting points and further diluted 1:5, incubating at 33°C overnight. The next day, the reaction was stopped by washing with the Washing Buffer E (see 1.b) diluted 1:400 in Triton-X (diluted 1:13 in distilled water) and the RT Product Tracer (see 1.b) previously reconstituted in 1% Triton were added per well. Plates were incubated at 33°C for 90 minutes and then washed. The alkaline phosphatase reaction was started by the addition of the AP substrate solution (AP Substrate Buffer P2 with dissolved AP Substrate Tablets P1, see 1.b) and incubation at room temperature, in the dark, for 30 minutes, 2 hours and overnight. A standard is provided with the kit to allow for absolute quantification of the RT values in the samples to be analyzed. Absorbance was measured in a Lucy 1 luminometer (Anthos-Labtech, UK) at 405 nm and analysed using Manta software (Dazdaq, UK) using a four parameter curve. RT values were used to standardize infection in different cell types.

2.h.ii.2 Flow cytometry

Viral titers of HIV-1, SIV, MLV vectors and chimeric viruses expressing eGFP were measured on HeLa, 293T, SIRC and OMK cells by flow cytometry. Three days after infection cells were trypsinized, resuspended in 500 μl of isotonic buffer and transferred to Falcon FACS tubes (Beckton Dickinson, UK). Ten thousand events were collected on a LSR flow cytometer (Becton Dickinson) and the percentage of eGFP positive cells was determined using Cellquest software. Uninfected cells were always analysed as negative controls and used to gate the infected population.

2.h.ii.3 Abrogation assay

Abrogation assays are used to test for the presence of a saturable restriction factor. The principle is that treatment of target cells with a restricted virus or virus-like particles can saturate the restriction factor and facilitate infection by a second restricted virus. Abrogation assays were performed by infecting 2×10^5 cells/well in 6 well plates with ten-fold serial dilutions of HIV-1-Puro virus (8000-0.008 pg/ml RT activity) to saturate any restriction factor, together with a constant amount of HIV-GFP virus (enough to infect 1% of the respective cell type). Cells were analyzed by fluorescence activated cell scanning (FACS) 48 hours after infection. For SIRC cells, up to 11,000 pg/ml RT activity of HIV-1 Puro virus were used (enough to infect $\geq$70% of SIRC cells).

2.h.ii.4 Antibody detection of virus infection

To detect infection with SIV/HIV RT chimeras, cells were fixed with cold (-20°C) methanol-acetone at a ratio of 1:1 for 10 minutes at room temperature and washed twice with PBS containing 1% FCS An anti-SIVgagp55/p27 antibody (see 1.d) was then added at a dilution of 1:200 in PBS +1% FCS and incubated for 1 hour at room temperature. Cells were washed three times for 5 minutes each wash with PBS +1% FCS and then incubated with a secondary anti-mouse antibody f(ab)'2 fragments conjugated to ß–galactosidase (Southern Biotechnology Associates, Inc) at a dilution of 1:400 in PBS +1% FCS. Cells were washed twice for 5 minutes each wash with PBS +1% FCS and twice with

serum free PBS. Infected cells were immunostained blue by adding X-gal (see 1.b). Blue stained cells were considered infected and counted (Clapham et al., 1992).

2.h.iii Long term infection

1.5×10^6 SIRC or HeLa cells were plated in T-75 flasks. Twenty-four hours later, cells were infected with an HIV-1 vector, the HIV-1 integrase-mutant vector p64Δ8.2 or an NB-MLV vector all at an MOI of 0.3 in the presence of 8μg/ml polybrene. Forty-eight hours later an aliquot of infected cells was analysed by FACS. After 2 weeks of continuous culture, cells were trypsinised and resuspended in PBS. An aliquot of the cells was again analysed by FACS and total DNA was extracted with the Qiamp® DNA Minikit (see 2.b.i) and subjected to Real-Time TaqMan PCR analysis (see 2.e).

2.h.iv Fusion assay and analysis of syncitia by immunolabeling

Cells were plated in 6-well trays (2×10^5 cells/well) at different ratios and combinations: 293T and HeLa (1:1), only HeLa, only 293T, 293T and SIRC (1:1) or only SIRC in a volume of 2 ml of media. Twenty-four hours later, media was changed for 2 ml of fresh DMEM + 10% FCS. Cells were transfected using 24.5 μl of Optimem with 2.2 μl of Fugene-6 and 1.5 μg of pV1-HTLV1env, per well (Cowan et al., 2002). Cells were incubated at 37°C overnight. Twenty-four hours later, fusion was assessed by direct observation under the light microscope.

Twenty-four hours after transfection with pV1/HTLV, syncitia were infected with enough HIV-eGFP to infect ~1 % of SIRC cells (according to previous titration of the viral stock) in the presence of 8 μg/ml polybrene. Two days after infection, media was removed and cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature, permeabilized with permeabilization solution (see 1.a), incubated 30 minutes at room temperature and blocked with blocking solution (see 1.a) 10% FCS in PBS for 30 minutes at room temperature. Immunolabeling was performed with an anti-human promyelocytic leukaemia (PML) protein antibody (see 1.d) diluted 1:25 in PBS and TOTO-3 (1:10,000)

(see 1.b). Cells were incubated for 1 hour at room temperature in the dark. Samples were washed with PBS three times 5 minutes each and analyzed on a Bio-Rad MRC 1024 confocal microscope equipped with a krypton-argon laser. Images were acquired sequentially and merged later by using Lasersharp confocal assistant software (Bio-Rad) (Nermut and Fassati, 2003).

2.h.v Fusion assay and analysis of syncitia by FACS

SIRC and OMK cells were trypsinized, washed with PBS and incubated with BODIPY 630/650 cell tracker (see 1.b) diluted 1:2000 in Optimem (4 ml total volume) for 50 minutes at 37°C in the dark. SIRC and OMK cells were washed twice with PBS and plated in 6-well trays on their own or in combination with 293T cells at a ratio of 1:1 and a total density of 2×10^5. Eight hours after plating, cells were transfected with 1.5 μg of pV1/HTLV (Cowan et al., 2002) using Fugene-6 (as described in 2.h.i). Thirty-six hours after transfection, cells were infected with an amount of virus sufficient to infect 1% of SIRC or 0.5% OMK cells. Forty-eight hours after infection, cells were analyzed by FACS. Cells were first analysed according to their size and granularity in a forward scatter (FSC-H) and side scatter (SSC-H) density plot. A population of cells with increased size appeared only when 293T were present and pV1/HTLV was transfected. This population was gated and analysed in a dot plot for GFP expression (FL-1H) and BODIPY 630/650 signal (FL-5H). Double positive cells were considered as infected syncytia containing SIRC or OMK cells fused with 293T cells. This population of cells was not present in the wells where only SIRC or OMK cells had been plated and transfected with pV1-HTLV-1 as controls

2.h.vi Cell fractionation

5 X 10^6 SIRC or HeLa cells were plated in medium flasks. Twenty-four hours later, the cells were infected with a multiplicity of infection (MOI) of 0.3 in the presence of 8 μg/ml polybrene. Samples were incubated for 2 hours at 4°C to allow virus binding but not internalization and then for 4 hours at 37°C. Cells were then trypsinized and washed in PBS. All subsequent manipulations were carried out at 4°C. The pellet containing the infected cells was resuspended in 5 volumes of hypotonic buffer (see 1.a) plus DTT, aprotinin and leupeptin (see 1.b) and centrifuged for 5 min at 1100 *xg* in a tabletop microcentrifuge. Supernatant was kept in a separate tube and the pellet was resupended in 5 volumes of hypotonic buffer and incubated for 10 minutes on ice. Cells were homogenized with 10-15 strokes in a Dounce homogenizer. Disruption of cell membrane and integrity of nuclei was monitored by Trypan blue staining diluted 1:1. Samples were centrifuged at 3,300 *xg* for 15 minutes. The supernatant was clarified by centrifugation at 7,500 *xg* for 20 minutes. The pellet was resuspended in isotonic buffer (see 1.a) plus DTT, aprotinin and leupeptin (see 1.b) and centrifuged at 4°C at 3,300 *xg* for 15 minutes. The supernatant was collected and the pellet was resuspended in high salt buffer (see 1.a) plus DTT, aprotinin and leupeptin (see 1.b) and centrifuged as before. The supernatant from this step was collected and the pellet was resuspended in a 1% Triton-X buffer (see 1.a) plus DTT, aprotinin and leupeptin (see 1.b) and centrifuged at 4 °C at 7,500 *xg* for 10 minutes. The supernatant from this step was collected and the pellet was resuspended in SDS buffer (see 1.a). Aliquots of all samples were digested with proteinase K (see 1.b) in SDS buffer (see 1.a) at 55°C for 16-18 hours followed by phenol-chloroform extraction and ethanol precipitation of nucleic acids (see 2.c.i). For extraction of RNA of the cell fractions, the same procedure was carried out in RNase-free conditions.

2.h.vii Purification of nuclei

To purify intact nuclei, cells were lysed by Dounce homogenization in hypotonic buffer (see 1.a) as described. The resulting pellet was gently resuspended in ice-cold 0.4% NP-40 (IGEPAL CA-630, SIGMA) in isotonic buffer (see 1.a) plus DTT, aprotinin and leupeptin (see 1.b) and quickly centrifuged at 700 *xg* for 5

minutes. The supernatant was collected and the pellet washed once in isotonic buffer (see 1.a) plus DTT, aprotinin and leupeptin (see 1.b). The integrity of nuclei was monitored by light microscopy at high magnification using trypan blue staining. Thirty μl of each fraction were collected and mixed in a proportion of 1:1 with Western blot loading buffer (see table in 1.a) for Western blotting (see below). The remaining sample was further fractionated into a soluble and insoluble part by the Hirt method (Hirt, 1967), digested by proteinase K (see 1.b) and nucleic acids purified by phenol-chloroform extraction and ethanol precipitation (see 2.c.i).

2.h.viii HIRT DNA extraction

HIV-infected HeLa and SIRC cells were lysed in HIRT lysis buffer (see 1.a) and incubated 10 minutes at room temperature. 5M NaCl was added drop wise to a final concentration of 1M while gently swirling the dish. The lysate was transferred into 12 ml polypropylene Sasstedt tubes with a cell scraper, incubated overnight on ice and centrifuged at 17,000 xg for 60 minutes at 0 °C in a Sorvall centrifuge RC26plus using the SS-34 rotor. Nucleic acids were extracted from pellet and supernatant by proteinase K digestion (see 1.b), extraction with phenol/chloroform and ethanol precipitation (see 2.c.i). They were then treated with 5 μg RNAse A (2.1.b) for 1 h at 37°C. Four microliters were used for analysis by PCR and 20 μl to analyse by Southern blot (see 2.l).

2.h.ix RTC extraction

Cytosolic extracts were prepared by Dounce homogenization in hypotonic buffer as described above from $5x10^6$ acutely infected HeLa cells. After Dounce homogenization, cells were centrifuged at 3,300 xg for 15 minutes at 4°C. The pellet was frozen at -70°C and the supernatant was recovered and clarified at 4950 xg for 20 minutes at 4°C. The pellet of this centrifugation was discarded and the supernatant collected and analyzed for the presence of RTCs.

Continuous linear sucrose gradients (5 ml total volume) were prepared with the Biocomp gradient maker using 20% sucrose solution (w/w) in 50 mM phosphate buffer pH 7.4 (see 1.a) buffer and 70% sucrose solution in D_2O (w/w) and kept

on ice. Gradients were overlaid with ~0.5 ml of cytoplasmic extracts and centrifuged at 35,000 rpm at 4°C for 20 h in a Sorvall AH-650 rotor. Gradients were fractionated by puncturing the bottom of the tube and collecting 12 fractions. The density was calculated by weighing 100 µl of each fraction (Fassati and Goff, 2001). Fractions were kept frozen at -70°C. Of each fraction, 300 µl were taken to precipitate proteins by mixing with the Western blot loading buffer and boiling for 5 minutes, and 5µl were used to run a PCR.

2.i Endogenous Reverse Transcription assay

Reactions were carried out in 60 μl of endogenous reverse transcription buffer (see 1.a). Fifteen microliters from the density equilibrium fractions were added to the buffer and incubated for > 6 h at 37°C. The products of reverse transcription were detected by PCR using 5 μl of the endogenous reaction as template (Fassati and Goff, 2001). As a control, the same mix was prepared for each sample but without dNTPs. PCR products were separated onto a 7.5% polyacrylamide gel and stained with SYBR-Gold Nucleic Acid Gel Stain (see 2.j) and analyzed by the phosphorimager STORM 860 (Molecular Dynamics). The products of reverse transcription were also analysed by Real Time PCR (see 2.e).

2.j Protein SDS-PAGE and Silverstaining

Protein samples from cell fractionation assays were concentrated 3-fold by centrifugation using Vivaspin 20 3000 MWCO PES columns (Vivascience, Hanover), loaded onto NuPAGE® Novex Bis-Tris Gels 4-12% (Invitrogen, UK) and ran at 200V in MOPS Buffer (see 1.b) for approximately 45 minutes. These gels were then either stained with Silver Stain Plus (see 1.b) or used for analysis by Western Blot (see 2.k). For silver-staining, gels were fixed in 100 ml of fixative enhancer solution with 50 ml of methanol, 10 ml of acetic acid, 10 ml of fixative enhancer concentrate and 30 ml of deionized distilled water for 20 minutes with gentle agitation at room temperature. Gels were then rinsed twice for 10 minutes with at least 400 ml of deionized distilled water with gentle agitation at room temperature. Gels were then stained and developed with a freshly prepared solution of 5 ml of Silver Complex Solution, 5 ml of Reduction Moderator solution and 5 ml of Image Development Reagent, added to 35 ml of deionized distilled water, in that order. Immediately before use, 50 ml of Development Accelerator Solution were added and the gels were then immersed in this for 15-20 minutes until the bands had the desired intensity. To stop the staining reaction, gels were transferred to a tray with 5% acetic acid for 15 minutes and then rinsed in deionized, distilled water. They were scanned in an Epson Perfection 4870 Photo scanner and stored at room temperature in distilled water.

2.k Western blot

For a list of the antibodies used for Western blot, see 1.d. Thirty microliters aliquots from the cell fractionation samples were resuspended directly in 30 µl 2X SDS loading buffer. For the density assay, 300 µl of each fraction from the density equilibrium gradients were first diluted in 1.2 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.4) in the presence of 2 µg /ml of bovine serum albumin (Sigma) and 10% (v/v) trichloroacetic acid. Fractions were incubated at -20°C for 16 h and centrifuged for 30 min at 4°C at maximum speed in a microcentrifuge. Pellets were washed once in a solution of ice-cold 80% acetone in distilled H_2O and resuspended in 20 µl SDS loading buffer and the pH was adjusted to ~ 7.0 by addition of 1 µl of 1.5 M Tris-HCl (pH 8.8). Samples were resolved onto a 12.5% SDS-polyacrylamide gel followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, Calif.). Membranes were incubated with primary antibodies for 1 h at room temperature after blocking with 10% non-fat dried milk (Tesco, London, UK). A horseradish peroxidase-conjugated secondary antibody was used diluted 1:3,000 (see 1.d). Enhanced chemiluminescence (ECL) was used to develop the blots (see 1.b). When necessary, blots were developed with ECL Plus (see 1.b). Autoradiography films were exposed for different periods of time to ensure linearity of the signal.

2.I Southern Blot

Samples were loaded onto 1% agarose gels and ran at 20V for 18 hours in 1X TAE buffer (see 1.a). The gel was then washed twice in 0.25M HCl for 7 minutes and samples were then denatured in 0.4M NaOH twice for 15 minutes and transferred to a Hybond N membrane (Amersham Biosciences) by capillary transfer in 0.4M NaOH. The next day, the gel was checked by UV light to make sure transfer was complete and the membrane was washed in 2X SSC (see 1.a) and UV cross linked (0.1 Kjoules for 1 minute). A single stranded, radioactive probe was used. It was prepared by PCR using a reverse complementary primer for the appropriate DNA fragment (previously sub cloned or prepared by PCR and purified) and ^{32}P-dCTP. The probe was generated by PCR in a final volume of 50 µl containing 1x PCR buffer (MgCl$_2$-free), 100 µM dATP, 100 µM dGTP, 100 µM dTTP, 0.25 mM MgCl$_2$, 5 U of Taq polymerase (see 1.b), and 30 pmol of SSRC primer. This was mixed with 1 ng of the template DNA, added 5 mM 32P-dCTP and 70 µl of mineral oil. Cycle parameters were as follows: 94°C for 2 min, 55°C for 2 min, and 72°C for 5 min for 30 cycles. The probe was then cleaned with Sefadex columns following the manufacturer's instructions. The membrane was prehybridized with 10 ml of Rapid-hyb solution (see 1.b) at 55°C for an hour. The probe was added to the hybridization solution and incubated with the membrane for two hours at 55°C. The membrane was washed twice with 10 ml of 2X SSC at 55°C for 30 minutes each wash and then washed with 2XSSC 0.1% SDS for 30 minutes at 65C followed by a final wash in 2XSSC. After this, the membrane was exposed to a Storage Phosphor Screen (Kodak, Rochester, NY) overnight. The screen was analyzed by a Storm 860 phosphorimager (Molecular Dynamics).

Chapter 3. Results

Part 1.

1.a Introduction

Inefficient HIV-1 infection of rabbit cells has been previously described. In one study, Hofmann et al. infected cell lines from different animal species with HIV-1, SIVmac and MLV replication-defective vectors and found different levels of infection in some primate and non-primate species. Among the several cell lines showing a specific block to HIV-1 infection, SIRC cells (rabbit corneal epithelial cells) appeared to have the strongest phenotype (Hoffman et al. 1999). Another study also found that SIRC cells restrict HIV-1 infection but are permissive to SIVmac infection (Besnier et al 2002). The same study reported that, in SIRC cells, HIV-1 shows a two-hit kinetics of infection at high doses of viral input but still remains 2 logs less infectious than SIVmac, which has linear kinetics of infection, and concluded that the block in rabbit cells might only partially be due to a restriction factor (Besnier et al., 2002; Hofmann et al., 1999). Both studies used VSV-G pseudotyped vectors, and thus assumed that the block was unlikely to reside at the viral entry level (Hoffman et al. 1999; Besnier et al. 2002). However, further investigation of this issue was required because of earlier reports showing efficient infection of rabbit cells by wild type HIV-1 1 (Speck et al., 1998)

Viral entry is the first step in the HIV-1 life cycle. It involves a series of tightly regulated events that ultimately lead to the fusion of the viral and cellular membranes. Two pathways can be followed to enter the cell: pH-dependent endocytosis or direct fusion (pH-independent).

Viruses that enter the cell via the endocytic pathway, such as alphaviruses, orthomyxoviruses (e.g. influenza), rhabdoviruses (e.g. vesicular-stomatitis virus) and the non-enveloped adenoviruses are delivered to the cytoplasm inside a late endosome and an increase of the acidity in this compartment allows fusion of the viral envelope with the membrane and release of the core into the cytoplasm (Marsh and Pelchen-Matthews, 2000).

Clathrin-coated pits are an important part of the mechanism of endocytosis. They are specialized plasma membrane domains that recruit lipids, proteins and other molecules from the cell surface. After assembly in the plasma membrane, they bud as coated vesicles, through a GTP-dependent process facilitated by dynamin (van der Bliek et al., 1993) as indicated by the fact that mutations of dynamin inhibit the formation of coated pits (Damke et al., 2001). Studies with HeLa cells that express dominant negative dynamin that cannot load or hydrolize GTP indicated that clathrin-coated vesicles are important for internalization of enveloped viruses such as Semliki Forest virus (SFV) and Sindbis virus and the non-enveloped human rhinovirus 14 (HRV 14).

However, other viruses, such as the non-enveloped poliovirus, do not require the clathrin pathway to enter the cells by endocytosis (DeTulleo and Kirchhausen, 1998). Studies using immunofluorescence labeling and confocal microscopy as well as dynamin mutants have suggested that the clathrin-coated pit-mediated endocytic pathway is not required by ecotropic Moloney MLV either to infect HeLa cells (Lee et al., 1999).

On the other hand, some viruses enter the cell by direct fusion of their envelopes with the cellular plasma-membrane and are delivered directly into the cytoplasm. Examples of this are paramyxoviruses and some retroviruses such as HIV-1 and SIV. These viruses have type I fusion proteins (protein F in the case of Newcastle disease virus and gp160 in the case of HIV) that are activated by binding to their receptor (Colman and Lawrence, 2003). The interaction of HIV gp120/gp41 with CD4 induces a conformational change which increases the avidity of gp120/gp41 for its co receptor: CXCR4 or CCR5 (Sattentau and Moore, 1991; Trkola et al., 1996; Wu et al., 1996). The interaction with CXCR4 or CCR5 leads to dissociation of gp120, exposure of the fusion protein and refolding of gp41 into a helical hairpin domain all of which leads to virus-cell membrane fusion (Weissenhorn et al., 1996).

It has been reported that the mechanism of entry into the cell determines at least to some extent the fate of the incoming virion in the cytoplasm. Trafficking to different cellular compartments depending on the nature of the receptor used for cell entry has already been shown with some retroviruses, such as avian

sarcoma-leukosis virus (ASLV) (Narayan et al., 2003). Uptake into endocytic vesicles, for example, may allow the virus to bypass the cytoskeleton. For HIV-1, some viral proteins (Nef) and cellular factors (Cyp A) are required for viral replication when the virus enters by direct fusion but not by the endocytic pathway (Aiken, 1997). Furthermore, cellular tropism can be influenced by the route of entry, as has been shown for Lv2 restriction which can be avoided by inhibiting the lipid-raft dependent and pH-independent endocytic pathway (Marchant et al., 2005). On the other hand, pseudotyping with different types of viral envelopes has also been used as a technique to study the effect of route of entry on the retroviral integration pattern (Barr et al., 2006).

In this section I confirm that rabbit cells have a strong block to HIV-1 infection and show that neither viral entry per se nor the viral envelope is involved in this block. I also show that the block is specific for HIV-1, does not involve SIVmac or MLV-NB and that it is also present in another rabbit cell line, EREp (embryonic skin epithelial).

1.b Results

1.b.i The block to HIV-1 infection in rabbit cells is post entry.

