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**LATE RESTRICTION INDUCED BY AN  
ENDOGENOUS RETROVIRUS**

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**August 2007**

**Thesis presented to the School of Veterinary Medicine at the University of  
Glasgow for the degree of Doctor of Philosophy**

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## Abstract

Exogenous retroviruses (i.e. horizontally transmitted) must integrate their proviral DNA into the host's genome as part of the retroviral cycle. Therefore, infections of the germ line can give rise to vertically transmitted endogenous retroviruses (ERVs). ERVs are