To assess if the previously described block to HIV-1 infection in rabbit cells was dependent on the cell receptor, viral vectors pseudotyped with VSV-G were produced by transient transfection of 293T cells and HIV-1 vectors pseudotyped with MLV amphotropic envelope were collected from a stable producer cell line (Ikeda et al., 2003). Viral stocks were normalized for RT activity and used to infect SIRC, HeLa and 293T cells at serial dilutions. As shown in Figure 11, HeLa and 293T cells were up to a few hundred folds more permissive to HIV-1 infection than SIRC cells. The restriction in SIRC cells was partially overcome when more than 650 pg/ml RT of VSV-G-pseudotyped virus was used (Figure 11). However, equivalent values of infection in SIRC and HeLa or 293T cells were never achieved, even with 11,000 pg/ml RT. This rather modest two kinetics rate of infection at high viral doses has been observed previously in SIRC cells (Besnier et al., 2002) and it suggests the existence of a saturable factor that might account for a small part of the restriction. Cells were then infected with HIV-1 pseudotyped with the amphotropic MLV envelope because it is a retroviral envelope that uses a pH-independent pathway for entry (Yang et al., 2005) Infection of SIRC cells with HIV-1 pseudotyped with amphotropic MLV envelope was also impaired compared to both 293T and HeLa cells (Figure 11). This result indicated that the block to HIV-1 infection did not depend on the receptor used for entry.
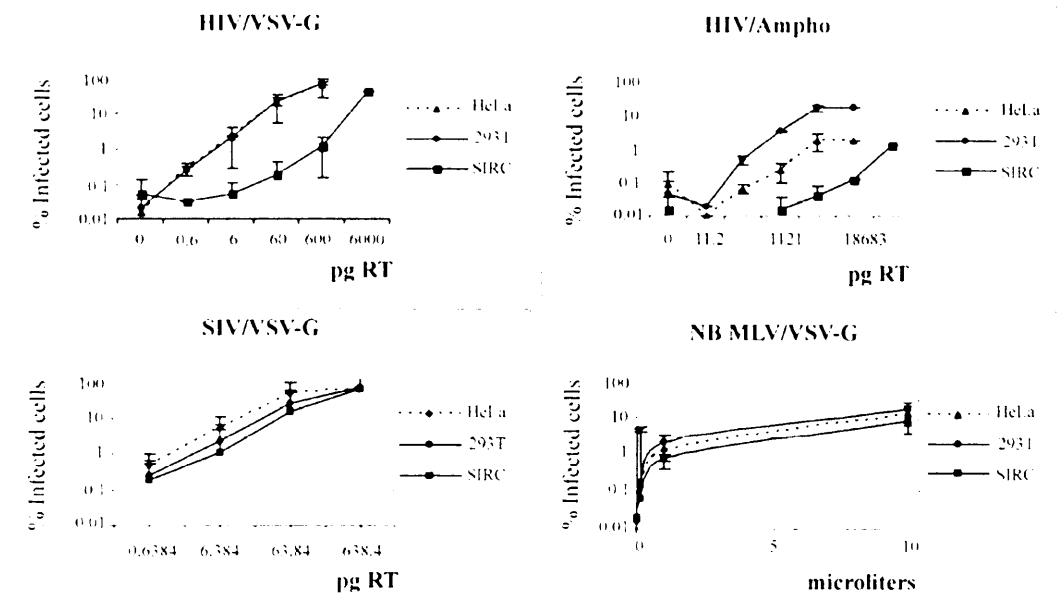
Figure 11. HIV-1 is restricted in SIRC cells. HeLa, 293T and SIRC cells were infected with increasing amounts (RT normalized) of HIV-1 vector pseudotyped with VSV-G (top left panel), HIV-1 vector pseudotyped with MLV amphotropic envelope (top right panel), SIVmac vector pseudotyped with VSV-G (bottom left panel) and NB MLV vector also VSV-G pseudotyped (bottom right panel). All vectors expressed GFP driven from a CMV promoter. The percentage of GFP+ cells (y axis) was counted by FACS 48 hours after infection and plotted against the amount of input virus. Results of two independent experiments are shown as mean values +/- standard error. NB MLV X axis shows the volume used to infect cells in microliters as the pg of RT could not be measured for this virus.

To see if inefficient HIV-1 infection in SIRC cells occurred with different retroviruses, the three cell types were infected with serial dilutions of VSV-G pseudotyped SIVmac and MoMLV vectors. Titers of SIVmac were approximately 5 fold lower in SIRC compared with HeLa and 293T cells at low virus input but the same percentage of infection was achieved at higher virus input. A modest reduction (2-3 fold) in infection efficiency was observed in SIRC compared to 293T and HeLa cells with the NB MLV vector, presumably as a consequence of the slower division rate of SIRC cells (Figure 11) as MLV can only infect dividing cells and presumably it needs dissolution of the nuclear membrane to get access to the nucleus and integrate into the host's DNA (Roe et al., 1993).

1.b.ii Genotyping of SIRC cells

A previous study showed that SIRC cells could be efficiently infected by HIV-1. Thus, to confirm that the SIRC cells used in this study were of rabbit origin, primers specific for a small region of the rabbit genome (*Oryctolagus cuniculus*, accession number AC158743.4) were designed and used to amplify SIRC total genome. Total DNA from HeLa cells was also used as a control and no amplification was observed (Figure 12). The SIRC-specific PCR fragment was cloned, sequenced and analyzed by a BLAST search. The results confirmed that our cells were of rabbit origin. To test if the block was limited to SIRC cells or was a feature of rabbit cells in general, the embryonic rabbit epithelial cell line, EREp, was infected with HIV-1, SIVmac and NB-MLV, all pseudotyped with VSV-G envelope. HIV-1 and SIV were slightly less restricted in EREp than in SIRC cells and NB MLV was about 3 times less infectious in EREp than in SIRC. However, overall similar levels of infection were observed in EREp and in SIRC cells with the three viruses. Low virus input was used in these experiments to avoid saturation effects and ensure that infection was in the linear range (Figure 13). It would have been interesting to infect as well some lymphocytic cell line which would be more relevant to the real-life situation but due to lack of availability this was not done. However, after this thesis was written the rabbit lymphocytic cell line RL-5 was infected with similar viral doses as the ones above and infection was as inefficient as in SIRC and EREp.

A HeLa SIRC MWM

500 bp

B

BLASTN 2.2.12

Fields: query id, subject ids, % identity, alignment length, mismatches, gap opens, q.star

101 hits found

| 1_4921 | gi 76573979 gb AC158743.4 | 98.24 | 511 | 8 | 1 | 604 | 35699 |

Figure 12. Genotyping of SIRC cells' genomic DNA. Primers specific for the rabbit genome were used to amplify SIRC and Hela genomic DNA. (A) An expected 500 bp band was amplified with SIRC DNA and no amplification was observed with HeLa DNA. MWM: molecular weight marker. The amplified fragment was cloned and sequenced. (B) The sequence was then used to search the rabbit genome database by BLAST: clone AC158743.4 corresponds to *Oryctolagus cuniculus*.

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1.b.iii There is a small difference in total viral RNA content between HIV-1-infected HeLa and SIRC cells.

VSV-G does not seem to require a specific cell receptor, but interacts with phospholipids and perhaps a number of cell surface proteins and has a very broad tropism (Burns et al., 1993). However, there are reports of variation in the transduction efficiency of VSV-G-pseudotyped HIV-1 in B and T cell lines if they are mitogen-stimulated (Kahl et al., 2005) presumably because this could lower expression of the phospholipids that act as receptors for VSV-G. Because of this variable and the possibility of a block immediately after entry, such as rapid and specific viral RNA degradation, it was important to measure viral entry and to control for non-specific uptake of virus particles (i.e. independent of env). HeLa and SIRC cells were infected with the same dose of an HIV-1 vector with or without VSV-G envelope (HIV Env-). Cells were incubated with the virus for 2 hours at 4°C and then infection was allowed to proceed for 4 hours at 37°C. The 4 hours post-incubation period was chosen because it allows sufficient time for entry and part of reverse transcription to occur. It was kept the same in all experiments, so they could be comparable between each other, although in some occasions such as this entry assay, an earlier time-point for RTC extraction could have been useful. We also chose it so results in this work can be compared to the existing literature which has used this time-lapse (Fassati and Goff, 1999). Nucleic acids were extracted from infected cells, DNA was degraded by treatment with RNAse free-DNase and total viral RNA was measured by quantitative PCR after oligo-dT primed *in vitro* reverse transcription. The specificity of the reaction was controlled by omitting RT in control samples. An aliquot of the cells was re-plated and the percentage of infection of each cell type was analysed by FACS 48 hours later. As shown in Figure 14A, viral RNA levels in HeLa cells were at most twofold higher than those in SIRC cells and they were reduced by more than 10 fold in both HeLa and SIRC cells infected with HIV (Env-) compared to cells infected with HIV-1/ VSV-G. Taken together, these data indicate that there is at most a twofold entry

Figure 13. HIV-1 is restricted in the rabbit cell line EREp. EREp cells were infected with increasing amounts (RT normalized) of HIV-1 vector pseudotyped with VSV-G (top panel), SIVmac vector pseudotyped with VSV-G (middle panel) and NB MLV vector also VSV-G pseudotyped (bottom panel). All vectors expressed GFP driven from a CMV promoter. The percentage of GFP+ cells (y axis) was counted by FACS 48 hours after infection and plotted against the amount of input virus. NB MLV X axis shows the volume used to infect cells in microliters as the pg of RT could not be measured for this virus.

defect for HIV-1 in SIRC cells, which does not account for the approximate 80-fold infectivity difference in these conditions as measured by flow cytometry (Figure 14B).
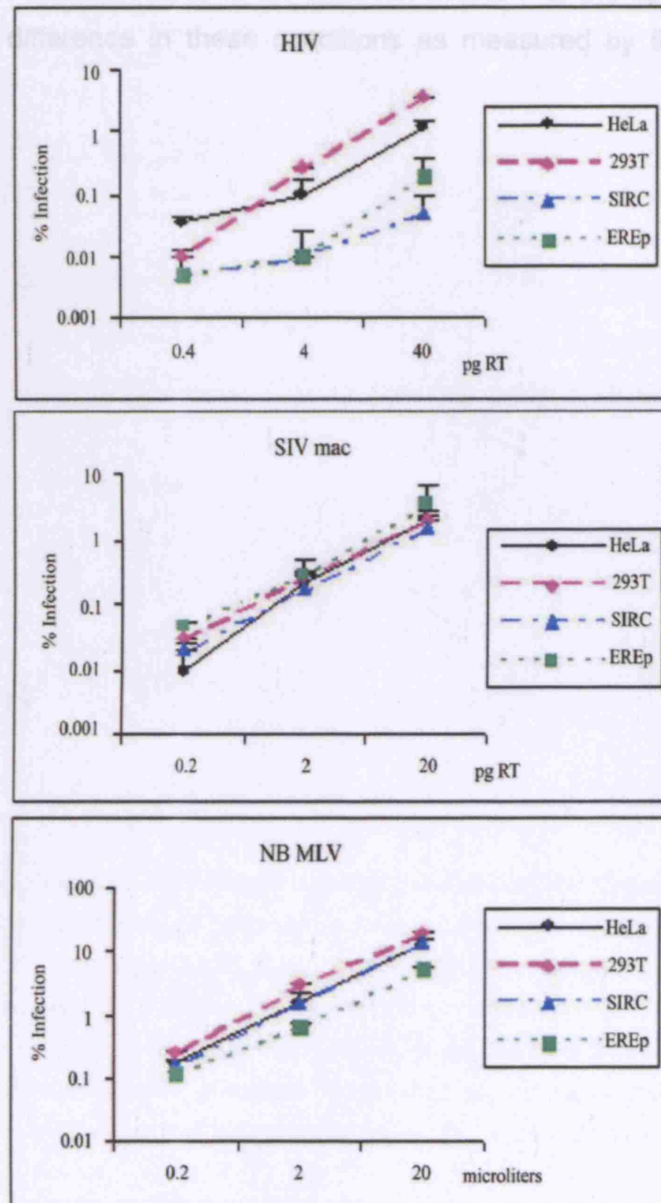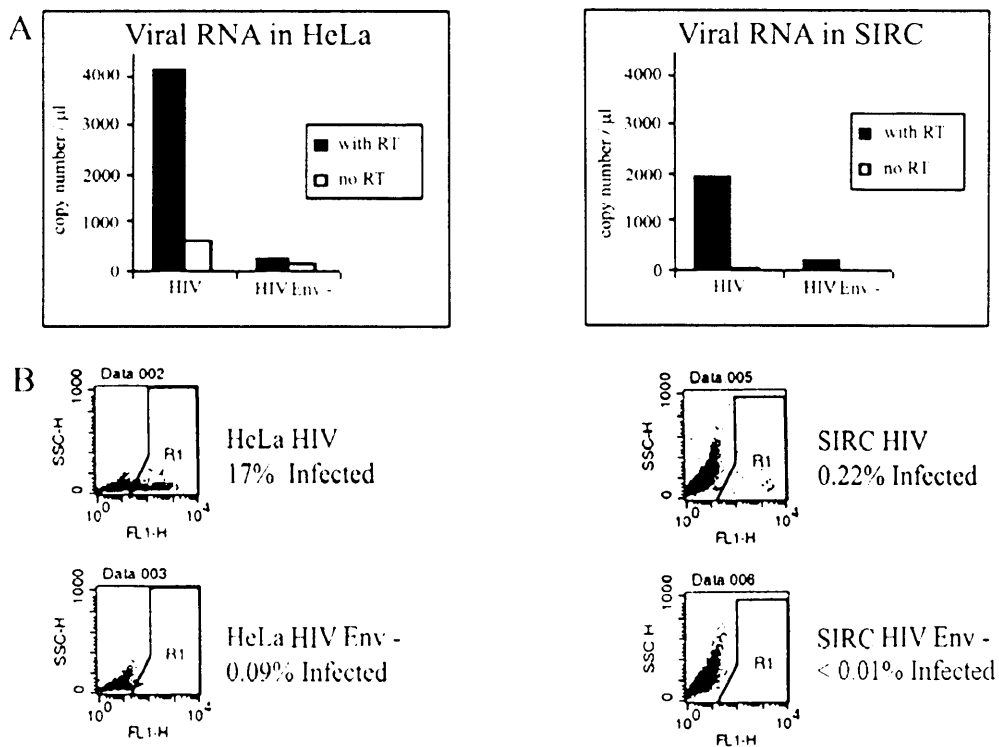
Figure 14. A defect in viral entry cannot account for reduced HIV-1 titers in SIRC cells.
RNA was extracted from HeLa and SIRC cells 4 hours after infection with the same dose (RT normalized) of HIV-1 (VSV-G) or HIV-1 (Env-) virus. RNA was subjected to oligo-dT primed in vitro reverse transcription (after DNase I treatment) and quantified by Taqman PCR using GFP primers. With RT, reaction performed in the presence of reverse transcriptase, no RT, control reaction performed in the absence of reverse transcriptase. (A) An aliquot of the cells used for RNA extraction was re-plated and analyzed by FACS 48 hours later to measure infection efficiency (B).

1.c Discussion

There are some conflicting studies concerning HIV-1 infection of rabbit cells. Two recent studies found a strong block to HIV-1 infection in SIRC cells, however older studies suggested that rabbit cells could somehow be infected by wild-type HIV-1 (Speck et al., 1998). One of these studies used SIRC cells expressing human CD4 and CCR5 molecules on their surface (Speck et al., 1998) and compared them with a human (HeLa) and a murine (3T3) cell line, also expressing CD4 and CCR5. In their study, Speck et al found that Nef, Tat and Rev viral proteins were functional in rabbit cells They also claimed that SIRC cells could support replication of a HIV molecular clone as efficiently as human cells. This was in fact tested by transfecting the HIV-1 clone into SIRC cells and using the supernatant to infect human PBMCs, in which virus was further passaged and p24 CA values monitored. No data on HIV-1 growth in rabbit cells was shown. The authors did infect SIRC cells expressing CD4/CCR5 with HIV-1 and measured both multinucleated cells formation and viral transcripts. However, there is no mention of the amount of input virus and the amount of cells used in each experiment so data are not quantitative. The authors did some more experiments with primary rabbit cells but they did not perform accurate titration of virus infectivity in any of them (Speck et al. 1998). It is essential to perform accurate titration curves when comparing infectivity in different cell types to avoid saturation effects and unspecific uptake of virus.

Another study by Kulaga et al. used a rabbit T cell line transformed with herpesvirus ateles 446, a rabbit T cell line transformed with HTLV-1 6083, a rabbit macrophage line transformed with SV40, and some other rabbit cell lines (Kulaga et al., 1988) and compared them with the human T cell lines SupT1 and A3.01 for their ability to sustain HIV-1 infection. They concluded that higher doses of input virus are necessary to productively infect rabbit cells as compared with human cells but that cellular factors necessary for infection are present in both species. The somewhat surprising implication of this study is that rabbit CD4 and CCR5 or CXCR4 are functional for HIV-1 infection.

In the light of the contradictory reports (Besnier et al., 2002; Hofmann et al., 1999; Kulaga et al., 1988; Speck et al., 1998), my first aim was to accurately

titrate HIV-1 infection in rabbit and human cells and to test if the route of entry (pH-dependent versus pH-independent) was important. HIV-1 infection was found to be 2 orders of magnitude lower in SIRC than HeLa and 293T cells, regardless of the envelope used.

Infection of target cells with viral vectors expressing a marker gene like GFP followed by flow cytometry to count GFP+ cells is arguably one of the most accurate ways to perform titration curves. Nonetheless, the accuracy of this system depends on GFP expression levels and ultimately on the promoter driving its transcription. I have used MLV, SIVmac and HIV-1-based vectors expressing GFP from the same CMV early promoter, thus differential expression of the marker gene in rabbit versus human cells was unlikely to explain my results. The *Oryctolagus cuniculus* origin of the SIRC cell line used in our laboratory was confirmed by genotyping. Moreover, both SIRC and EREp cell lines used in our laboratory have been previously confirmed to be of *Oryctolagus cuniculus* origin by Griffiths et al., who identified a new and specific rabbit endogenous retrovirus (Griffiths et al., 2002).

Thus, the results shown in this chapter strongly suggested that the block to HIV-1 infection in rabbit cells did not lie at the entry step nor depended on rapid and specific degradation of HIV-1 RNA. They indicated that some post entry step might be involved and this possibility was investigated further.

Part 2.

2.a Introduction

Having established that the block to HIV-1 infection in rabbit cells is at post-entry level, it was important to understand if this block had a dominant or recessive phenotype. Heterokaryons have been used to establish if blocks to viral replication were dominant or recessive in many studies. In one study on SV40 replication (Watkins and Dulbecco, 1967), the authors fused an embryonic fibroblastic mouse cell line transformed with SV40 virus (SV3T3) that does not sustain SV40 multiplication, to an SV40 susceptible cell line (BSC1) derived from African green monkey kidney cells, by addition of UV-inactivated Sendai virus, which promotes cell fusion. They found that infectious virus was produced from the heterokaryons of BSC1 and SV3T3 indicating that SV3T3 lacked a factor important for SV40 replication. In another study, the same assay was used to fuse irradiated Chinese hamster ovary (CHO) cells containing a RSV provirus with chick embryo fibroblasts. CHO cells cannot sustain RSV replication but the CHO/chick embryo fibroblast heterokaryons could. They proposed that the formation of heterokaryons between these cells allows transfer of RSV genetic material from CHO cells to the susceptible cell where the virus can replicate (Svoboda and Dourmashkin, 1969). More recently, cell fusion has been used as way to investigate the Vif: APOBEC circuit (Simon et al., 1998). In this case, the authors produced transient heterokaryons by expressing HIV-1 Env protein on the surface of 293T cells and CD4/CXCR4 on the surface of either human T-cells CEM-SS (permissive to vif-deficient HIV-1) or HUT78 (non-permissive to vif-deficient HIV-1). They found that vif- virions produced from heterokaryons of 293T and non-permissive cells were about 10-fold less infectious than Vif+ virions. Conversely, Vif- or Vif+ virions had similar infectivity when produced in heterokaryons of 293T and permissive cells (CEM-SS). They concluded that Vif probably counteracts an innate anti-viral factor, present in some non-permissive cells such as human T cells (Simon et al., 1998).

Heterokaryons assays have been used to investigate late blocks to HIV-1 infection in mouse cells (Bieniasz and Cullen, 2000; Trono and Baltimore, 1990)

and to study retroviral restriction in primate cells (Cowan et al., 2002; Munk et al., 2002). Munk et al used polyethylene glycol to fuse HeLa and CV-1 African green monkey kidney cells that are not permissive for HIV-1 infection, and found that simian cells contained an anti-HIV inhibitory factor. Similarly, Cowan et al fused 293T cells to OMK cells (restrictive to HIV-1) or HeLa cells (permissive to HIV-1) and found that OMK cells expressed a dominant restriction factor for HIV-1 infection, which they called Lv1.

To determine if the block to HIV-1 infection in SIRC cells had a dominant or recessive phenotype, a fusion assay was carried out with 293T and either SIRC or OMK cells. The latter were used as a validating control for the assay as they have been shown previously to have a dominant restriction factor (Cowan et al., 2002). If, after fusion, the 293T/SIRC heterokaryons were permissive to HIV-1 infection, it would suggest that there is a factor required by HIV-1, absent in the SIRC cells but provided by the 293T in the syncytia. On the other hand, if the heterokaryons were restrictive to HIV-1 infection, it would suggest that there is a dominant restriction factor in the SIRC cells that blocks infection.

2.b Results

2.b.i The heterokaryon assay

Two different fusion assays were used. In both assays, fusion was induced by transfection of cells with a plasmid coding for the highly fusogenic HTLV-1 envelope protein (pV1/HTLV) (Cowan et al., 2002). In the first assay, unlabelled 293T cells were plated with SIRC cells. As controls for the effect of fusion per se on infection, 293T cells were plated alone or in combination with HeLa cells. Cells were transfected 24 hours later with pV1/HTLV and 24 hours after transfection cells were infected with HIV-GFP using the viral dose previously seen to infect about 1% of SIRC cells. Forty-eight hours after infection, human nuclei were specifically detected with a monoclonal IgG1 anti-human PML antibody Texas-red conjugated and all nuclei were stained with the nucleic acid-binding dye TOTO-3. Cells were analyzed by confocal microscopy and syncytia containing both human and rabbit nuclei were scored for GFP expression. In all experiments untransfected and unfused cells were also infected and stained in the same way. When SIRC cells were fused to 293T cells, the percentage of GFP+ (infected) heterokaryons was always about 25 fold higher than that observed in SIRC cells individually or in SIRC cells homokaryons and about half of that seen in 293T cells homokaryons. This suggested that SIRC cells might lack a factor essential for infection, which is provided by 239T cells in the heterokaryons (Figure 15).

However, there were technical problems inherent to this assay: it was difficult and time-consuming to count a significant number of cells or syncitia in images acquired by confocal microscopy. The signal of the anti-PML antibody was rather weak when compared to the signal of TOTO-3 or GFP so it was difficult to compensate for this difference. For all these reasons and to confirm these results, an alternative assay was developed, which allowed a larger number of cells and syncitia to be counted.
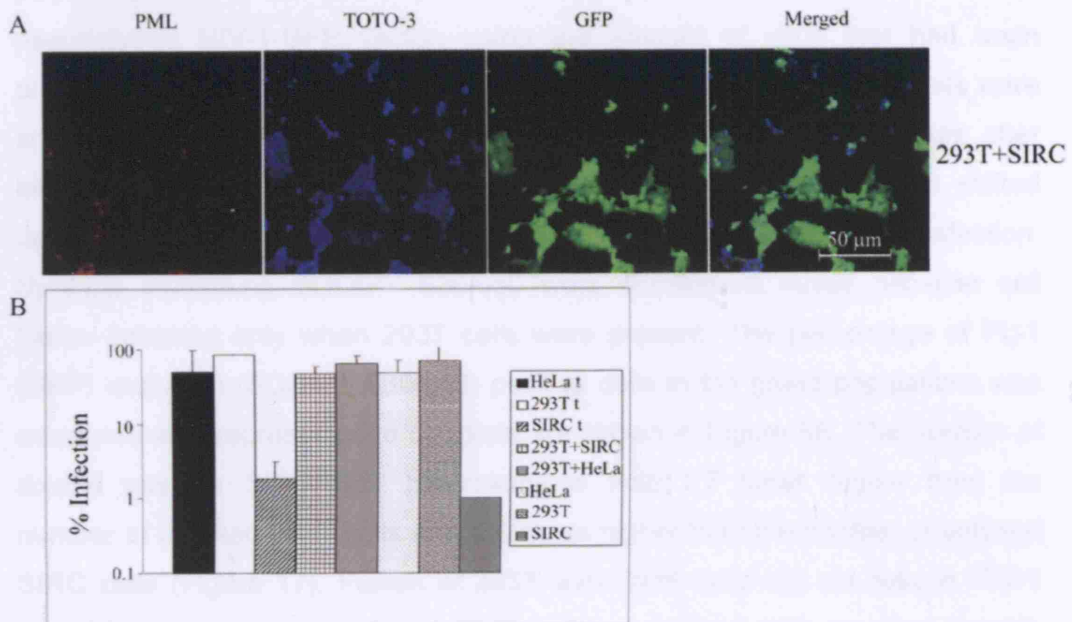
Figure 15. Fusion assay with immunolabeling. Cells were fused by transfection of a plasmid coding for HTLV-1 envelope protein. (A) 293T nuclei were labeled with an anti-human PML antibody (red), all nuclei were stained with TOTO-3 (blue) and heterokaryons were infected with an HIV-1 vector encoding GFP (green). Cells were analyzed and counted by confocal microscopy (top panels). (B) The levels of infection in pHTLV1-transfected HeLa (HeLa t), 293T (293T t) and SIRC (SIRC t) cells on their own and in heterokaryons of 293T + SIRC or 293T + HeLa cells as well as non-transfected cells are shown. Bar graphs represent the average of three independent experiments ± SD.

In the second assay, SIRC and OMK cells were labelled with the amine-reactive fluorescent dye BODIPY 630/650, plated alone or in combination with 293T cells and 24 hours later cells were induced to form syncytia by transfection with pv1/HTLV. Twenty-four hours after transfection, cells were infected with VSV-G pseudotyped HIV-1-GFP vector, using the amount of virus that had been previously found to infect around 1% of SIRC cells (see Figure 11). Cells were analyzed by confocal microscopy (Figure 16A) and by FACS 48 hours after infection (Figure 16B). Syncytia were apparent as a population that shifted upwards in the side scatter channel (SSC-H) after pV1/HTLV transfection. Syncytia containing BODIPY 630/650 were considered mixed because cell fusion occurred only when 293T cells were present. The percentage of FL-1 (GFP) and FL-5 (BODIPY 630/650) positive cells in the gated populations was measured and representative dot plots are shown in Figure 6B. The number of double positive SIRC/293T heterokaryons was 1.7 times higher than the number of unfused 293T cells and 210 times higher than the number of unfused SIRC cells (Figure 17). Fusion of 293T with OMK cells did not rescue HIV-1 infectivity compared to unfused OMK cells, consistent with previous reports (Cowan et al., 2002). The result with OMK cells also confirmed that significant infection did not take place in 293T cells before fusion occurred and further validated this assay (Figure 17).

This assay had several advantages over the previous one: it allowed me to count a larger number of cells and syncitia and used two equally intense cellular labels that were easier to detect and that could be analyzed in a more standardized way. Furthermore, this assay was less prone to subjective bias.
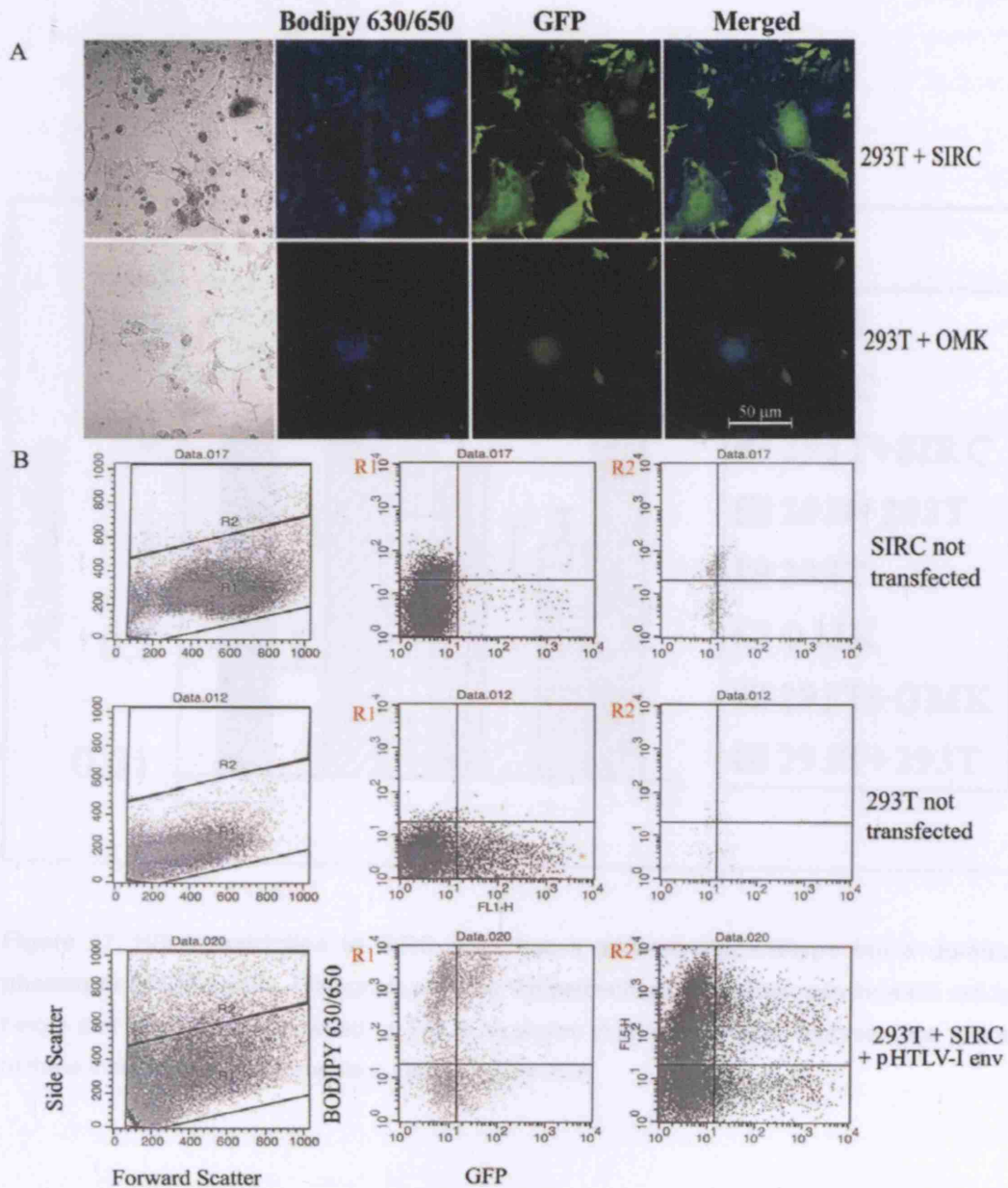
Figure 16. HIV-1 restriction in SIRC cells has a recessive phenotype. SIRC and OMK cells were labelled with the amine-reactive fluorescent dye BODIPY-630/650, fused with 293T cells expressing the highly fusogenic HTLV-1 envelope protein and twenty-four hours later infected with VSV-G pseudotyped HIV-1-GFP vector. (A) Approximately 24 hours after infection syncitia were analyzed by confocal microscopy: those containing SIRC or OMK cells (blue labelled) were considered mixed because cell fusion occurred only when 293T cells were present. (B) The same cells were analyzed by FACS and density plots of the forward (FSC-H) and side (SSC-H) scatter measurements are shown. Syncytia appeared as a population that shifted upwards in the R2 region of the SSC-H channel after transfection with HTLV-I envelope (bottom panel on the left). This shift was not apparent in mixed 293T and SIRC populations in the absence of HTLV-I envelope (not shown). Cells were analyzed for BODIPY staining and GFP expression and density plots of the FL-5H channel (BODIPY-630/650) and FL-1H channel (GFP) are shown in the middle panels (for the R1 region) and right panels (R2 region) respectively. SIRC cells were labelled with BODIPY but were poorly infected by the HIV-1 GFP vector (top, middle panel). 293T cells were not labeled with BODIPY but were efficiently infected by the HIV-1 GFP vector (middle, middle panel). Fusion of SIRC cells with 293T cells rescued HIV-1 infection as shown by the increase in the number of double BODIPY/GFP positive cells (bottom, right panel).
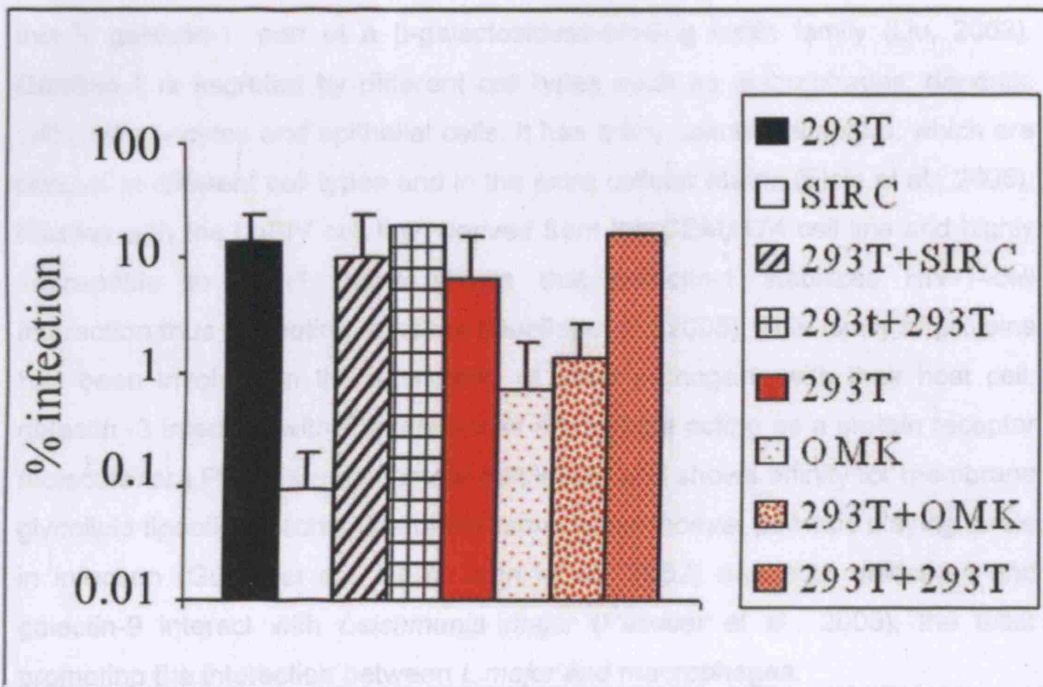
Figure 17. HIV-1 restriction in SIRC cells has a recessive phenotype but a dominant phenotype in OMK cells. Histogram showing the percentage of infected cells for each cell type before and after fusion calculated by FACS as shown in panel B. Values represent the average of three independent experiments ± standard deviation.

It was also important to determine if 293T cells provided to SIRC cells a soluble factor capable of increasing susceptibility to HIV-1 infection. Soluble factors secreted by infected cells have been reported to influence maturation of neighbouring cells (Kis et al., 2006), or promote HIV-1 infectivity. An example of this is galectin-1, part of a β-galactosidase-binding lectin family (Liu, 2002). Galectin-1 is secreted by different cell types such as macrophages, dendritic cells, lymphocytes and epithelial cells. It has many cellular receptors, which are present in different cell types and in the extra cellular matrix (Elola et al., 2005). Studies with the LuSIV cell line, derived from the CEMx174 cell line and highly susceptible to HIV-1, have shown that galectin-1 stabilizes HIV-1–cell interaction thus promoting infection (Ouellet et al., 2005). This family of proteins has been involved in the interaction of other pathogens with their host cell: galectin -3 interacts with *Pseudomonas aeruginosa* acting as a protein receptor molecule for LPS in human corneal epithelium and shows affinity for membrane glycolipid lipooligosaccharides of *Neisseria gonorrhoeae,* perhaps playing a role in infection (Gupta et al., 1997; John et al., 2002) and both galectin-1 and galectin-9 interact with *Leishmania major* (Pelletier et al., 2003), the latter promoting the interaction between *L.major* and macrophages.

To determine if 293T cells in the heterokaryons were providing a soluble factor secreted in to the media, I collected the supernatant from confluent 293T cells cultures, filtered it and adjusted the pH to 7.0 with HEPES. SIRC, HeLa and OMK cells were infected with VSV-G pseudotyped HIV-1 GFP in the presence of different dilutions of fresh 293T media. In addition, SIRC cells were also infected with SIVmac and N MLV, both VSV-G pseudotyped, and HIV-1 pseudotyped with MLV amphotropic envelope. No significant difference was found in the levels of infection in the presence of 293T media with any of the viruses tested (Figure 18).
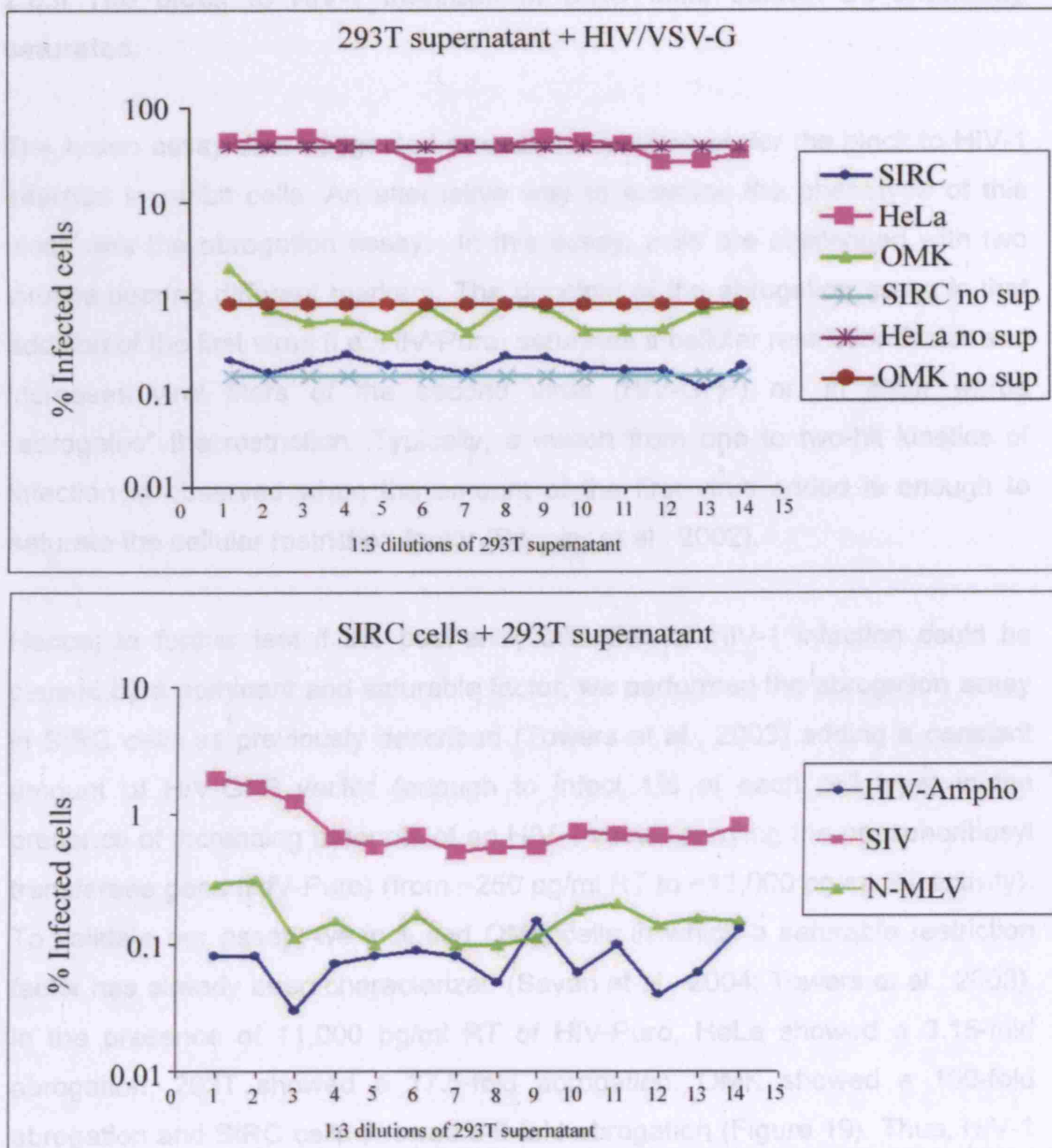
Figure 18. Effect of 293T supernatant on infection of SIRC, HeLa and OMK cells. (A) Cells were infected with the same dose (RT normalized) of HIV-1 GFP vector pseudotyped with VSV-G in the presence of three-fold serial dilutions of supernatant from confluent 293T cell cultures (point 1 on the x-axis is neat supernatant). *SIRC no sup, HeLa no sup, OMK no sup*: cells infected in the absence of 293T supernatant. (B) SIRC cells were infected with a constant amount of the indicated viral vectors expressing GFP (all pseudotyped with VSV-G) in the presence three-fold serial dilutions of supernatant from confluent 293T cell cultures (point 1 on the x-axis is neat supernatant). The percentage of GFP+ cells was measured by FACS 48 hrs after infection.

2.b.ii The block to HIV-1 infection in SIRC cells cannot be efficiently saturated.

The fusion assay data suggested a recessive phenotype for the block to HIV-1 infection in rabbit cells. An alternative way to examine the phenotype of this block was the abrogation assay. In this assay, cells are challenged with two viruses bearing different markers. The principle of the abrogation assay is that addition of the first virus (i.e. HIV-Puro) saturates a cellular restriction factor and increases viral titers of the second virus (HIV-GFP) or, in other words "abrogates" the restriction. Typically, a switch from one to two-hit kinetics of infection is observed when the amount of the first virus added is enough to saturate the cellular restriction factor (Besnier et al., 2002).

Hence, to further test if the post-entry inhibition of HIV-1 infection could be caused by a dominant and saturable factor, we performed the abrogation assay in SIRC cells as previously described (Towers et al., 2003) adding a constant amount of HIV-GFP vector (enough to infect 1% of each cell type) in the presence of increasing amounts of an HIV-1 vector carrying the phosphoribosyl transferase gene (HIV-Puro) (from ~250 pg/ml RT to ~11,000 pg/ml RT activity). To validate our assay, we included OMK cells in which a saturable restriction factor has already been characterized (Sayah et al., 2004; Towers et al., 2003). In the presence of 11,000 pg/ml RT of HIV-Puro, HeLa showed a 3.15-fold abrogation, 293T showed a 17.5-fold abrogation, OMK showed a 100-fold abrogation and SIRC cells showed a 9-fold abrogation (Figure 19). Thus, HIV-1 infection was similarly low in SIRC and OMK cells but was only weakly saturated in SIRC as compared to OMK cells. Strong saturation of restriction in OMK cells is in agreement with previous studies (Besnier et al., 2002). The saturation of restriction observed in 293T cells was puzzling because human cells express TRIM5alpha that is not able to block HIV-1 infection. The saturation was found in three independent experiments, even if it was quite variable (see the error bars in Figure 19). An apparent "saturation" in 293T cells could be due to the very high virus dose used in these experiments. Alternatively, because all our virus stocks are purified and contain surose, it may be possible that large amounts of sucrose could alter 293T susceptibility to HIV-1 infection. It will be interesting to address this hypothesis experimentally in

the future. The abrogation assay results are in agreement with a previous study, which showed modest saturation of the SIRC cells restriction upon addition of large amounts of HIV-1 vector (Besnier et al. 2002). These results together with the fusion assay indicated that the block in SIRC cells had a recessive phenotype, although it remained formally possible that 293T cells provided a factor able to inhibit a dominant restrictor in SIRC cells

2.b.iii 293T cells express TRIM5α

As mentioned above, one possible explanation for the rescue of HIV-1 infection in 293T and SIRC heterokaryons was the existence of a restriction factor that was partially inhibited by a dominant negative factor in 293T cells. This has been observed for TRIM5γ_{rh}, a splicing isoform of TRIM5α_{rh}, that when co-expressed with TRIM5α_{rh} in primary rhesus monkey lung fibroblasts increases HIV-1 infection (Stremlau et al., 2004). So, albeit rather unlikely, human TRIM5α or another TRIM variant could in principle suppress a TRIM5-like activity in rabbit cells and give a recessive phenotype in 293T/SIRC heterokaryons. It was therefore interesting to investigate if 293T cells did or did not express TRIM5α. 293T cells are human embryonic kidney fibroblasts transformed by adenovirus and contain the SV40 large T antigen, thus it was not unreasonable to hypothesize that they might not express or might express low levels of TRIM5α (Graham et al., 1977). CrFK cells were used as a control because they have not been found yet to express TRIM5α or any other TRIM-like restrictor and they are very permissive to many different retroviruses. Cells were infected with 10-fold dilutions of HIV-1, NB MLV and N MLV VSV-G pseudotyped vectors expressing GFP as a reporter gene. Forty-eight hours after infection, cells were analyzed by FACS and titration and infection efficiency plotted against input virus. The results from this experiment showed that HeLa and 293T cells were equally permissive to HIV-1 and NB MLV and they both potently restricted N MLV. SIRC cells were 10 times more permissive to N MLV compared to HeLa and 293T cells, but equally permissive to NB MLV. CrFK cells were infected as efficiently as HeLa and 293T cells with HIV-1, slightly more efficiently with NB MLV and 100 up to fold more efficiently with N MLV, depending on the viral dose (Figure 20). It was enough to use the same volumes of each virus to infect all cell types in this experiment, without

measuring picograms of RT, because the aim was only to compare infection levels in 293T cells with those achieved in CrFK using 10-fold dilutions of virus. These results suggested that 293T cells do express TRIM5α unlike CrFK cells. To see if ectopic TRIM5α expression could rescue HIV-1 infectivity, I have attempted to express it in SIRC cells. However, repeated attempts to express human TRIM5α in SIRC cells were unsuccessful, due to apparent toxicity.
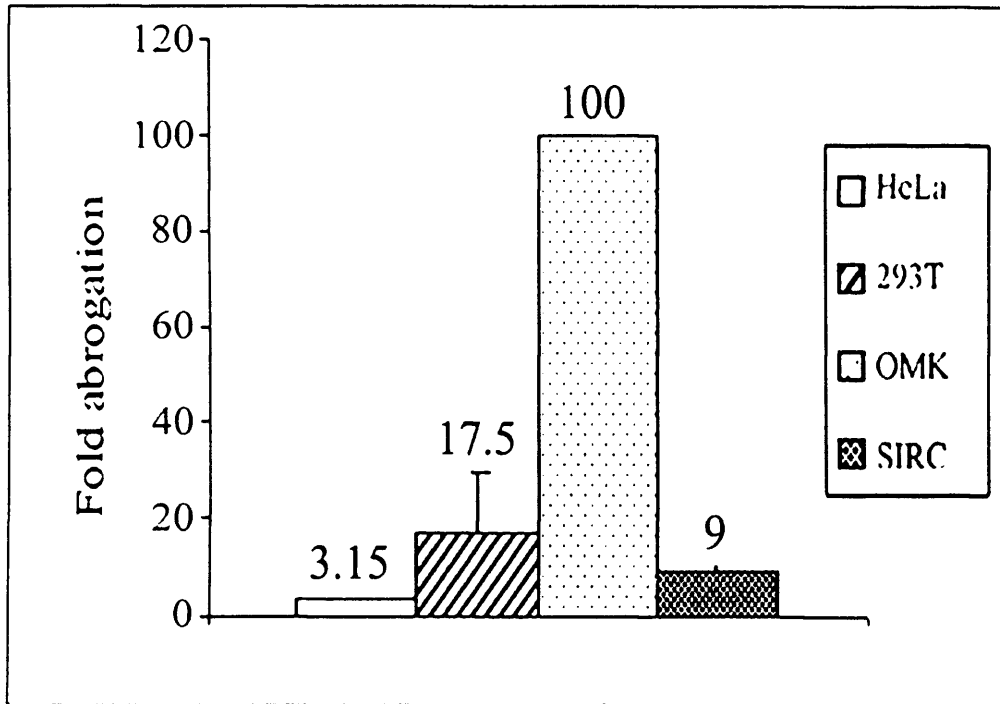
Figure 19. HIV-1 restriction in SIRC cells is not saturable. Abrogation assay on HeLa, 293T, OMK and SIRC cells. Cells were exposed to 11,000 pg/RT of HIV-1-puro vector, infected with a fixed amount of HIV-GFP vector and analyzed by FACS to count the percentage of infected cells. Rescue of infectivity by pre-exposure to HIV-1-puro is expressed as fold abrogation relative to cells that were not pre-exposed to HIV-1-puro. Data are representative of three Independent experiments ± SD.

Figure 20. 293T cells restrict N MLV. HeLa, 293T and SIRC cells were infected along with a cat cell line, CrFK, to compare infectivity of HIV-1, NB MLV and N-MLV GFP expressing vectors all pseudotyped with VSV-G. Forty-eight hours after infection, cells were analyzed by FACS and the percentage of infected (GFP+) cells plotted against input virus.
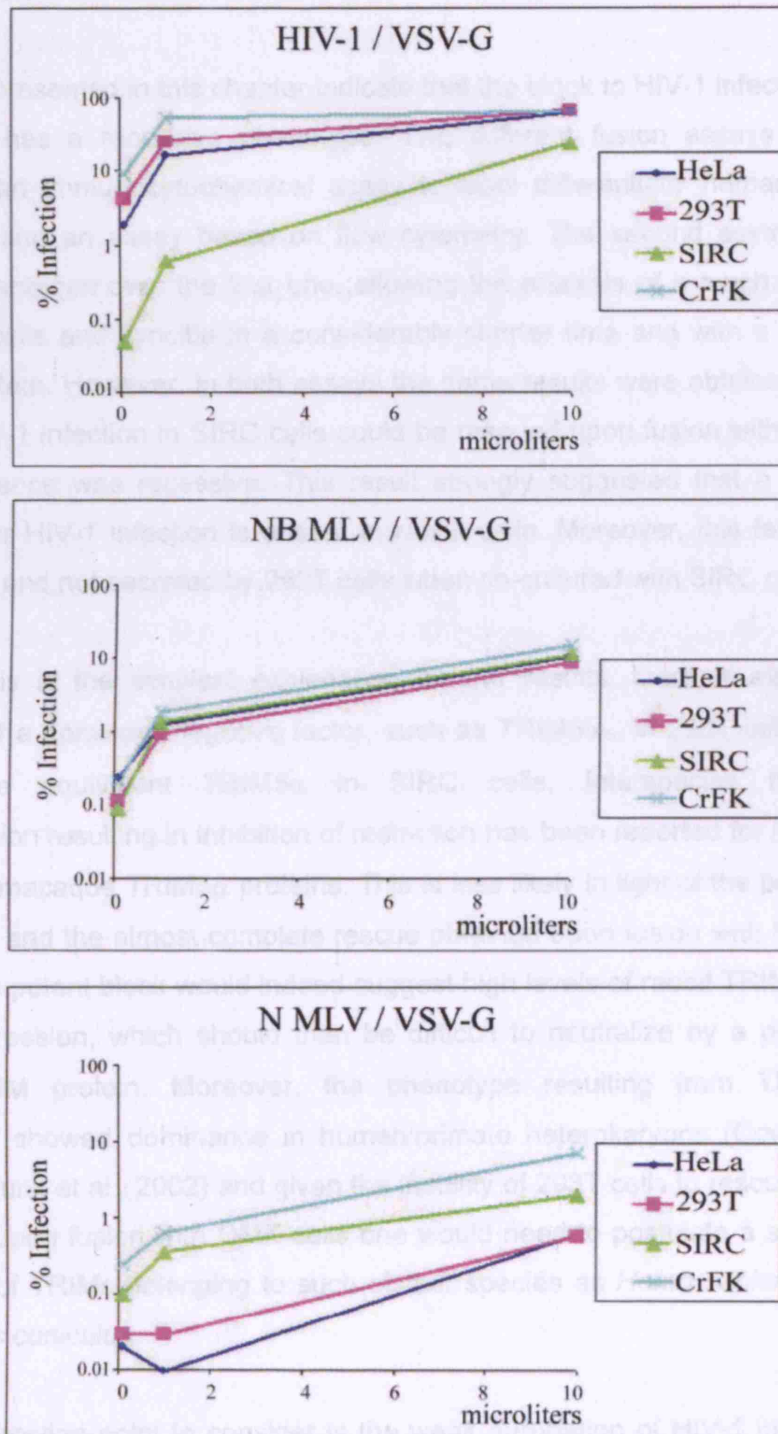
2.c Discussion

The results presented in this chapter indicate that the block to HIV-1 infection in rabbit cells has a recessive phenotype. Two different fusion assays were performed: an immunocytochemical assay to label differentially human and rabbit cells and an assay based on flow cytometry. The second assay had several advantages over the first one, allowing the analysis of a much larger number of cells and syncitia in a considerably shorter time and with a better labeling system. However, in both assays the same results were obtained: the block to HIV-1 infection in SIRC cells could be rescued upon fusion with 293T cells and hence was recessive. This result strongly suggested that a factor important for HIV-1 infection is absent in rabbit cells. Moreover, this factor is intracellular and not secreted by 293T cells when co-cultured with SIRC cells.

Although this is the simplest explanation for our results, there is also the possibility of a dominant negative factor, such as TRIM5α_{hu} in 293T cells that nullifies the equivalent TRIM5α in SIRC cells. Interspecies hetero-multimerization resulting in inhibition of restriction has been reported for human and rhesus macaque TRIM5α proteins. This is less likely in light of the potency of the block and the almost complete rescue obtained upon fusion with human cells. A very potent block would indeed suggest high levels of rabbit TRIM5-like protein expression, which should then be difficult to neutralize by a putative human TRIM protein. Moreover, the phenotype resulting from TRIM5α consistently showed dominance in human/primate heterokaryons (Cowan et al., 2002; Munk et al., 2002) and given the inability of 293T cells to rescue HIV-1 infection upon fusion with OMK cells one would need to postulate a specific interaction of TRIMs belonging to such distant species as *Homo sapiens* and *Oryctolagus cuniculus*.

Another interesting point to consider is the weak abrogation of HIV-1 infection that was achieved in SIRC cells. The modest (9 fold) saturation observed could be due to the presence of a TRIM5α like restrictor factor that partially contributes to the SIRC restrictive phenotype, albeit presumably to a lesser extent than the absence of the alleged factor necessary for infection. It should also be noted that certain primate TRIM5α variants, like New World squirrel

monkey TRIM5α, cannot be abrogated and they block HIV-1 infection at a step after reverse transcription (Ylinen et al., 2005). It is also noteworthy that HIV-1 infection in 293T cells could be abrogated 17.5 times using high viral doses. This is interesting as it suggests perhaps a difference in the expression levels of human TRIM5α. Differences in the expression of TRIM proteins in distinct tissues and developmental stages have been described (Berti et al., 2002; Reymond et al., 2001) and this could be an explanation for the results I have found in the abrogation assay with 293T cells, an embryonic cell line. Alternatively, other TRIMs with anti-HIV-1 activity might be expressed in 293T cells. In conclusion, the fusion assays and the abrogation assay indicate that the block to HIV-1 infection in rabbit cells is most likely recessive, although a minor block caused by a rabbit TRIM5-like factor is possible and would explain the weak abrogation observed. The first step towards finding this factor would be to carry out a BLAST search looking for TRIM-like sequences in the rabbit genome. A rabbit TRIM-like protein with distant homology to primate TRIM5 has been found and cloned and has been recently reported to have some (10-20 fold) ability to block HIV-1 infection, particularly if overexpressed in target cells (Greg Towers personal communication).

Part 3

3.a Introduction

Blocks to retroviruses infection can occur at different stages of the viral lifecycle. They can happen at entry if the cell receptor is absent or non-functional, after entry but before reverse transcription (as with Lv1 in rhesus macaque and African green monkey or Ref1 in human cells) after reverse transcription but prior to nuclear entry (as with Fv1 in murine cells) or after integration as in mouse cells due to the lack of functional CyclinT1 (Bieniasz et al., 1998; Browning et al., 1997; Chen et al., 1999; Garber et al., 1998; Kwak et al., 1999; Sun et al., 2006) or in rat cells, where expression of human CRM1 protein allows efficient infection (Hakata et al., 2001; Zhang et al., 2006) and in non-permissive human cells infected with vif-deficient virus (Sheehy et al., 2002; Sheehy et al., 2003).

The mechanisms of action of some of these blocks are poorly understood. For example, both the Fv1 and the Lv1 genes have been identified but how exactly they inhibit retrovirus replication remains an elusive issue. Fv1 is homologous to the gag of human HERV-L and murine MuERV-L (Benit et al., 1997) and the gene responsible for the Lv1 phenotype is Trim5α, which belongs to a family of proteins possessing a tripartite motif, a RING motif, a B-BOX and a coiled coil domain (Stremlau et al., 2004). It is interesting that although these two restricting proteins are very different, for both of them the viral determinant of restriction lies in the viral core. Fv1 and TRIM5α have been reported to act independently and to compete for incoming viral cores (Passerini et al., 2006). One hypothesis is that TRIM5α destabilizes prematurely the incoming viral core, blocking reverse transcription (Stremlau et al., 2006). However, reduced reverse transcription may not completely explain TRIM5α-mediated restriction (Wu et al., 2006).

Other blocks are better understood, albeit perhaps not completely so. Examples of these include APOBEC3G, previously known as CEM-15 (Sheehy et al., 2002), whose antiviral effect is counteracted by the viral protein Vif (Sheehy et al., 2003). One proposed mechanism of action for APOBEC3G is deamination

of deoxycytidine to deoxyuridine during HIV-1 DNA reverse transcription (Mangeat et al., 2003). This would then lead to accumulation of mutations in the provirus to a catastrophic level. It is likely that APOBEG3G is associated with the RTC but, in contrast with TRIM5α, it is incorporated in Δ-Vif virions during assembly. A strong block to HIV-1 infection in mouse cells is caused by differences in CyclinT1 that makes them non-functional with regard to HIV-1 replication (Bieniasz et al., 1998).

It was therefore important to gain a better mechanistic understanding of the block to HIV-1 infection in SIRC cells. In my case, experiments shown in chapter 1 indicated that HIV-1 in rabbit cells was blocked after entry. Since I have used viral vectors, which do not recapitulate the entire life cycle of the virus but only events up to integration and gene expression, and since HIV-1, SIVmac and MLV vectors all expressed the green fluorescent protein (GFP) driven by the same CMV promoter (Mangeot et al., 2000; Naldini et al., 1996; Soneoka et al., 1995), I summoned that the block was either at the level of reverse transcription or integration, or both.
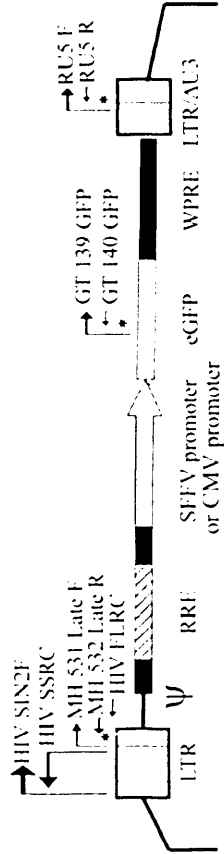
3.b Results

3.b.i Reverse transcription is impaired in SIRC cells *in vivo* but not *in vitro*.

To test if reverse transcription was affected in SIRC cells, human and rabbit cells were infected with the same dose (RT normalized) of HIV-1 vector and quantitative PCR was used to measure the amount of early (strong stop), intermediate (GFP) and late (U5/gag) viral DNA at 4, 7 and 16 hours post infection (Figure 21 and Table 1). A small aliquot of cells was analyzed by FACS 48 hours later to measure the percentage of infection in each experiment.

Details of the primers used for this analysis are shown in Table 1. Intermediate and late reverse transcription products in SIRC cells were 20 to 30 fold lower than in HeLa cells at all time points tested. Early reverse transcription products were 5 to 10 fold lower in SIRC than HeLa cells, with the least difference at 7 hours post infection (Figure 21, top panel).

All viral stocks (including SIVmac and MoMLV) used for infection were treated with DNAse I and purified through a two-step sucrose cushion. To monitor the effectiveness of the purification procedure and rule out any contamination with plasmid DNA used for virus production, viral stocks were tested by PCR using primers specific for the viral *pol* and late reverse transcription products. No DNA contamination was found in our viral stocks (Figure 22).

Purified virus was amplified with primers to detect late RT products and no signal was found. To confirm that viral DNA could be detected in our system, we amplified strong stop DNA, which is synthesized in virions in small amounts even before infection. In this case, a PCR band of the correct molecular weight could be detected. Taken together, these results demonstrated that I was indeed measuring endogenously reverse transcribed viral DNA by quantitative PCR.

Table 1. Primers and probes sequences for Taqman and standard PCR. The sequences of the primers and probes used for TaqMan PCR or regular PCR are shown. The diagram shows the primers binding sites with arrows. * indicates probe binding site.

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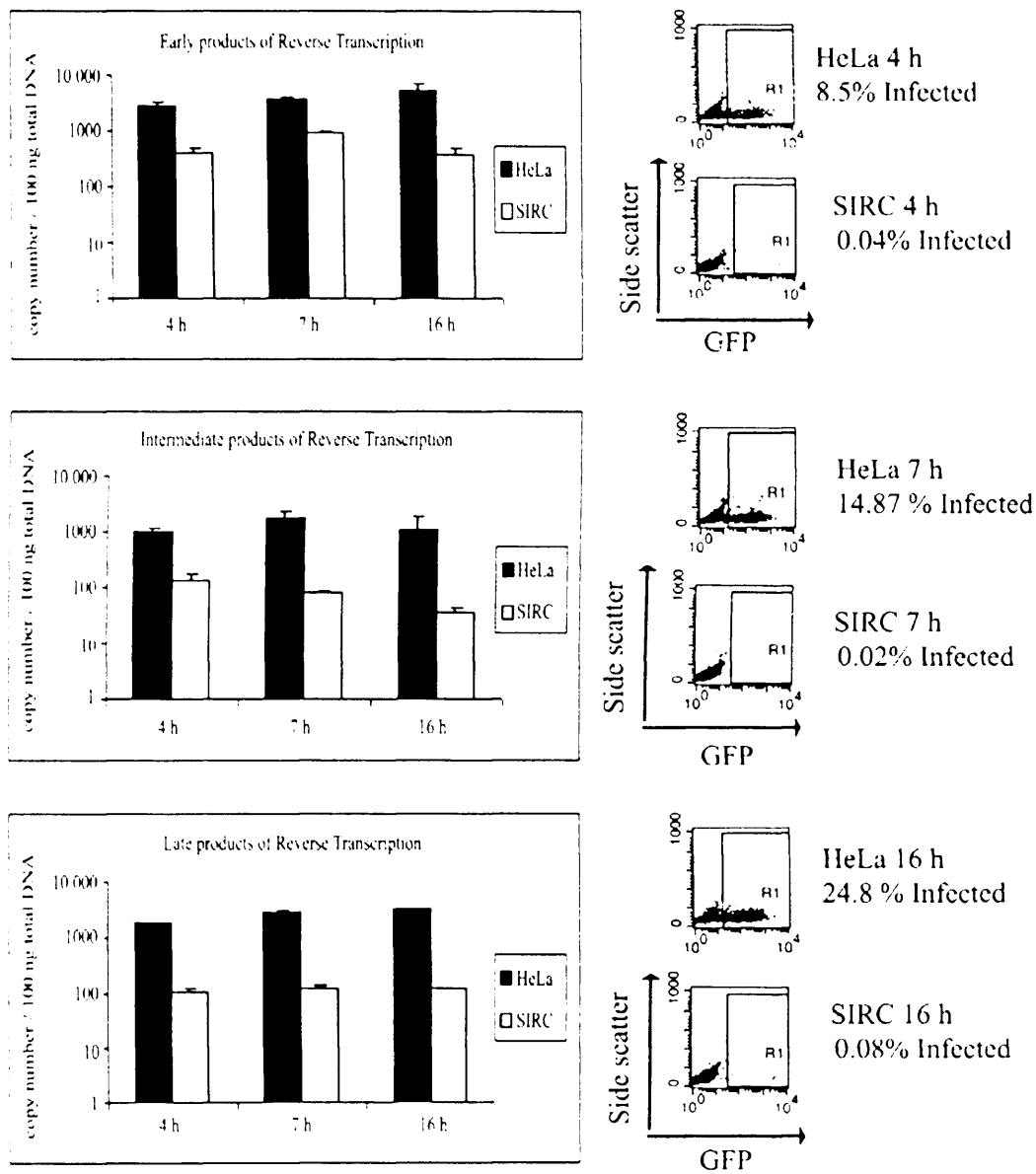
Figure 21. HIV-1 reverse transcription is defective in SIRC cells. HeLa and SIRC cells were infected with the same dose (RT normalized) of HIV-1 GFP vector pseudotyped with VSV-G envelope, incubated at 4°C for 2 hours to allow virus binding to the cell receptor but not internalization and then incubated at 37°C for 4, 7 and 16 hours. Total DNA was extracted and the number of viral DNA copies/100 ng total DNA was calculated by real-time PCR using R/U5 (early), GFP (intermediate) or U5/gag (late) primers. Values represent the mean ± standard deviation of triplicate experiments. An aliquot of infected cells was re-plated and analyzed 48 hours later by FACS to measure infection efficiency.

The results of the real-time PCR in infected human and rabbit cells demonstrated a defect in RT, although a 20 to 30-fold reduction in viral DNA copy number could not fully account for the potency of the block in SIRC cells.

3.b.ii The density of HIV RTCs extracted from HeLa and SIRC is different.

Since reverse transcription was impaired, the properties of intracellular RTCs in HeLa and SIRC cells were examined. RTCs generally have a high density in sucrose gradients, which reflects the ratio of lipids to proteins to nucleic acids, the latter being the densest components. HeLa and SIRC cells were infected with HIV-1 pseudotyped with VSV-G envelope. Cytoplasmic extracts from acutely infected cells were prepared by Dounce homogenization in hypotonic buffer as previously described (Nermut and Fassati, 2003) and subjected to equilibrium density centrifugation in linear sucrose gradients. Individual fractions were analyzed by PCR to detect viral strong stop DNA, an early product of reverse transcription and by Western blot to detect CA protein.

In HeLa cytoplasmic extracts, the peak of HIV-1 strong-stop DNA could be consistently detected in fractions with a density of approximately 1.30 g/ml, in agreement with previous studies (Fassati and Goff, 2001; Heinzinger et al., 1994). In SIRC extracts the peak of strong stop DNA was consistently detected in fractions with a density of 1.20 g/ml (Figure 23A and B). A weaker strong stop DNA band was also present in fractions with a density of 1.30 g/ml in SIRC cells and this distribution pattern was observed in another independent experiment, indicating that it was not due to experimental variation. Moreover, extraction of the nucleic acids from the density gradient fractions with phenol/chloroform followed by ethanol precipitation did not alter the results, demonstrating that the unusual two-peak distribution in SIRC cells was not due to an inhibitor of the PCR.

Figure 22. Test for DNA contamination in viral stocks. To control for DNA carry over from transfections performed to generate virus stocks, eight different virus stocks were subjected to 35 cycles PCR using primers specific for pCMVΔ8.2 *gag-pol* expression vector (see supplementary Table). Serial dilutions of plasmid down to 1pg were used to monitor the sensitivity of the PCR. Viral stocks were also subjected to PCR with primers specific for the strong stop DNA (+C) and late reverse transcription products (-C). MWM, DNA molecular weights markers.

The gradients were analyzed by Western blot to detect CA protein and results are shown in Figure 24. The p24 CA protein was detected with a peak having a density of 1.14 to 1.12 g/ml in both HIV-1 infected HeLa and SIRC cells and did not co-sediment with the p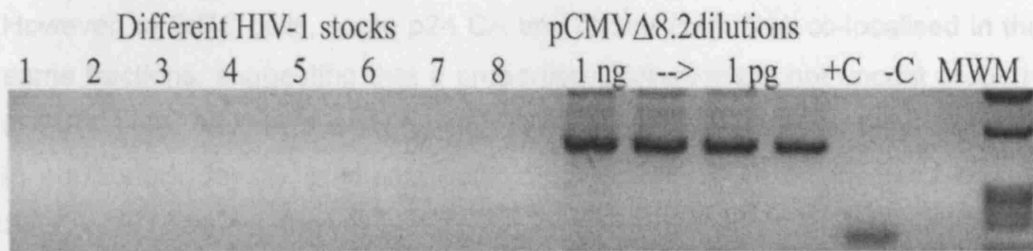eak of strong-stop DNA in significant amounts. However, in SIRC cells, some p24 CA and strong stop DNA co-localised in the same fractions, suggesting that a proportion of virus might not uncoat properly in SIRC cells. Neither the strong-stop DNA nor p24 CA proteins were detected in equilibrium density gradients containing extracts from uninfected HeLa or SIRC cells (data not shown).

To further control for the density fractionation procedure and to find out if there was some broad correlation between RTC density and inefficient retroviral infection, a combination of different viruses and cell types was examined. For example, OMK cells were infected with both HIV-1 (to which they are restricted) and SIVmac (to which they are permissive). HeLa cells were infected with HIV-1 (to which they are permissive), SIVmac (to which they are partially restricted) and N MLV (to which they are restricted). SIRC cells were infected with HIV-1 (restricted), SIVmac (permissive), N MLV (partially restricted) and NB MLV (permissive). Following infection, RTCs were extracted and analysed by equilibrium density sedimentation as described before. Data are summarized in Table 2. For a given cell type, a lower density of the strong stop DNA peak was associated with lower infectivity and a higher density with more efficient infection (see also Figure 1 in Chapter 1), suggesting that proper maturation of the RTC may be reflected in its higher density in linear sucrose gradients.

Figure 23. RTCs have a different density in HeLa and SIRC cells Cells were infected with the same dose of HIV-1 vector, incubated 2 hours at 4°C to allow virus binding to the cell receptor and then 4 hours at 37°C. Cytosolic extracts were prepared from infected cells by Dounce homogenization in hypotonic buffer and centrifuged through a 20-70% linear sucrose gradient. Individual fractions were subjected to PCR with primers specific for the strong stop DNA (expected size 140 bp). (A) Density fractions from HeLa cells; (B) density fractions from SIRC cells. The arrow indicates the direction of the gradient (top = low density, bottom = high density). Low molecular weight bands are PCR artifacts. Plasmid DNA was used as a positive control (+C). (C) Regression plot showing the density of each individual fraction measured as described in Matherials and Methods.
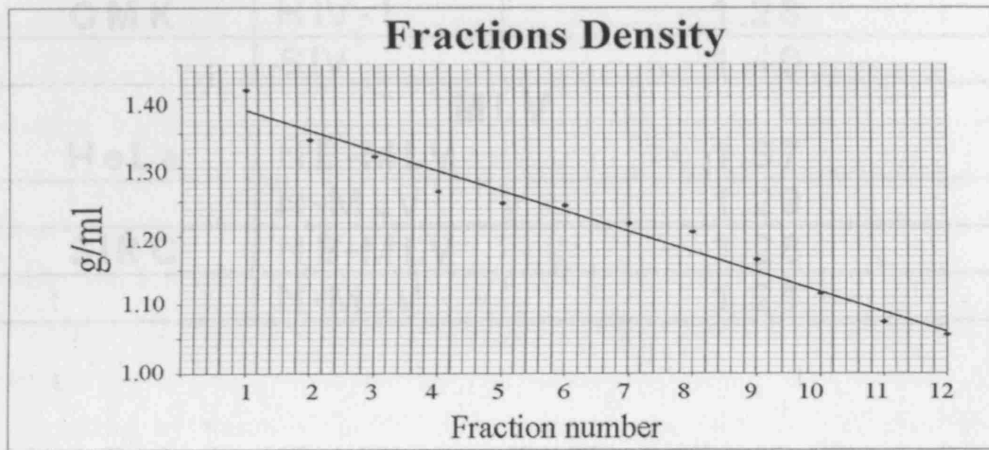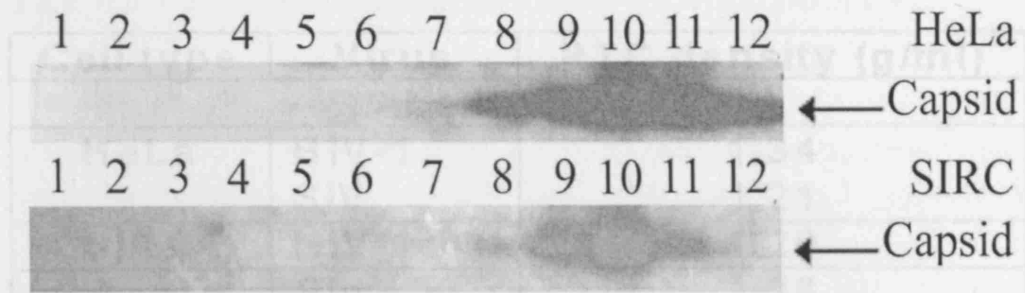
Figure 24. Western blot of density gradient fractions. Fractions from a density gradient of HIV-1 infected HeLa and SIRC cells were analyzed by Western blot using a monoclonal antibody to anti-p24.

Cell type	Virus	RTC density (g/ml)
Lentivirus		
HeLa	HIV-1	1.34
	SIV	1.21
SIRC	HIV-1	1.20
	SIV	1.28
OMK	HIV-1	1.28
	SIV	1.40
MLV		
HeLa	NB-MLV	1.37
	N-MLV	1.29
SIRC	NB-MLV	1.28
	N-MLV	1.21

Table 2. The density of HIV-1, SIVmac and MLV RTCs is different in different cell types. Cells were infected with VSV-G pseudotyped vectors, incubated for 2 hours at 4°C to allow virus binding but not internalization and then for 4 hours at 37°C. Cytoplasmic extracts prepared were prepared by hypotonic lysis and Dounce homogenization, subjected to equilibrium density sedimentation in linear sucrose gradients and the position of the strong stop DNA was analysed by PCR. For SIRC cells infected with HIV-1, the density of only the major peak is indicated in the table.

3.b.iii HIV-1 RTCs extracted from SIRC cells are functional *in vitro*

Given that HIV-1RTCs extracted from SIRC cells had a lower density than HeLa cells and that HIV-1 RT was defective in SIRC cells, it was important to test if HIV-1 RTCs isolated from SIRC cells were intrinsically defective. An endogenous reverse transcription (ERT) assay to compare the *in vitro* activity of the 1.30 g/ml RTC peak found in HeLa cells with the 1.20 g/ml RTC peak found in SIRC cells was performed. Fractions containing the peak of the strong stop DNA were incubated with dNTPs at 37°C for 6-7 hours to allow completion of reverse transcription of viral RNA and late RT products detected by PCR (Fassati and Goff, 2001). In both HeLa and SIRC cells, synthesis of near full length HIV-1 DNA (Figure 25A) was observed. As a control, the same ERT assay was carried out in the absence of dNTPs and, as shown in Figure 15A, there was no near full length HIV-1 DNA in these conditions. To further confirm the activity of the 1.20 g/ml RTC extracted from rabbit cells, samples were normalized for strong stop DNA concentration and examined by quantitative PCR the amount of endogenously synthesized late viral DNA (Figure 25B). RTCs extracted from rabbit cells had the same or even greater relative ability (+dNTPS/ –dNTPs) to synthesize late viral DNA products than RTCs extracted from HeLa cells, though SIRC complexes yielded approximately 6-fold fewer late RT products than HeLa cell RTCs (Figure 25B).
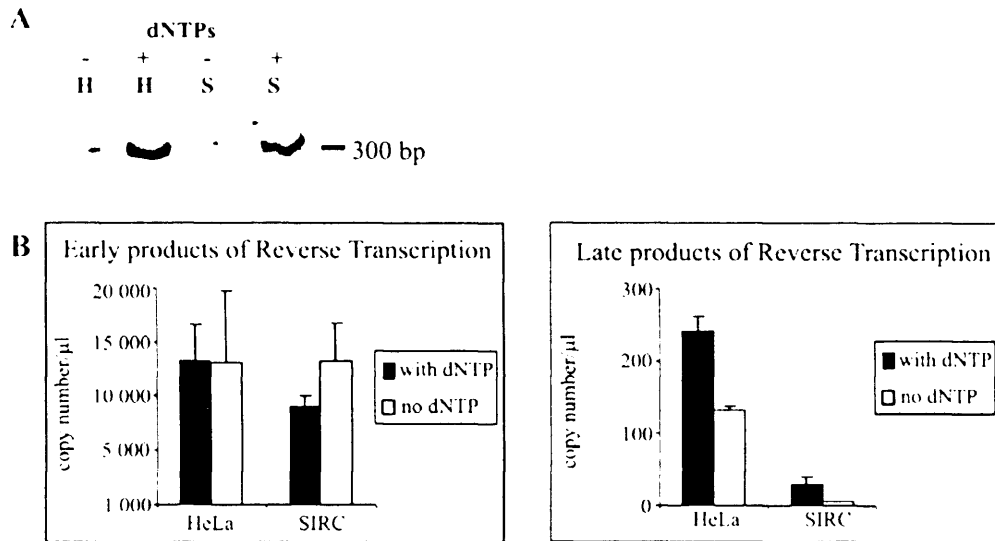
Figure 25. HIV-1 RTCs extracted from HeLa and SIRC are competent for reverse transcription *in vitro*. (A) The fractions containing the peak of strong stop DNA (fraction 5 for HeLa and fraction 9 for SIRC cells) were subjected to an endogenous RT reaction in the presence (+) or absence (-) of exogenous dNTPs and analyzed by PCR with primers specific for late reverse transcription products (HIV SIN2F and HIV FLRC). H, HeLa; S, SIRC; MWM. (B) Quantification of endogenous reverse transcription activity by qPCR. Samples were normalized for strong stop DNA concentration (left panel) and subjected to an endogenous reaction as before in the presence or absence of exogenous dNTPs. Products of the reaction were then analyzed by qPCR using primers for late viral DNA products (right panel).

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3.b.iv Introduction

Since HIV-1 RTCs were not intrinsically defective for reverse transcription, it was possible that they were incorrectly localised within cells in compartments not conductive to viral DNA synthesis so it was necessary to look for methods that would help analysing the intracellular distribution of RTCs in human and rabbit cells.

Different methods of sub cellular fractionation have been used for different purposes (Fassati and Goff, 2001; Jensen et al., 1968; Marchant et al., 2005; Ostoa-Saloma et al., 1989; Zhang et al., 2003). For example, in a study by Gao and Goff, two mutant Rat2 cells clones were identified to block MLV infection. A cell fractionation procedure was used to study the intracellular distribution of the blocked virus (Gao and Goff, 1999). I decided to use a similar sub cellular fractionation procedure in which buffers with increasing salt concentrations are followed by buffers with different detergents such as Triton-X and SDS. Low (hypotonic) and physiological (isotonic) salt extractions are used mainly to obtain cytoplasmic soluble proteins. High salts extraction is used mainly to release proteins with stronger bonds to the cytoskeleton and other intracellular structures. Different detergents have been shown to be able to extract different proteins from cell lysates: Triton-X extracts proteins from the cellular membrane and internal organelles while SDS extracts the more insoluble proteins and the chromatin-bound nuclear content (McCarthy et al., 2005). Thus, a fractionation procedure with different salt concentrations and detergent composition was designed to cause stepwise disruption of increasingly stronger protein-protein interactions as well as extraction of different intracellular compartments and allow some crude examination of viral trafficking (Gao and Goff, 1999).

In this section, HIV-1 viral DNA and RNA are shown to have a different intracellular distribution in HeLa than in SIRC cells.

3.b.iv.1 HIV-1 viral DNA has a different intracellular distribution in HeLa and SIRC cells.

For sub cellular fractionation, Hela and SIRC cells were infected with the same amount of recombinant VSV-G pseudotyped HIV, SIV or MLV vectors. In the case of HIV-1, the amount of virus used resulted in infection of approximately 2% HeLa cells and ≤ 0.07% SIRC cells as measured by FACS analysis for GFP expression 48 hours post infection. Infection was synchronized by pre-incubation of cells at 4°C for 2 hours to allow virus binding to the receptor but not internalization. Cells were then incubated for 4 hours at 37°C and subjected to sequential lysis in hypotonic, isotonic, high salt, 1% Triton X and 0.5% SDS - containing buffers (Figure 26).

The effectiveness of the fractionation procedure was monitored by SDS-PAGE and silver staining of the protein content of each individual fraction and by Western blot with a monoclonal antibody against alpha-tubulin. As shown in Figure 27A, silver staining after SDS-PAGE revealed distinct protein patterns in each fraction and the Western blot showed that similar amounts of tubulin were recovered in the same HeLa and SIRC cells fractions, demonstrating near-equivalent cell lysis (Figure 27B).

Each fraction was then analyzed for the presence of viral DNA by standard and quantitative PCR (Figure 28). The PCR results confirmed that the overall efficiency of HIV-1 reverse transcription at 4 hours post-infection was reduced in SIRC cells. Importantly, a different intracellular distribution of HIV-1 DNA in HeLa and SIRC cells was found. HIV-1 reverse transcription products appeared mainly in the hypotonic fraction in infected SIRC cells. In HeLa cells, most of HIV-1 reverse transcribed DNA appeared in the isotonic and high salt fractions (Figure 28A). These results were confirmed by quantitative PCR in an independent fractionation experiment (Figure 28B). When the SIVmac vector was used for infection, viral DNA had a similar distribution in both HeLa and SIRC cells, with most DNA being recovered in the hypotonic fraction. Importantly, MLV also had a similar distribution in both HeLa and SIRC cells, and most viral DNA was consistently recovered in the hypotonic fractions (Figure 28B).

Infection of cells → Incubation at 4 °C for 2 hrs + 37 °C for 4 hrs

Hypotonic buffer → Dounce homogenisation

CENTRIFUGATION → Supernatant → PCR

↓

Pellet → Isotonic extraction

CENTRIFUGATION → Supernatant → PCR

↓

Pellet → High Salt extraction

CENTRIFUGATION → Supernatant → PCR

↓

Pellet → Isotonic 1% Triton extraction

CENTRIFUGATION → Supernatant → PCR

↓

Pellet → SDS extraction
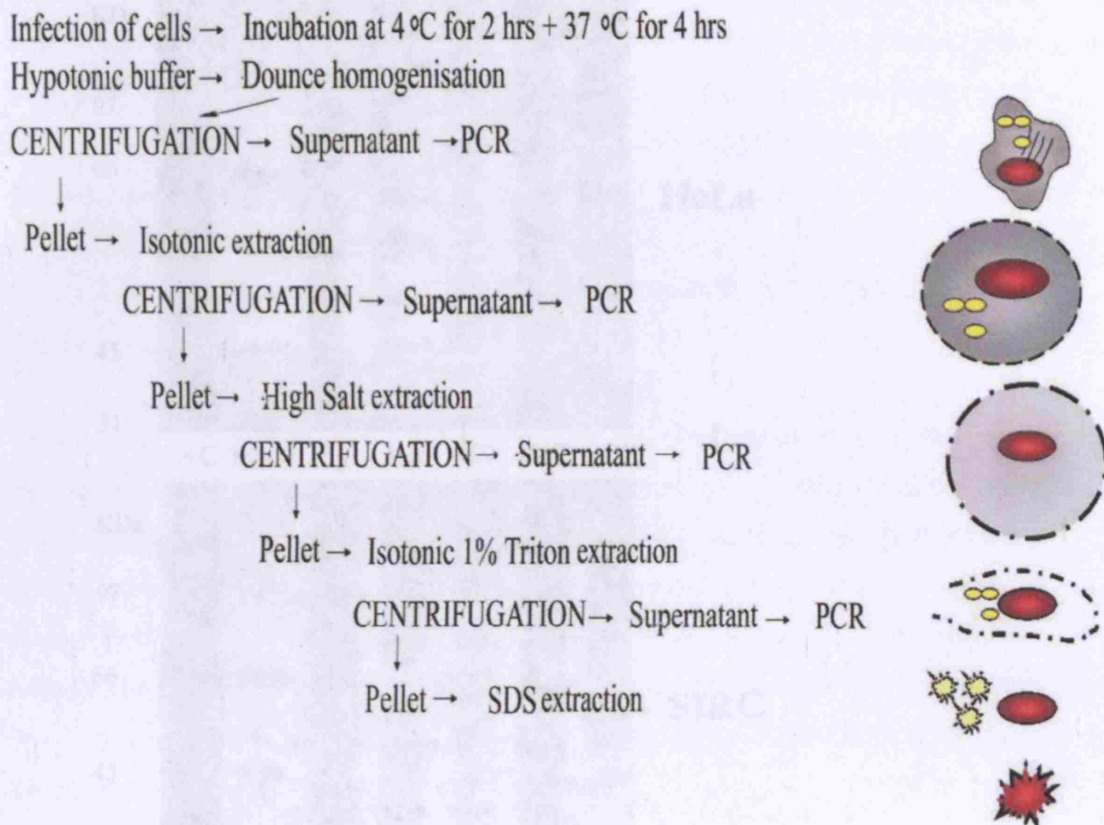
Figure 26. Stepwise fractionation of HeLa and SIRC cells. Diagram showing the various steps of the sub cellular fractionation procedure used to examine viral trafficking.

Figure 27. Cell fractionation extracts different cellular proteins. Proteins extracted by cell fractionation were concentrated and analyzed by SDS-PAGE and Silver stain. Hy, hypotonic fraction; Is, isotonic fraction; HS, high salt fraction; 1% T, 1% Triton-X fraction, SDS, SDS fraction. MWM, molecular weight markers. +C, total (unfractionated) cell extracts. Asterisks mark protein bands present in specific fractions (B) Western blot analysis of the same fractions with an anti-α tubulin monoclonal antibody.

These results suggested that there was a correlation between virus infectivity and intracellular distribution of viral DNA. Indeed the SIVmac and MLV vectors infected SIRC cells at higher efficiency than the HIV-1 vector and most of their DNA was recovered in the same fractions in both cell types. Conversely, most of HIV-1 DNA was recovered in the hypotonic fraction in restricted cells and in the high salt fraction in permissive cells.

3.b.iv.2 HIV-1 viral RNA is found in the same cellular fractions as the viral DNA.

To exclude the possibility that the intracellular distribution of only the non-restricted virus population was being analyzed, the same fractionation procedure was carried out in RNase-free conditions: samples were treated with RNase-free DNAse to reduce background from endogenously reverse transcribed RTCs and the viral RNA in each fraction was used as template for first-strand cDNA synthesis. Control reactions were carried out in the absence of RT and viral cDNA copy number was measured by quantitative PCR (Figure 29). The data revealed two noteworthy aspects.

First, in the hypotonic fraction the amount of viral RNA in SIRC cells was higher than in HeLa cells. This suggested that RTCs were not degraded in SIRC cells, at least at 4 hours post-infection. Second, the distribution of viral RNA in both HeLa and SIRC cells mimicked the distribution of endogenously reverse transcribed viral DNA (compare Figures 28B and 29 top two panels), with the exception of the viral RNA found in the SDS fraction. However, because the bulk of viral RNA was found in the SDS fraction of both HeLa and SIRC cells, it must represent a dead-end pathway unrelated to the infection block. Control reactions with no RT contained fewer than 100 viral cDNA copies, demonstrating that bona fide viral RNA was being measured in test samples (Figure 29). These results suggested that distinct virus populations, one restricted and the other not restricted, may not be present in SIRC cells.
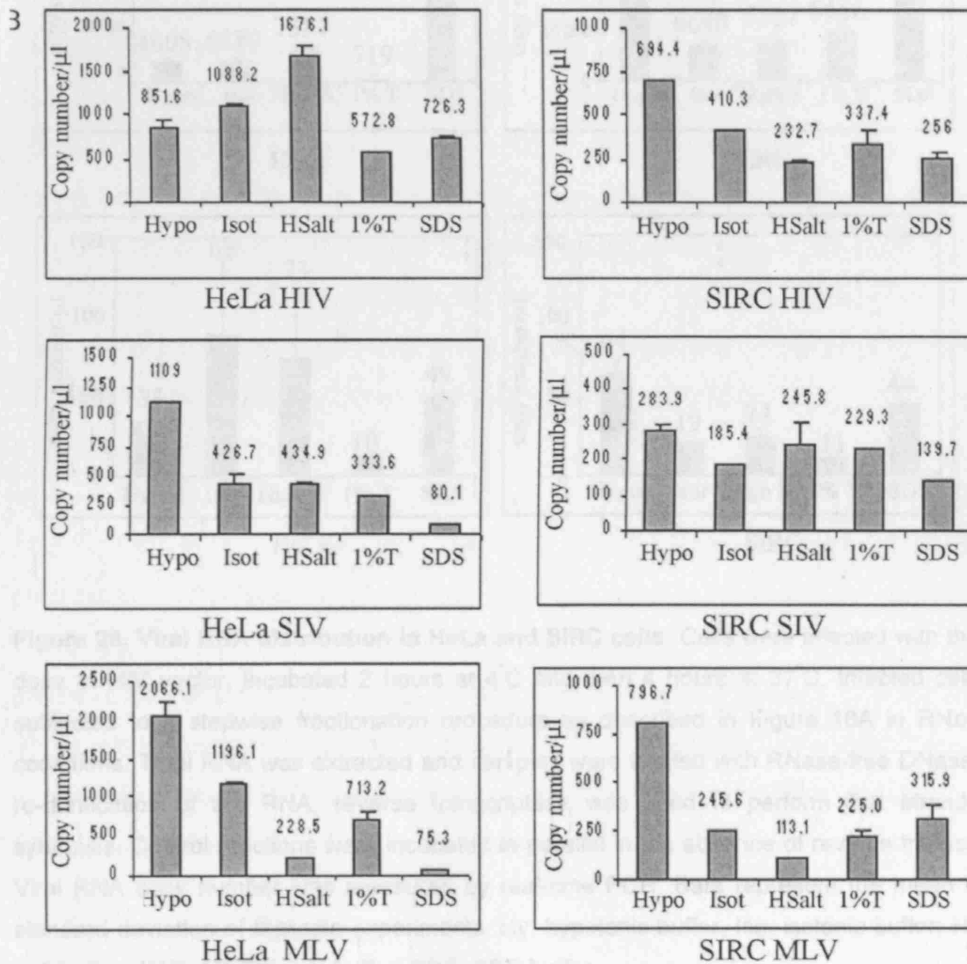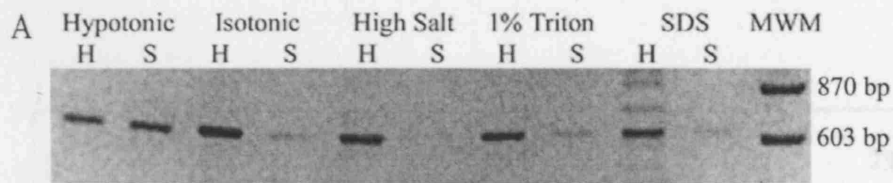
Figure 28. HIV-1 DNA has a different intracellular distribution in HeLa and SIRC cells. Cells were infected with the same dose of HIV-1, SIVmac or MLV vectors pseudotyped with VSV-G, incubated 2 hours at 4°C and then 4 hours at 37°C and subjected to the stepwise fractionation procedure described in Figure 5A. (A) Total DNA was extracted from each fraction and analyzed by PCR with primers specific for late reverse transcription products. H, HeLa cells, S, SIRC cells. MWM, molecular weights markers. (B) The entire procedure was repeated and fractions analyzed by real-time PCR (GFP primers) to measure viral DNA copy number. Bars represent the mean ± standard deviation of triplicate experiments.
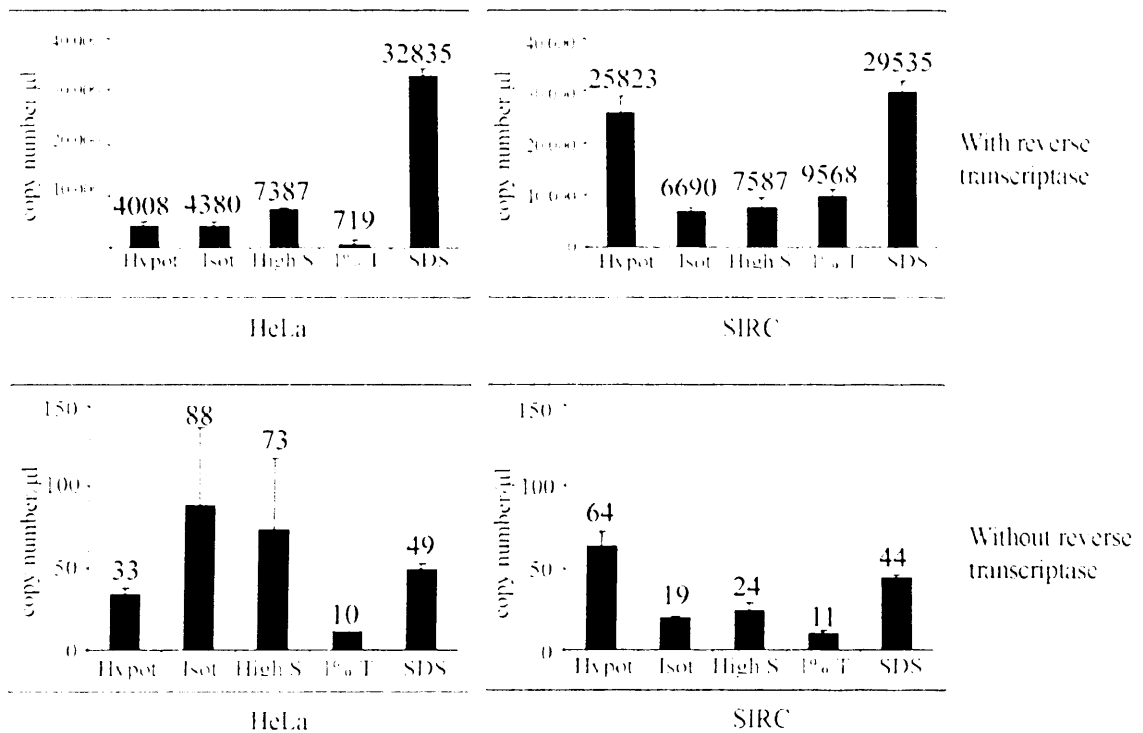
Figure 29. Viral RNA distribution in HeLa and SIRC cells. Cells were infected with the same dose of HIV vector, incubated 2 hours at 4°C and then 4 hours at 37°C. Infected cells were subjected to a stepwise fractionation procedure as described in Figure 18A in RNase free conditions. Total RNA was extracted and samples were treated with RNase-free DNaseI. After re-purification of the RNA, reverse transcriptase was used to perform first strand cDNA synthesis. Control reactions were incubated in parallel in the absence of reverse transcriptase. Viral RNA copy number was measured by real-time PCR. Bars represent the mean value ± standard deviation of triplicate experiments. Hy, hypotonic buffer; Iso, isotonic buffer; HS, high salt buffer; 1%T, 1% Triton-X buffer; SDS, SDS buffer.

The next thing to investigate was if viral DNA could reach the nucleus or if it remained in the cytoplasm as a consequence of aberrant trafficking. To do this, nuclei and cytoplasm must be separated after infection and each fraction analysed for viral DNA content.

Nuclei extraction from cells can be carried out with different methods, one of them is cellular lysis with NP40 buffer (Bandyopadhyay et al., 1986; Fischle, 2005; Nicolaides and Stoeckert, 1990) which disrupts cytoplasmic and intracellular membranes but preserves the nuclear envelope. Nuclei Isolated by this procedure have been used for a wide range of assays, to study the early association of SV40, the nuclear structures after infection (Watson and Gralla, 1987), the nuclear export of influenza ribonucleoprotein complexes (Elton et al., 2001) and the in nucleo study of histone modification (Fischle, 2005) to mention a few examples.

After isolating the nuclei from the rest of the cell's components, a further separation of the nuclear content can be obtained by the method described by Bernhard Hirt in 1967 to extract polyoma virus DNA from mouse cells. This method consists of cell lysis by the addition of SDS and precipitation of unsheared genomic DNA with NaCl at 0°C. Cell extracts are centrifuged and viral DNA stays in the supernatant because of its smaller size while chromatin DNA precipitates (Hirt, 1967).
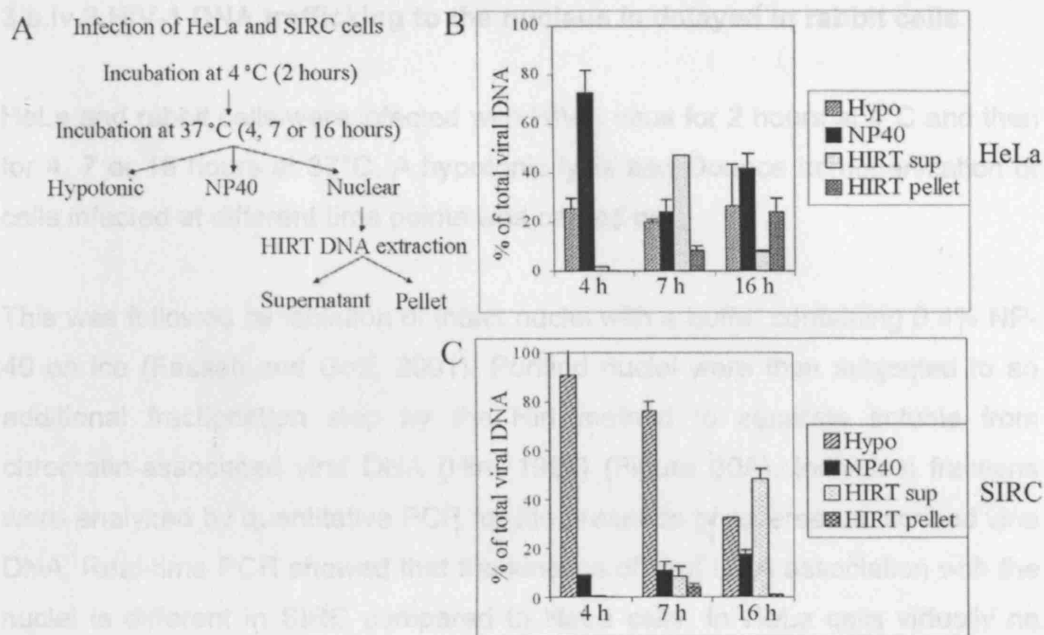
Figure 30. Association of viral DNA with the nuclei is delayed in SIRC cells. HeLa and SIRC cells were infected with the same dose of HIV-1 vector, incubated 2 hours at 4°C and then 4, 7 and 16 hours at 37°C. (A) Schematic representation of the fractionation procedure designed to separate the cytosol from intact nuclei. Nuclei were then processed into a soluble and chromatin fraction by the Hirt method. DNA was extracted from each fraction and analyzed by real-time PCR (GFP primers) to measure viral DNA copy number. (B), distribution of viral DNA in HeLa cells. (C) Distribution of viral DNA in SIRC cells. Bars represent the mean value ± standard deviation of triplicate experiments. Hypo, hypotonic buffer; NP40, buffer containing 0.4% NP-40.

3.b.iv.3 HIV-1 DNA trafficking to the nucleus is delayed in rabbit cells.

HeLa and rabbit cells were infected with HIV-1 virus for 2 hours at 4°C and then for 4, 7 or 16 hours at 37°C. A hypotonic lysis and Dounce homogenization of cells infected at different time points was carried out.

This was followed by isolation of intact nuclei with a buffer containing 0.4% NP-40 on ice (Fassati and Goff, 2001). Purified nuclei were then subjected to an additional fractionation step by the Hirt method to separate soluble from chromatin-associated viral DNA (Hirt, 1967) (Figure 30A). Individual fractions were analyzed by quantitative PCR for the presence of reverse transcribed viral DNA. Real-time PCR showed that the kinetics of viral DNA association with the nuclei is different in SIRC compared to HeLa cells. In HeLa cells virtually no viral DNA was nucleus-associated at 4 hours after infection however viral DNA could be found associated with HeLa nuclei (Hirt sup) 7 hours after infection and was chromatin-associated (Hirt pellet) in significant amounts 16 hours post-infection (Figure 30B). In SIRC cells HIV-1 DNA was found mainly in the hypotonic fraction 4 and 7 hours after infection. Viral DNA was associated with the nuclei (Hirt sup) only 16 hours after infection and was not chromatin-associated (Figure 30C). Cytoplasmic and nuclear extracts were analyzed by Western blot with antibodies against a protein mainly located at the plasma membrane and in the cytoplasm like Na/K ATPase to control for cross-contamination during fractionation. The Western blot with anti-Na+/K+ ATPase antibody showed only very modest cross-contamination between plasma membrane/cytosol and nuclear fractions (Figure 31).

These results suggested that in SIRC cells a small proportion of HIV-1 DNA could access the nucleus but an additional block to HIV-1 infection might occur after nuclear entry, perhaps a block to integration. To confirm that viral DNA could access the nucleus in SIRC cells, the accumulation of 2LTR circular DNA in nuclear extracts from HeLa and SIRC cells infected with HIV 4, 7 and 16 hours post infection was analyzed.

Figure 31. Cross contamination levels between cytoplasmic and nuclear fractions during nuclei extraction. Cytoplasmic and nuclear fractions were prepared as described in Figure 20 and tested by Western blot using an anti-Na+/K+ ATPase monoclonal antibody. +C, positive control (unfractionated HeLa cells); H, HeLa cells; S, SIRC cells

Viral infection results in different types of DNA molecules derived from the viral genomic RNA: a double-stranded, linear DNA molecule with an LTR at each end and two different circular forms of DNA, present inside the nucleus. The most abundant circular form has a single LTR and the less abundant form has two LTRs in tandem, (2LTR circular viral DNA) formed by ligation or autointegration (Farnet and Haseltine, 1991). The linear form is a precursor of the integrated provirus but the circular forms are a dead-end product (Brown et al., 1989). 2LTR circular forms are considered a hallmark of nuclear entry and measurement of 2LTR circular DNA has been used to assess HIV-1 nuclear entry blocks in murine T cells (Baumann et al., 2004) and in human monocytes (Neil et al., 2001) among many examples.

No 2LTR circular DNA could be detected at 4 hours post-infection and only very little amounts could be detected in HeLa nuclei 7 hours post-infection. However, at 16 hours post-infection, 2LTR DNA was found mainly in the Hirt supernatant in both HeLa and SIRC cells (Figure 32). Small amounts of 2LTR DNA were detected in the NP-40 fraction in HeLa cells and this could be due to some contamination between fractions or to the fact that a small proportion of infected HeLa cells entered mitosis at the time of fractionation, thus had no defined nucleus. A smear was detected in the NP40 probably due to the different sizes that 2LTRs can have as they form by recombination or ligation so it was imposible to obtain a single band of amplification. It was also impossible to detect 2LTR circles by Real-Time PCR probably because the binding site for the probe is not always present in the 2LTR sequence for the same reason mentioned above. It is important to mention that low virus input was used for these experiments to maximise the phenotype and obtain clearer results (stay in the linear range of infection) but this made it more difficult to detect low abundance 2LTR circular DNA. To bypass this problem, a very sensitive system was used: PCR products were resolved in polyacrylamide gels and visualized with SYBR gold staining. Nevertheless, these results were consistent with the earlier fractionation data on linear viral DNA and indicated that HIV-1 could, albeit inefficiently, enter the nucleus in SIRC cells.
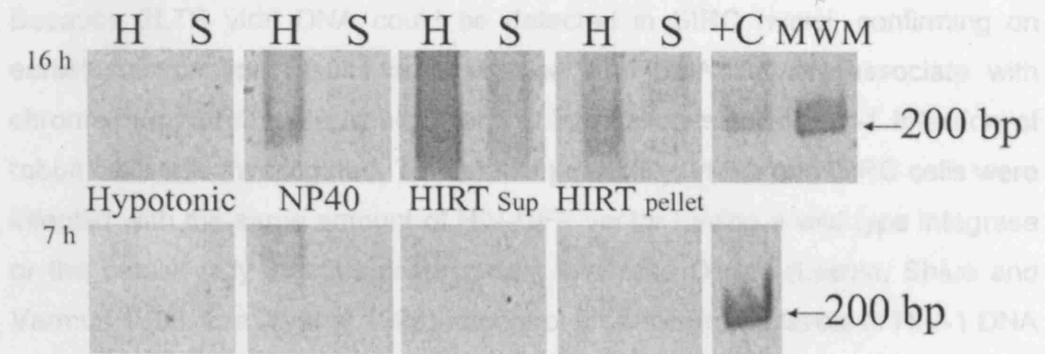
Figure 32. 2LTR circles in infected SIRC cells are slightly fewer than in HeLa. HeLa and SIRC cells were infected with the same amount of VSV-G pseudotyped HIV-1 vector and fractionated 7 and 16 hours post-infection as depicted above. DNA was extracted and PCR was performed on each fraction to detect 2LTR circular forms (approximate size 200bp). Total DNA extracted from HIV-1 infected HeLa cells was used as positive control (+C). *MWM* DNA molecular weight marker

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3.b.iv.4 Long term infection

Because 2LTR viral DNA could be detected in SIRC nuclei, confirming on earlier fractionation results and because viral DNA did not associate with chromatin in SIRC cells, a block at the integration step in HIV-1 infection of rabbit cells was investigated. To test this possibility, HeLa and SIRC cells were infected with the same amount of HIV-GFP vector having a wild type integrase or the catalytically inactive point mutant integrase D64V (Leavitt, Shiue and Varmus 1993, Leavitt et al 1996) to control for background levels of HIV-1 DNA integration and persistence. Infected cells were cultured for two weeks: In these conditions, the vast majority of viral DNA detected is integrated since non-integrated DNA is progressively lost by degradation and dilution. Infected cells were first analyzed by FACS to assess the percentage of GFP+ cells and then DNA was extracted and analyzed by quantitative PCR to measure provirus copy number. As shown in Figure 33A, the percentage of GFP+ HeLa cells was greatly reduced upon infection with HIV-1 having a defective integrase compared to the normal HIV-1 vector. Consistent with previous results, SIRC cells showed very low levels of infection with both the normal and the integration defective HIV-1 vector. The amount of viral DNA in SIRC cells was substantially reduced compared to HeLa cells, and was comparable with the levels measured in cells infected with an integration defective virus (Figure 33B). HeLa cells contained up to 500 times more viral DNA copies than SIRC cells, which fully accounted for the difference in infection efficiency as measured by GFP expression (Figure 33A). It is worth emphasizing here that linear viral DNA at 4, 7 and 16 hours post infection was reduced in SIRC compared to HeLa cells to an extent that could not fully account for the block in infection as measured by FACS. On the other hand, provirus copy number in long-term cultures was reduced in SIRC cells to levels that matched infectivity. Given that HIV-1 linear DNA could access the nucleus in SIRC cells, these results suggested that some step leading to efficient HIV-1 integration might also be defective in SIRC cells.
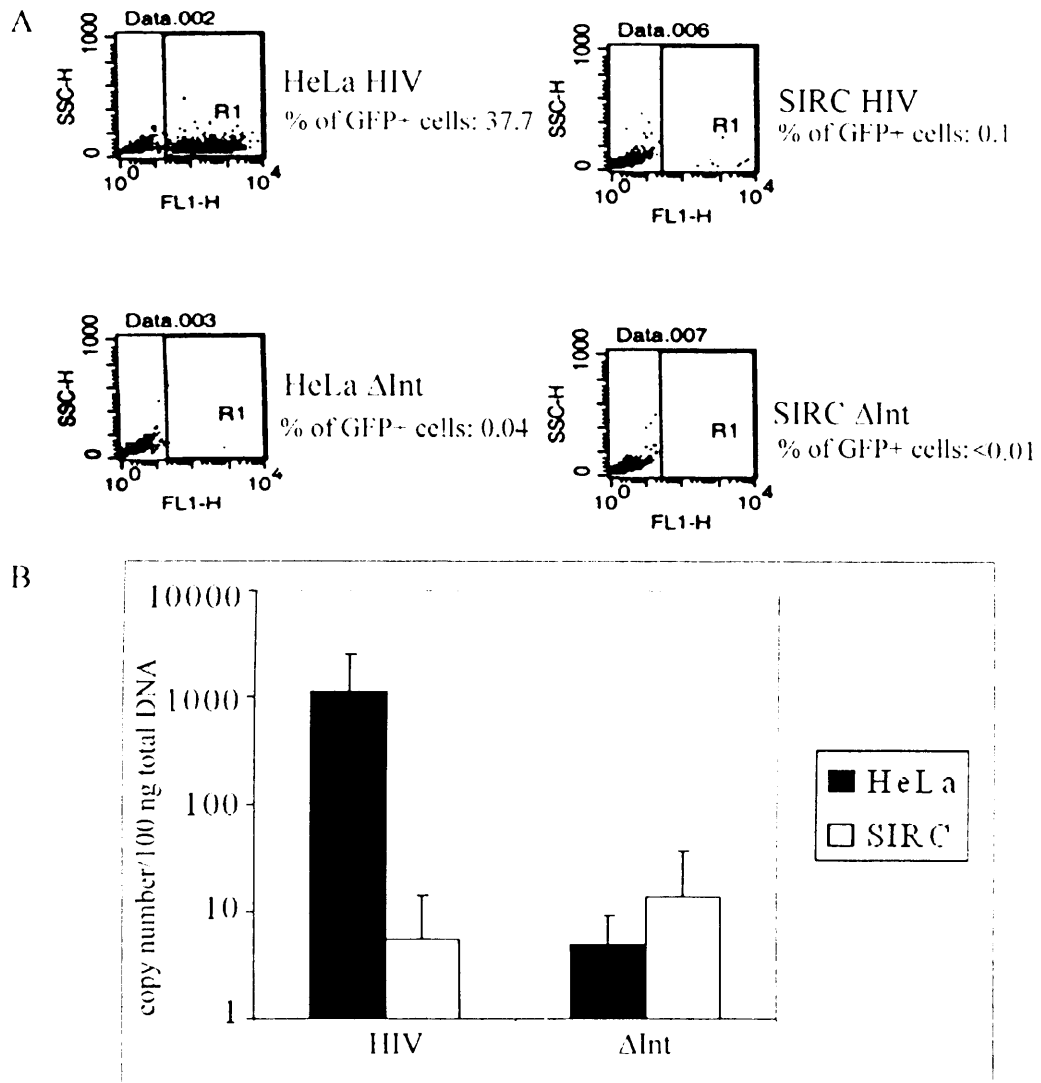
Figure 33. Integration might be defective in SIRC cells. (A) HeLa and SIRC cells were infected with the same amount of a VSV-G pseudotyped HIV-1 vector or with a mutant HIV-1 GFP vector having a catalytically inactive point mutant integrase (ΔInt) and passaged for 2 weeks. Cells were analyzed by FACS to detect GFP+ (infected) cells. (B) Total DNA was extracted from the same cells and analyzed by quantitative real time PCR to measure proviral DNA copy number.

3.c Discussion

The experiments described in this chapter demonstrate that reverse transcription and probably integration of HIV-1 in SIRC cells are impaired. The amount of late viral DNA in SIRC cells was 20 to 30 fold lower than in HeLa cells at 4, 7 and 16 hours post infection. This suggested that reverse transcription in SIRC cells was irreversibly blocked rather than delayed. This block to reverse transcription could be due to defective RTCs, rapid RTC degradation and/or aberrant intracellular RTC trafficking.

The first possibility was examined by partial RTC purification in linear sucrose gradients. This technique has been used previously to characterize HIV-1 and MLV RTCs from acutely infected cells and is suitable to detect major differences in the biophysical properties of RTCs (Bowerman et al., 1989; Fassati and Goff, 1999; Heinzinger et al., 1994; Yuan et al., 2002). Most RTCs isolated from acutely infected SIRC cells had a significantly lower density compared to RTCs isolated from HeLa cells, a result found in two independent experiments. Analysis of RTC of different viruses in different cell types revealed a correlation between RTC density and infectivity. Higher RTC density correlated with higher infectivity and vice versa. This suggests that some maturation step of the RTC may be required, similar to the maturation step, which takes place after virus budding. Proteomics analyses of purified complexes from infectious and non-infectious viruses and genetic manipulation of HIV-1 followed by biochemical studies of RTC behavior in infected cells may help understand this aspect in the future.

Despite different biophysical properties, RTCs isolated from HeLa and SIRC cells were both competent for reverse transcription in vitro when provided with exogenous dNTPs. Thus, the block to HIV-1 reverse transcription in SIRC cells was unlikely to be caused by an intrinsic defect of intracellular RTCs.

Rapid degradation of the viral genome was also unlikely to explain the poor HIV-1 DNA accumulation in SIRC cells because the amount of viral DNA measured in SIRC cells was low but did not decrease significantly with time and the amount of HIV-1 RNA in the hypotonic fraction was higher in SIRC

than in HeLa cells 4 hours post infection. Moreover, RTCs recovered from SIRC cells 4 hours post infection were competent for reverse transcription if provided with exogenous dNTPs. Nevertheless, on the basis of this data it cannot be excluded that RTC degradation may involve its protein components and may take place at a later stage and further work is needed to address this issue.

Western blot of density fractions with antibodies against p24 CA showed efficient dissociation of the viral capsid from viral DNA at 4 hours post infection in HeLa cells and to an extent also in SIRC cells, but this assay cannot establish if the uncoating was accelerated, which has been postulated as the mechanism of action of TRIM5α to inhibit viral replication (Stremlau et al., 2006). Optimal core stability has been shown to be important for viral replication (Forshey et al., 2002) and timed uncoating may have something to do with the RTC maturation step and increase in density discussed above.

The hypothesis that aberrant RTC trafficking could be linked to reduced levels of HIV-1 reverse transcription in SIRC cells was investigated by stepwise cell fractionation in different lysis buffers. This technique has been used to monitor intracellular organelle and protein distribution in different cell types (Lim et al., 2001; Ramsby et al., 1994; Wang et al., 1997) and to perform crude examination of intracellular virus trafficking (Gao and Goff, 1999). The cell fractionation procedure revealed that most of HIV-1 DNA is found in isotonic and high salt fractions in HeLa cells and in the hypotonic fraction in SIRC cells. This difference of HIV-1 distribution in SIRC cells is likely to be related to inefficient infection because it was not observed in the same cell types infected with SIVmac and MLV, two viruses that could infect SIRC cells quite efficiently. Furthermore, significant amounts of HIV-1 RNA were recovered in the hypotonic fraction in SIRC cells, suggesting that RTCs in that fraction were poorly active. The same RTCs were competent for reverse transcription if provided with exogenous dNTPs, reinforcing the view that their intracellular location was not conductive to viral DNA synthesis. Although the fractionation procedure is too crude to draw a firm conclusion, it can be speculated that in SIRC cells HIV-1 RTCs do not associate with some cytoskeleton component but remain trapped in an easily extractable compartment. Indeed, there is

evidence that HIV-1 RTCs associate with cytoskeleton components at some point after cell entry, making them more difficult to extract by low salt buffers (Bukrinskaya et al., 1998; McDonald et al., 2002). Another unanticipated result of the cell fractionation was the different intracellular distribution of HIV-1 compared to MLV and, to a lesser extent, SIVmac DNA in the same cell type. All vectors were pseudotyped with VSV-G, thus in this case different viral trafficking was likely to be determined by viral proteins other than Env. It is unclear at present why HIV-1 appears to require an intracellular trafficking distinct from the one required by MLV for efficient infection.

The magnitude of the reverse transcription defect (20-30 fold) could not fully account for the block to HIV-1 infection in SIRC cells as determined by FACS analysis (100 to 300 fold). Provirus silencing was considered very unlikely because inefficient infection was observed with HIV-1, SIVmac and MLV vectors expressing GFP from the same CMV promoter. Cell fractionation experiments showed that HIV-1 DNA association with the nuclei was delayed in SIRC compared to HeLa cells. Moreover, nuclear DNA was found in both a soluble and a chromatin-associated form in HeLa cells but only in the soluble form in SIRC cells. This suggested that at least some HIV-1 DNA could access SIRC nuclei, as confirmed by the presence of 2LTR circular forms in the HIRT supernatant, but could not integrate efficiently. Accordingly, proviral DNA copy number was reduced in SIRC cells compared to HeLa cells in long-term cultures to an extent that matched infection levels measured by FACS. These data point to a further block after nuclear entry but before integration in rabbit cells. This integration block could be due to a defective pre-integration complex (PIC), to aberrant intranuclear trafficking or to the inability of a rabbit cellular factor to interact with HIV-1 PICs.

Part 4

4.a Introduction

Having gained some insight into the nature of the block to HIV-1 infection in rabbit cells, it was important to determine if any specific viral element played a role. Clearly, rabbit cells were susceptible to infection by MLV and also by SIVmac, albeit probably at lower efficiency. Thus it was likely that some HIV-1 element influenced infection efficiency in our model. One way to screen for viral determinants is to use chimeric viruses in which a portion of the genome of a non-infectious virus is substituted by a portion of the genome of an infectious virus. Chimeric viruses have been used extensively to identify viral proteins important for certain parts of the lifecycle (Purdy and Chang, 2005), viral determinants of tropism (Delebecque et al., 2005), neutralization (Roy et al., 2005), drug resistance (Ambrose et al., 2004), viral fitness (Padow et al., 2003) and even for therapeutic or diagnostic aims (Falkner and Holzer, 2004).

In HIV research, among many examples, HIV-1/RSV chimeras have been useful to study gag as determinant of virion size, morphology and assembly (Ako-Adjei et al., 2005). An SIV virus containing HIV-1 RT has been used to study the effect of HAART in AIDS treatment, using a macaque model (North et al., 2005). HIV-1/SIV gag chimera have been used to map the viral determinant for Lv-1 restriction in primate cells and to other blocks in mouse cells (Cowan et al., 2002; Hatziioannou et al., 2004).

In this chapter, HIV-1 determinants of restriction in SIRC cells are identified using different chimeric viruses. In the first instance, genome cross-packaging between HIV-1 and SIVmac239 was tested. Then, chimeric HIV-1 constructs with different portions of MLV gag in place of HIV-1 gag regions (Yamashita and Emerman, 2004) were tested. These chimeric viruses were used previously to study nuclear import of HIV-1 (Yamashita and Emerman, 2003). These constructs contained either the MA p15 (with or without p12) or the MA, p12 and CA p30 sequences from of the amphotropic MLV clone pAMS (Miller D, Law M, Verma I, 1985) in an otherwise intact HIV-1 provirus clone pLai (Peden et al., 1991). Gag junctions of parental viruses were precisely conserved in the

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chimeric viruses. A diagram of the genomic organization of these chimeras is shown in Figure 34. In this section, the gag region is shown to be important for the block to HIV-1 infection in SIRC cells.

4.b Results

4.b.i Titration of Chimeras

To test if some element of the viral genome itself played a role in the block to HIV-1 infection in rabbit cells, I tried to package HIV-1 genome in MLV cores, SIVmac genome HIV-1 cores, and vice versa by transfecting 293T cells with the appropriate constructs and pMDG (encoding for VSV-G envelope). Supernatant was collected, filtered and concentrated. Viral stocks were normalized for RT activity and used to infect SIRC and HeLa cells at serial dilutions. Cells were fixed and analyzed by FACS 48 hours after infection.

Whole core chimera experiments were done in duplicate and the results are described in Figure 35 and Figure 36. Since it has been shown before that NB MLV Gag cannot package HIV RNA (Berkowitz et al., 1995; Zhang and Barklis, 1995) the MLV/HIV chimera, not surprisingly, did not work. However, the SIVmac genome in HIV core chimera infected 6.79% of HeLa and 0.11% SIRC cells, the parental HIV infected 6.14% of HeLa and 0.07% SIRC cells and the parental SIVmac infected 1.35% and 0.14% of HeLa and SIRC cells respectively (Figure 35). These results are also expressed as a ratio of infection of HeLa and SIRC cells (Figure 36). The results indicated that the SIV genome in HIV cores was 67.3 times more infectious in HeLa as compared to SIRC cells, while the parental HIV-1 and SIVmac were 66.4 and 9 times more infectious in HeLa than in SIRC cells, respectively. It is worth mentioning that the percentage of SIV-infected cells observed in these experiments is similar to that one observed in previous experiments (see Figures 11 and 13) when the viral dose used infects ~1% of HeLa cells. The ratio of HeLa/SIRC changes slightly depending on the viral dose used as can be seen in titration curves using 10-fold dilutions of SIV: the maximum difference (almost one log) can be seen when 1-10% of HeLa cells are infected and the difference decreases with higher viral doses (Figure 11). Thus, the viral genome was irrelevant for the block in SIRC cells. In fact these results showed that Gag-Pol was likely a determinant for the block to HIV-1 infection in rabbit cells (Figure 36).

HIV / MLV CA chimeras
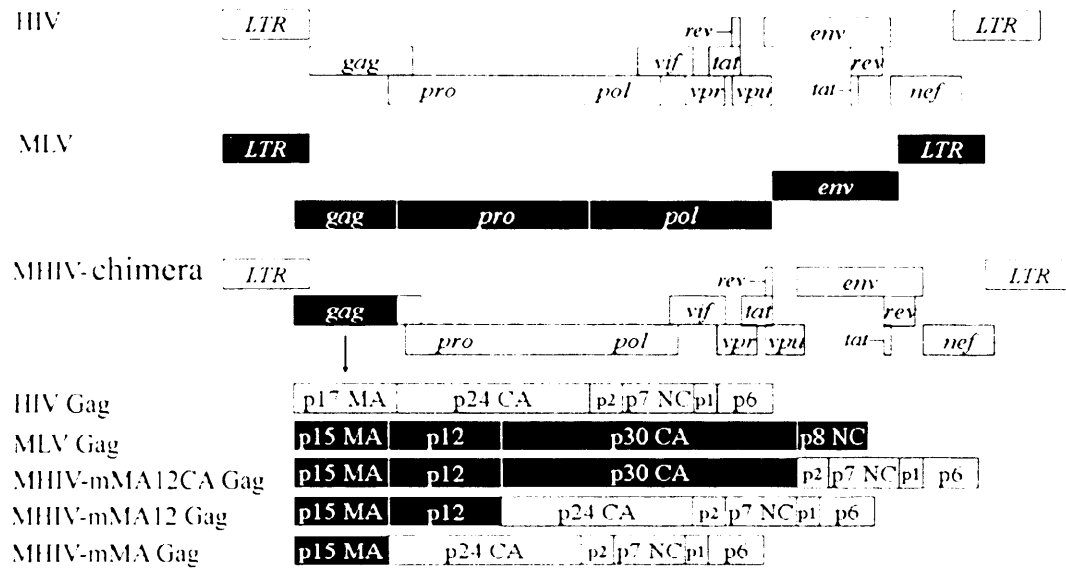
Figure 34. Diagram of the MHIV gag chimeras genomes organization. Diagram depicting the the MLV/HIV chimeric viruses genomes. *Adapted from Yamashita and Emerman. 2004 J Virol 78: 5670-8.*.

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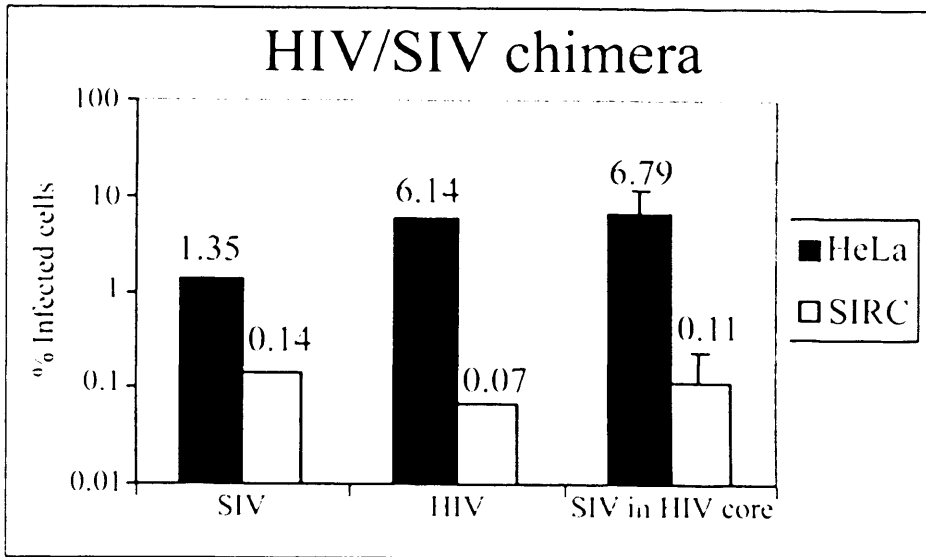
Figure 35. Analysis of infected cells with SIV vector genome packaged into HIV core. Chimeric viruses were produced by transfecting 293T cells with HIV *gag-pol* expressor plasmid, an SIV transducing vector and a VSV-G envelope expressor plasmid. Viruses were used to infect HeLa and SIRC cells along with the parental viruses and infected cells were analyzed by FACS 48 hours later. Bars represent the average value ± SD of two duplicate experiments.
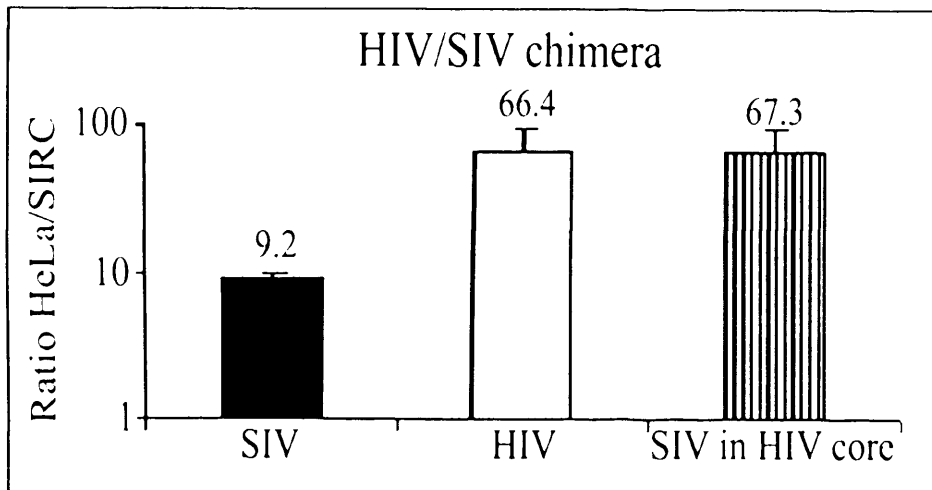
Figure 36. SIV genome in HIV core is blocked to the same extent as the HIV parental virus. Values shown in Figure 35 are represented here as the ratio between infected HeLa and SIRC cells. Higher ratio means stronger block in SIRC cells.

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Participation of Gag in the block to HIV-1 infection in SIRC cells was investigated. To this end, three different constructs (kind gift of Michael Emerman, Fred Hutchinson Cancer Research Center, Seattle, USA) were used, each having MLV MA, MAp12, and MAp12CA replacing HIV-1 MA or MA and p24 CA. To facilitate scoring of infection efficiency viral plasmids were co-transfected in 293T cells with HIV-1 vectors coding for GFP. Because of their low titers, all viruses were concentrated through a 25% sucrose cushion.

The HIV chimera with MLV MA infected 0.68% and 0.04% of HeLa and SIRC cells respectively while the HIV chimera with MLV MA and p12 infected 0.32% and 0.02% of HeLa and SIRC cells respectively. Because the HIV chimera with MLV MACAp12 was much less infectious, the experiment was repeated infecting both HeLa and SIRC cells with 10 times more virus than before. In this experiment, the HIV parental virus infected 21.49% HeLa and 0.74% SIRC cells, the NB MLV infected 3.04 HeLa and 1.19% SIRC cells, and the HIV chimera with MLV MA, p12 and CA infected 4.35% HeLa and 1.6% SIRC cells (Figure 37).

When the ratio of HeLa and SIRC infection was calculated for all these viruses, it emerged that the HIV and NB MLV were, respectively, 29 and 2.5 times more infectious in HeLa cells as compared to SIRC cells. HIV was 17 times more infectious in HeLa than in SIRC when MLV MA was substituted, 16 times more infectious when MA and p12 were substituted and 2.7 times more infectious when MA, p12 and CA were substituted (Figure 38). Thus, the presence of NB MLV MA, CA and p12 proteins in HIV-1 overcame the restriction in SIRC cells (Figure 38). A more modest effect was observed with MA substitution.

4.b.ii MLV CA influences intracellular trafficking.

Because results in Chapter 3 Part 2 showed that HIV-1 was trafficked in a different way in rabbit and HeLa cells, the effect of MLV MA, CA and p12 in changing the trafficking of HIV during infection was analyzed. Work had to be carried out in a containment level III laboratory because the MACAp12 HIV/MLV chimera is a full-length virus and needed to be pseudotyped with VSV-G to infect SIRC cells. Cells were infected as usual, incubated for 2 hours at 4°C and

then for 4 hours at 37°C and stepwise fractionation procedure was carried out. Unexpectedly, however, cells broke suddenly and completely in hypotonic buffer and the stepwise fractionation protocol could be performed. After several failed attempts to control the conditions of the infection and fractionation process, only one possible interpretation was left for these results: over swelling and cell lysis was prematurely induced by the negative pressure in the level III safety laboratory. So in an attempt to avoid this problem, the concentration of salt in the hypotonic buffer was increased from 10 to 50 mM KCl. This appeared to solve the problem of over swelling and sudden lysis and the fractionation procedure could eventually be completed. Fractions were then analyzed by quantitative PCR to measure the amount of viral DNA in each fraction. When the results were compared to those of Figure 28, it was apparent that a larger amount of HIV-1 DNA was found in the hypotonic fraction in both HeLa and SIRC cells, possibly as a result of the higher content of salt in the hypotonic buffer. Nonetheless, it appeared that HIV had a different trafficking in HeLa as compared with SIRC cells, with a trend similar to that one observed in previous experiments, (more viral DNA detected in the high salt fractions of HeLa cells and in the hypotonic fraction of SIRC cells). MLV also had a similar pattern as that one observed previously, with most viral DNA recovered in hypotonic fractions of both HeLa and SIRC cells.
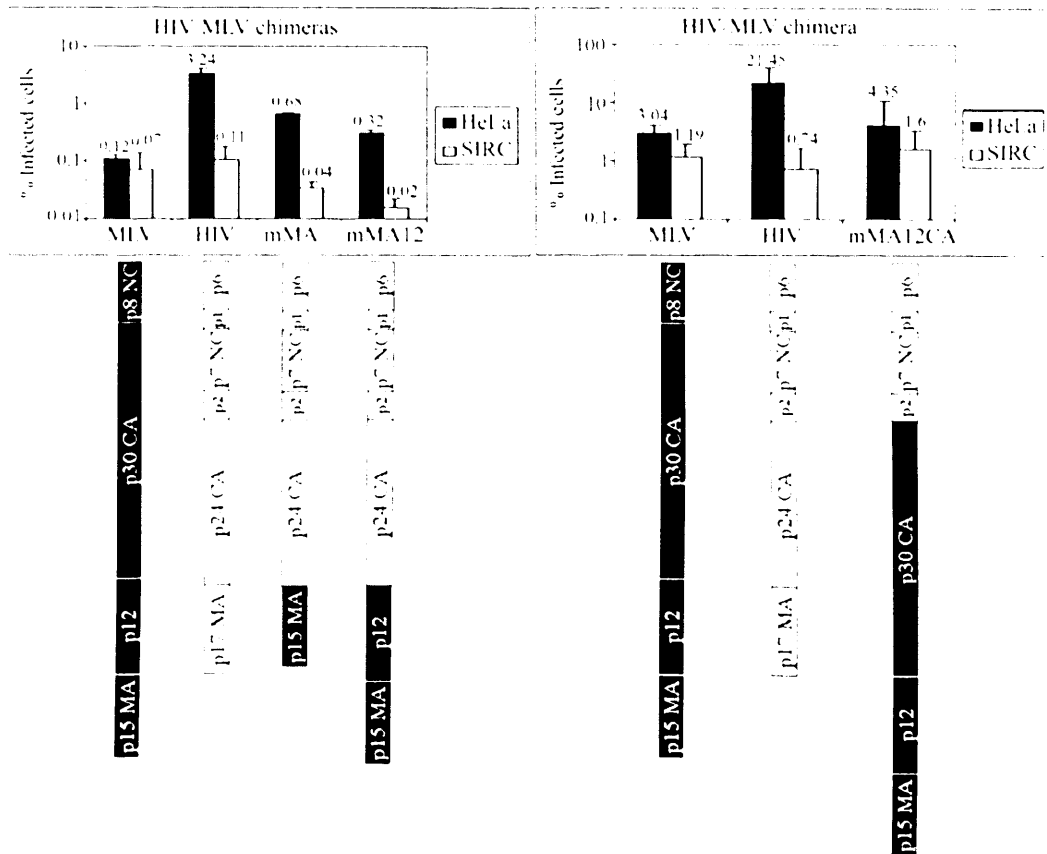
Figure 37. FACS analysis of HeLa and SIRC cells infected with HIV/MLV chimeras. MLV chimeras were produced by transfection of 293T cells with a plasmid containing the HIV sequence (deleted for envelope) and MLV matrix protein (mMA), matrix and p12 proteins (mMA12) or matrix, p12 and capsid proteins (mMA12CA) (see Figure 26) and pMD.G encoding for VSV-G envelope. The chimera and parental HIV and NB MLV viruses were used to infect HeLa and SIRC cells. Infected cells were analysed by FACS 48 hours after infection and the average of two independent duplicate experiments ± SE is shown.
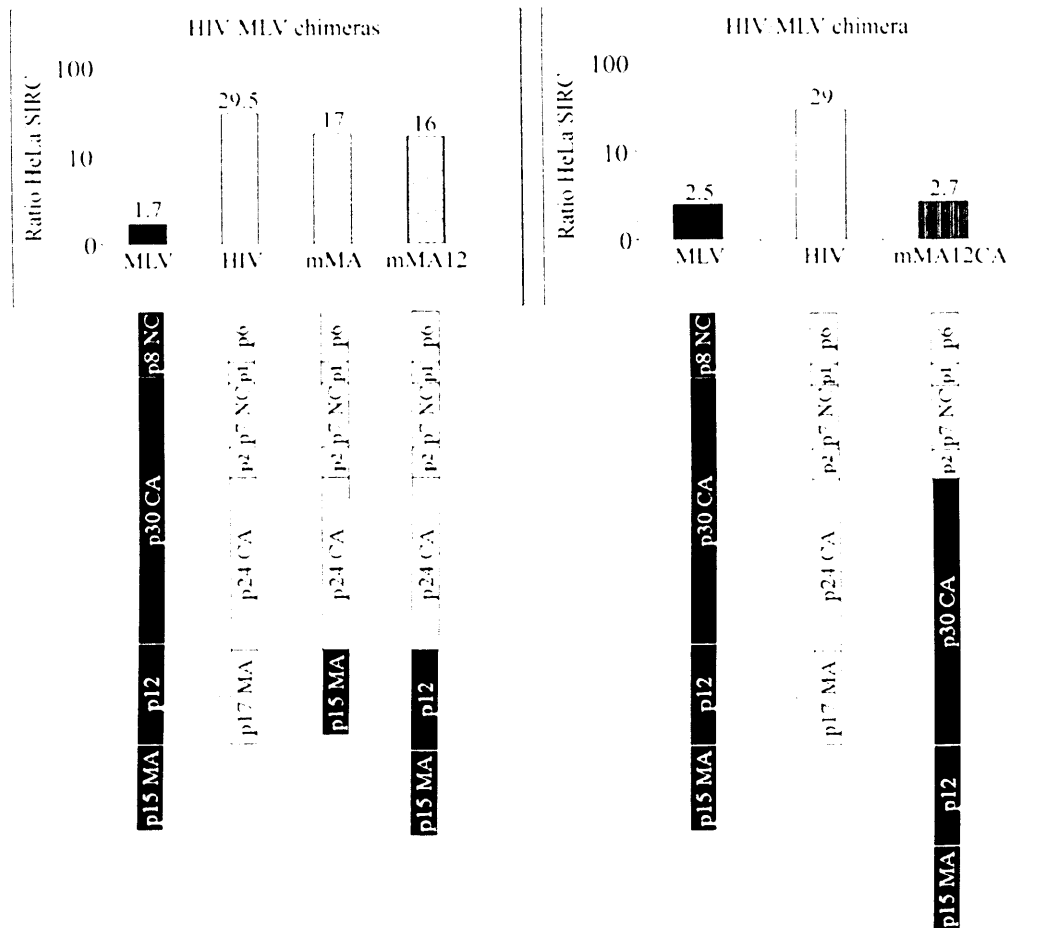
Figure 38. MLV capsid rescues HIV infection in SIRC cells. Ratio of infection in HeLa and SIRC cells infected with different MHIV chimera is shown. Ratios are calculated based on data shown in Figure 29.

Interestingly, the pattern of viral DNA distribution was very similar in HeLa and SIRC cells infected with the HIV chimera mMA12CA and a higher amount of HIV-1 DNA was found in the high salt fractions in both cell types (Figure 39).

These results suggested that replacing HIV-1 gag with MLV gag may at least partially bypass the intracellular trafficking defect seen with wild type HIV-1 in rabbit cells. Remarkably, MLV gag did not re-direct chimeric HIV-1 viral DNA into the hypotonic fraction, in which most MLV DNA ends up.

Unfortunately, these experiments proved very difficult to repeat. In particular, I was unable to control the degree of cell lysis in hypotonic buffer, presumably due to fluctuations in the negative pressure of the level III laboratory. Thus, results shown in Figure 39 should be interpreted with caution.
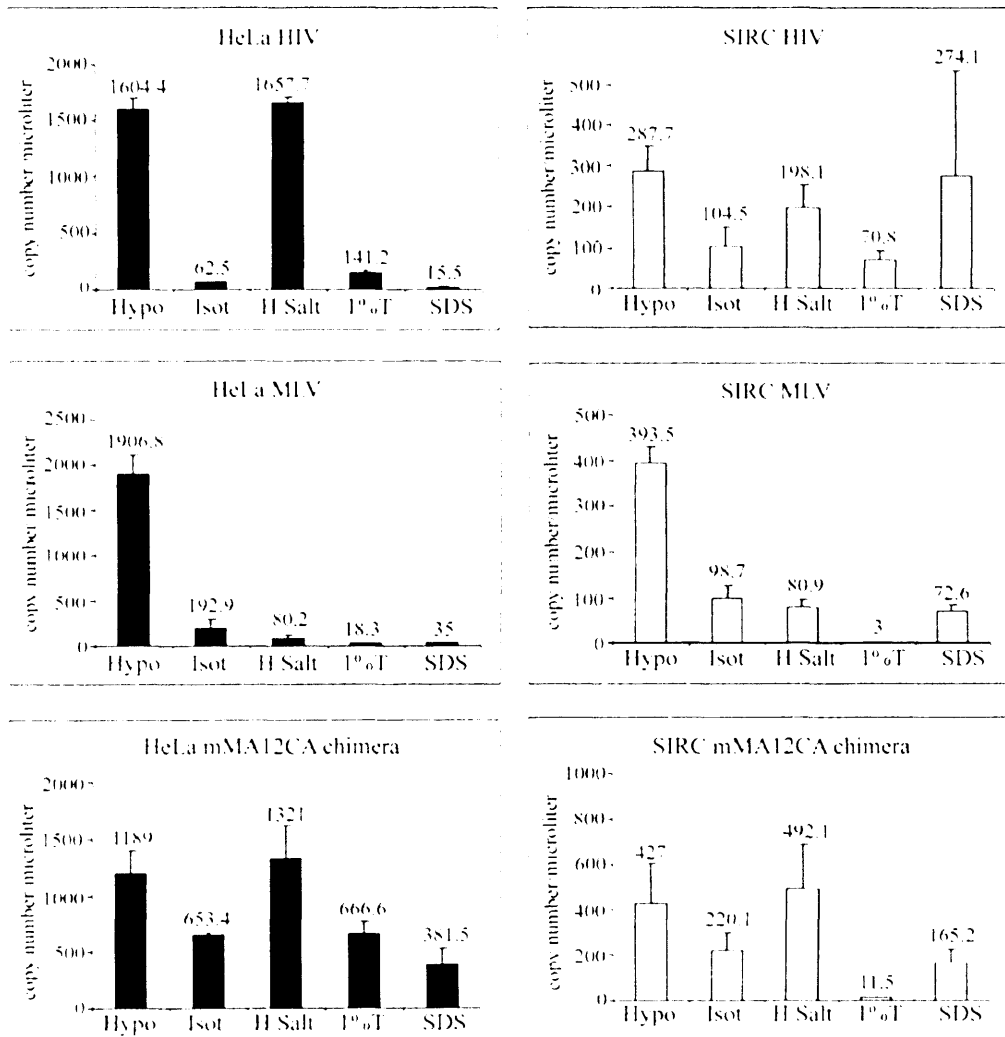
Figure 39. Cell fractionation with HIV-1, NB MLV and mMA12CA HIV/MLV chimera. Cells were infected and subjected to stepwise cell fractionation as described for Figure 17, except that hypotonic buffer contained 50mM KCl. DNA was extracted from each fraction and analyzed by TaqMan PCR. Results are averages of wells run in triplicate ± SD. Hypo: Hypotonic, Iso: Isotonic, HSalt: High Salt, 1%T: 1%Triton, SDS: SDS buffer.

4.c Discussion

In this chapter, the viral determinant for the block to HIV-1 infection in rabbit cells has been mapped to the gag region and CA. A role for the vector genome was excluded by cross packaging of SIVmac into HIV-1 core. These preliminary experiments suggested that gag or gag-pol or some of the accessory proteins could be involved in the SIRC cells block. To systematically analyse if gag regions in HIV-1 could participate in the block, MLV/HIV gag chimera were chosen since MLV is not or only very weakly blocked in SIRC cells. Results using these chimera viruses indicated that MA might only play a marginal role but CA or the entire gag region played a more important role. It is worth noting that the infectivity of the MLV/HIV chimeras is up to 100 times lower than that of wild type HIV. Ideally chimera and parental virus should have the same infectivity to draw more solid conclusions and the apparent rescue of infection that I see in SIRC cells when compared to HeLa may be the result of infection levels specifically falling in HeLa cells.

I next asked how chimeric MHIV virus containing MLV gag was trafficked in SIRC cells. Results in Chapter 3 indicated that HIV-1 intracellular trafficking was altered in SIRC compared to HeLa cells, possibly leading to reduced reverse transcription and integration and results in this chapter showed that the chimeric MHIV was much less blocked than HIV-1 in rabbit cells. Thus it was interesting to correlate intracellular trafficking and infectivity using the mutant virus. One problem was the substantial degree of attenuation of the mutant virus compared to the parental HIV-1, even in HeLa cells. This problem was partially overcome by infecting cells with higher virus input (as determined by RT activity), although this in turn might have affected the outcome of the experiment by, for example, titering out a putative partial saturable block in SIRC cells (see Chapter 2, abrogation assay in Figure 19). A second, unexpected, problem was the very variable degree of cell lysis obtained in hypotonic buffer when experiments were performed in the level III safety laboratory. Most cells lysed completely in this buffer and nuclei were also broken and lost. All conditions, which could lead to such a result were tested, including possible detergent contamination of the buffer, wrong pH, quality of the tubes, growth conditions of the cells etc. However, in the end the only possibility left was the negative pressure in the

level III laboratory, which most likely induced excessive swelling of cells in hypotonic buffer. To circumvent this problem, the osmotic pressure of the buffer was increased, which seemed to solve the problem, at least for the experiment shown in Figure 39. Based on the results shown in Figure 39, and considering all the caveats, it still may be possible that CA does play a role in the early stages of intracellular trafficking of HIV-1. The reason for a cautious optimism comes from the profile of the DNA distribution in HeLa and SIRC cells of the two parental viruses, MLV and HIV-1, which is not too different from that one observed earlier (see Figure 28, Chapter 3).

Clearly, more work is needed to confirm and expand this observation by, for example, making a gag-pol vector with the same MHIV mutation to allow working in a level II laboratory without negative pressure. It will be important to repeat all these experiments with a chimera that contains only MLV CA to determine if it is only CA that is responsible for the results shown in this chapter or if the presence of MA, CA and p12 are all necessary to rescue HIV-1 infection in SIRC cells and to change its post entry trafficking. If confirmed, the fractionation studies will suggest a role for CA in an early, post-entry step of HIV-1 infection required for the correct trafficking of the RTC, although elements other than CA, which could be disrupted by high salt concentration, could mediate interaction of the RTC with cellular components.

It would not be unprecedented that CA is the main element responsible for virus trafficking. It has been shown that capsid associates with the cellular cytoskeleton and so determines the viral trafficking of many other viruses, such as herpes simplex virus (Dohner et al, 2002; Douglas et al., 2004; Sodeik et al., 1997), parvovirus (Suikkanen et al., 2003) and cytomegalovirus (Ogawa-Goto et al., 2003) among others. A role of CA in the early post-entry trafficking of HIV-1 would also be consistent with the fact that TRIM5α, which targets capsid, directs the incoming viral particles to the proteasome (Wu et al. 2006) and thus may function by altering the early trafficking of HIV-1 in primate cells. In this model, CA would direct HIV-1 to the right cellular compartment shortly after penetration into the cell and then, following its dissociation from the RTC, additional viral and cellular components would be recognised by the nuclear import machinery. TRIM5α would block the initial trafficking and deviate RTCs

in a different compartment not productive for infection. It is also possible that TRIM5α induces capsid disassembly too early after virus entry (Stremlau et al. 2006) hence causing aberrant RTC trafficking. Thus, the putative cellular factor missing in rabbit cells and TRIM5 would have two opposing effects on the very same element important for early post entry trafficking of HIV-1.

Chapter 4.

Discussion and conclusions

There are some conflicting studies concerning HIV-1 infection of rabbit cells. Two recent studies found a strong block to HIV-1 infection in SIRC cells, however older studies suggested that rabbit cells could be efficiently infected by HIV-1 (Besnier et al., 2002; Hofmann et al., 1999; Kulaga et al., 1988; Speck et al., 1998). I found that HIV-1 infection is blocked in rabbit cells and that this block is not dependent on the cell receptor used by the virus for entry, appears to be recessive, and is characterized by an aberrant intracellular trafficking of the RTC and by a further impairment before or at integration.

The results of the abrogation and the fusion assays point to the idea that SIRC cells lack a factor that specifically allows efficient HIV-1 infection. The heterokaryons assays were useful to point the direction of my work but it is important to remember that, upon cell-cell fusion the overall cell architecture and perhaps many of its functions may not be normal so results should always been interpreted with caution. Even assuming that in the heterokaryons the cell functions necessary for HIV-1 infection were preserved, an alternative explanation for my results would be that a factor present in 293T cells could inhibit a restrictor in SIRC cells or that the restriction factor in SIRC is diluted in the heterokaryon and so its effect is extinguished. Overall these latter possibilities are less likely in light of the potency and the relatively poor saturation of the restriction in SIRC cells, the almost complete rescue obtained upon fusion with human cells and the fact that rabbit and human are very distant species as to expect efficient hetero-multimerization of their TRIM proteins. Finally, it is quite possible that two independent blocks to HIV-1 infection exist in rabbit cells: a TRIM-like activity, which is saturable and would account for an approximately 10 fold restriction to HIV-1 infection and a recessive block, which could account for the remaining block.

A time-course analysis of viral DNA synthesis in HeLa and SIRC cells showed that reverse transcription was irreversibly blocked rather than delayed in rabbit cells. The block to HIV-1 RT in SIRC cells was unlikely to be caused by an

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intrinsic defect of intracellular RTCs. Most RTCs isolated from acutely infected SIRC cells had a significantly lower density in linear sucrose gradients compared with RTCs isolated from HeLa cells but were still competent for RT in vitro when provided with exogenous dNTPs. Rapid degradation of the viral genome was also unlikely as the amount of viral DNA measured in SIRC cells was low but did not decrease significantly with time and the amount of HIV-1 RNA in the hypotonic fraction was equal or higher in SIRC than in HeLa cells 4 h post-infection. Western blot of density fractions with antibodies against p24 CA showed efficient dissociation of the capsid proteins from viral DNA at 4 hours post-infection in HeLa cells and to an extent also in SIRC cells, although this assay can not determine the exact timing in which this occurs.

Interestingly, I have found an association between infectivity and RTC density in cells of different species with different viruses, suggesting that RTCs must undergo a "maturation" step. Because HIV RTCs from SIRC cells also showed a lower density than RTCs extracted from HeLa cells, a block in RTC maturation may also be causing reduced HIV-1 infectivity. In fact RTC maturation and intracellular trafficking may be two tightly interconnected events.

Because an intrinsic defect of the RTC and rapid RTC degradation were excluded, intracellular trafficking of RTCs has been investigated. To this end, a fractionation procedure with different salt concentrations and detergent composition was carried out to cause stepwise disruption of increasingly stronger protein-protein interactions as well as extraction of different intracellular compartments and allow some crude examination of viral trafficking (Gao and Goff, 1999). Results in Chapter 3 Part 2 suggested that HIV-1 was trafficked in a different way in rabbit and HeLa cells, and results in Part 4 showed that the block to HIV-1 infection could at least in part be rescued by substituting HIV-1 gag with MLV gag. Thus, the effect of MLV gag in changing the trafficking of HIV during infection was analyzed biochemically. Work had to be carried out in a containment level III laboratory with negative pressure and cells were lysed suddenly and completely in hypotonic buffer. In an attempt to avoid this problem, the concentration of salt in the hypotonic buffer was increased and the fractionation procedure was completed. Fractions were then

analyzed by quantitative PCR to measure the amount of viral DNA in each fraction. When the results were compared to previous cell fractionation experiments' results, it was apparent that a larger amount of HIV-1 DNA was found in the hypotonic fraction in both HeLa and SIRC cells, possibly as a result of the higher content of salt in the hypotonic buffer. Nonetheless, it appeared that HIV had a different trafficking in HeLa as compared with SIRC cells, with a trend similar to that one observed in previous experiments: most of HIV-1 DNA was found in isotonic and high salt fractions in HeLa cells and in the hypotonic fraction in SIRC cells. However, the DNA distribution profile of HIV-1 in HeLa and SIRC cells was quite similar upon substitution of the CA region in HIV-1.

Surprisingly, intracellular DNA distribution of the mutant HIV-1 (with MLV CA) was closer to the HIV-1 wild-type distribution rather than to MLV DNA distribution. In other words, MLV CA rescued infectivity and induced accumulation of HIV-1 DNA in the high salt fraction but did not modify trafficking towards a more MLV-like profile. It is therefore possible that CA influences a very early event post-infection. If this event can proceed correctly, HIV-1 is then trafficked into a high salt compartment, which presumably leads to high infectivity. If not, HIV-1 is trafficked in a different compartment, which leads to abortive infection. This then raises the question: why MLV does not behave like HIV-1? Why should the intracellular trafficking of the two viruses be different? No clear answer can be provided at present, however one may speculate that HIV-1 has additional signals that allow its rapid association with some cytoskeletal component and that MLV lacks such signals. One must remember that the composition of the HIV-1 and NB MLV RTCs is different: the CA remains in the latter while it is shed in the former and this may influence their trafficking. A more detailed analysis of the components to which HIV-1 RTCs associate in the high salt fraction may help clarify this point.

All these results were obtained with RTCs extracted 4 hours post infection. This time point was the earliest used in all experiments. This was done initially to compare the density assay and cell fractionation results with those already reported in the literature that use the same time point after infection. Subsequently, the same time point was used in other types of experiments to have a constant that allowed me to compare my own results between them.

Clearly this is a limitation and depicts a particular stage in infection. To gain more insight into the dynamics of HIV-1 trafficking it will be interesting to repeat some of these experiments at earlier and later time points, from 30 minutes to 6 hours post infection. Such an approach may reveal the time at which the trafficking and RTC structure/density of HIV-1 in SIRC and HeLa cells start to diverge and allow formulation of new hypothesis and more focussed research.

It should be noted as well that although the distributions of MLV and HIV in SIRC cells are similar, the comparison should also be made between HeLa and SIRC cells with the same virus. The reason for this is that a certain distribution (reflecting its trafficking) for a virus might mean a productive infection while the same distribution means block to infection for a different virus. In this case, as mentioned before, the presence of different viral proteins in the RTCs of MLV and HIV-1 possibly require interactions with different cellular proteins at different times post infection and this is reflected in the cell fractionation results.

The cell fractionation assay has several limitations: biochemical separation of the cellular compartments is never absolute and some degree of cross contamination must be expected. Most importantly my fractionation assay was not informative enough on the precise location of the RTC within the cell. To this end one may use alternative approaches to investigate the intracellular trafficking of a virus: immunofluorescence tagging of viral proteins and of cellular compartments is one of them (Le Blanc et al., 2002) and a more sophisticated one is using GFP tagged viral particles and costaining intracellular structures such as the cytoskeleton. This has been done by live cell imaging, labelling cellular organelles and infecting them with HIV-1 viral particles with GFP fused to the NH_2 terminus of the Vpr protein (McDonald et al., 2002).

I obtained the GFP-Vpr expression vector from Dr. Tom Hope, Northwestern University and tried to produce tagged virus but I was unsuccessful. The main problem was to obtain enough purified and tagged virus for my experiments. Other authors using live cell imaging have used lipophilic dyes to stain influenza virus envelope (Lakadamyali et al., 2003). Time-lapse microscopy has been used to study labelled adenoviral capsid interactions with dynein for nuclear translocation (Suomalainen et al., 1999). This technique also has limitations

mainly becasue many events must be collected to ensure statistically significant and meaningful results.

An interesting alternative to obtain more information from the cell fractionation assay would be to combine it with drugs targeting specific cytoskeletal structures or overexpressing dominant-negative proteins that disrupt actin, microtubules or cytoplasmic motors. Examples of this are cytochalasin D, nocodazole and the anti-dynein intermediate chain mAb or dynamitin respectively, which have been used extensively to study viral intracellular trafficking (Döhner et al., 2002; Lakadamyali et al., 2003; McDonald et al., 2002; Suomalainen M et al., 1999).

Cell fractionation experiments showed that HIV-1 DNA association with the nuclei was delayed in SIRC compared to HeLa cells. Moreover, nuclear DNA was found in both a soluble and a chromatin-associated form in HeLa cells but only in the soluble form in SIRC cells. This suggested that at least some HIV-1 DNA could access SIRC nuclei, as confirmed by the presence of 2LTR circular forms in the HIRT supernatant, but could not integrate efficiently. Accordingly, proviral DNA copy number was reduced in SIRC cells compared to HeLa cells in long-term cultures to an extent that matched infection levels measured by FACS pointing to a further block after nuclear entry but before integration in rabbit cells. Quantification of viral DNA in the Hirt pellet and supernatant is an indirect and not ideal way to measure integration. Other techniques were thought of before, such as Alu PCR. This would have allowed me to quantify the number of integrated proviruses in the chromatin of infected SIRC or HeLa cells directly. Alu sequences are short interspersed elements of which there are more than a million in the genomes of primates and hence are a source of simple sequence repeats in primates' genomes (Batzer and Deininger, 2002). However, adequate primers could not be designed because there are no Alu sequences known for rabbits so attempts were made to do Alu PCR using primers for human Alu sequences but they were unsuccessful. However, I consider that there is enough evidence in my experiments to say that integration of HIV-1 in SIRC cells is inefficient when compared to HeLa as the results from the HIRT separation, the integrase mutant HIV-1experiment and the long term cultures all point to the same conclusion.

In the future, it would be interesting to carry out the in vitro integration assay (Brown et al., 1987, Ellison et al., 1990) with PIC from human and rabbit cells, although it probably will be very difficult to obtain enough PICs from SIRC cells for this assay. Nevertheless, this assay is important as it can tell if the PIC extracted from SIRC cells has an intrinsic defect or if there is a problem with the host cell, such as lack or incompatibility of co-factors important for integration. Indeed, the block to integration could be due to incompatibility between the HIV-1 IN protein and some rabbit cellular factors required for integration, such as BAF, LEDGF/p75 or other unknown factors (Li et al., 2000; Lin and Engelman, 2003; Sorin et al., 2006).

Interestingly, some mutated forms of TRIM5α were reported to block integration as well as reverse transcription (Yap et al., 2006) and more recently, rhTRIM5α has been shown to block HIV-1 infection in two phases: inhibiting reverse transcription and altering trafficking to the nucleus (Wu et al., 2006). Inhibition of reverse transcription can be rescued by treatment of infected cells with inhibitors of the proteasome. However the same proteasome inhibitors have no effect on nuclear trafficking or infectivity (Wu et al. 2006). This suggests that rhTRIM5α recruits RTCs to the proteasome, which has several consequences like blocking reverse transcription and altering RTC trafficking. Recently, a cellular factor called *MRI* or modulator of retrovirus infection has been shown to act by modulating the proteasome activity to allow HIV-1 uncoating and then quickly releasing the RTC from the proteasome itself (Agarwal et al., 2006). Thus rhTRIM5α and *MRI* might work as antagonists on the proteasome and modulate retroviral infectivity. It will be interesting to investigate the *MRI* gene in rabbit cells and the effect of the proteasome on HIV-1 infectivity. As mentioned earlier, it may be possible that a combination of a missing/defective gene and a TRIM-like activity co-exist in rabbit cells, thus resulting in a particularly strong block to HIV-1 infection.

I propose a model in which HIV-1 enters SIRC cells but is unable to bind a factor that is necessary for early sorting of the virus into the right intracellular "track". This factor somehow does not recognise HIV-1 CA. As a consequence, HIV-1 is not trafficked normally in rabbit cells. It slowly travels to the nucleus,

perhaps via an alternative pathway, but it cannot integrate efficiently. CA would provide an early sorting signal and other elements in the HIV-1 RTC would then direct it to the high salt compartment. The early factor could be a motor protein that allows it to bind the cytoskeleton such as those of the dynein and kinesin families; these proteins transport cargos using ATP (Mallik and Gross, 2004). Furthermore, Herpes Simplex virus I, Adenovirus and HIV-1 have already been found to latch onto dynein motors to traffic to the cell nucleus (Kelkar et al., 2004; McDonald et al., 2002; Sodeik et al., 1997; Suomalainen et al., 1999).

Another possibility could be an incompatibility between the rabbit myosin proteins that transport cargoes along actin filaments immediately after the entry of the virus to the cell. Interaction with actin has been reported to be necessary to mobilize HIV-1 virions towards the microtubules (Fackler and Krausslich, 2006) although the interaction with actin is no longer necessary when virions are pseudotyped by VSV-G as this envelope uses endocytosis to enter the cell (Komano et al., 2004).

The lack of an association to the cytoskeleton would cause inability of the RTC to reverse transcribe efficiently (Bukrinskaya et al., 1998) and perhaps, to mature in such a way that later in the life cycle, the provirus can integrate (Figure 40).

In conclusion, the phenotype that I have described in rabbit cells could be the result of the disruption of one necessary step in the viral life cycle that affects both reverse transcription and integration or could be caused by two consecutive blocks.

In the future, it will be important to try to determine what is missing in SIRC cells that blocks HIV-1 replication. One way to investigate this would be the production of a cDNA library from 293T cells to transform SIRC cells, infect them with HIV-1 and then screen clones. This strategy has already been used to identify proteins that have antiviral restriction activities (Gao et al., 2002; Sheehy et al., 2002; Stremlau et al., 2004). This experimental approach would pose some problems, such as the partial abrogation that we and others have observed when high viral doses are used to infect rabbit cells. Another problem

to this approach would be the fact that there might be more than one block to HIV-1 replication in SIRC cells. It will also be important to determine if there is a TRIM5α-like restriction factor in rabbit cells, which could explain the approximately 10-fold saturation of the block that I have observed.

Understanding what determines the block to HIV-1 infection in rabbit cells can bring some light in the way in which HIV-1 traffics within the cell. Viral trafficking is an important part in the viral life cycle and our data indicate that correct viral trafficking in the early phases post-entry is relevant to RTC maturation and overall efficiency of infection. Examples of this phenomenon have been observed already for other viruses, such as the modification that parvovirus capsids seem to require intracytoplasmically to allow trafficking to the nucleus (Sonntag et al., 2006), or the escape from endosomes of adenovirus serotype 5 capsids that is necessary for intranuclear localization, (Leopold et al., 2000) and the effect of the fiber proteins on the different trafficking routes that different subgroups of adenovirus follow after entry (Miyazawa et al., 1999). The block to HIV-1 integration that is apparent in SIRC cells could also help shed some light on the host cell proteins necessary for this process, although known factors like LEDGF/p75 and BAF will need to be investigated first.

Furthermore, understanding the nature of this block could enable us to engineer rabbits as animal models for HIV-1 infection and perhaps AIDS. This work contributes to the understanding of the HIV-1 infection process and might facilitate in the future the development of a small animal model that will aid us to understand HIV-1 pathogenesis and clinical course and will improve therapeutics and prevention through vaccines.
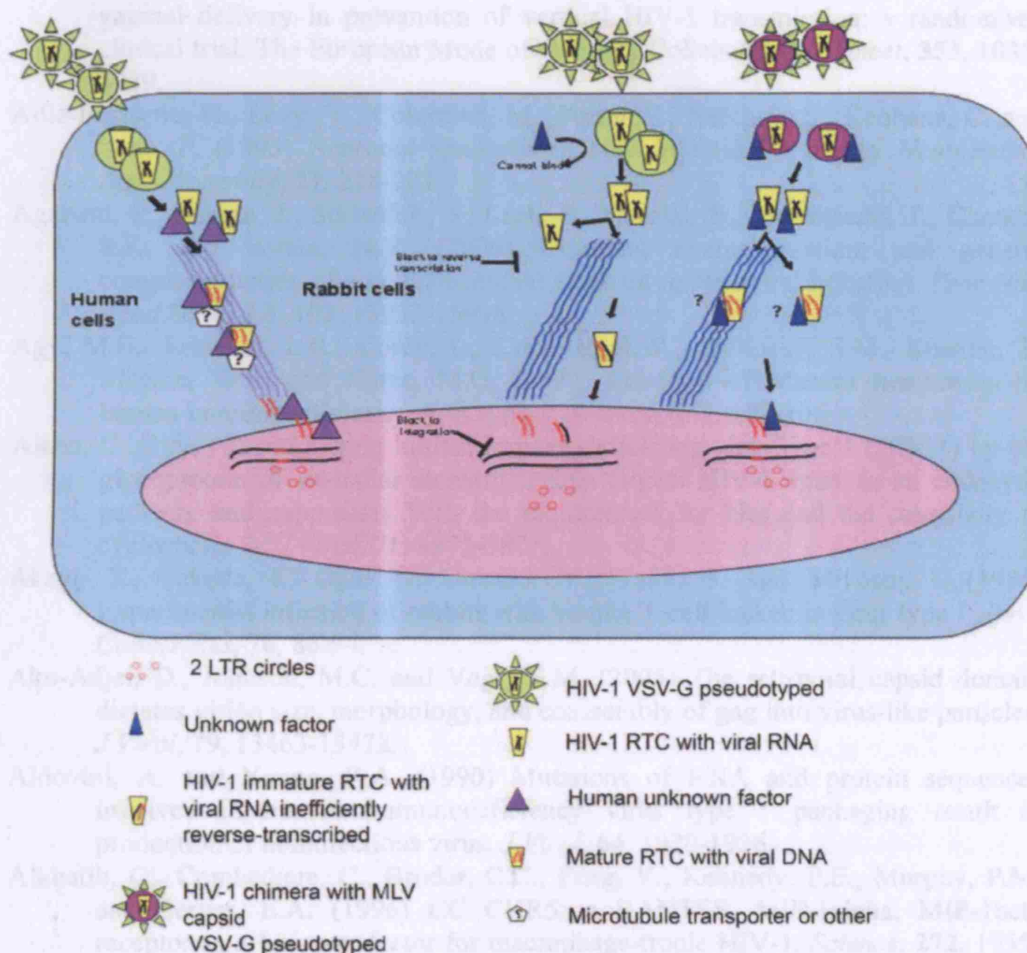
Figure 40. Model of restriction in SIRC cells. After HIV-1 enters human cells it uncoats and initiates reverse transcription while it associates with some factor that allows early sorting. After this step, additional signals in the RTC direct it to the cytoskeleton where the RTC starts its rapid translocation to the nucleus by a potential association with some motor protein such as dynein. RTCs mature, enter the nucleus of the host cell (with the production of 2LTR circles) and integrate. In rabbit cells, the factor is missing or it cannot bind the viral capsid so HIV-1 may not be properly sorted and engage with the cytoskeleton. Some RTCs are sequestered in a dead-end pathway in the cytoplasm while others eventually reach the nucleus but cannot mature so after entering the nucleus they do not integrate efficiently. When HIV-1 has the MLV capsid, after entering the cell it can bind the putative factor for early sorting, then it can associate with the cytoskeleton and translocate to the nucleus. The trafficking pathway might be different to that one followed by HIV-1 in human cells but allows appropiate maturation of the RTC and reverse transcription so that proviral DNA can integrate efficiently into the host cell genome.

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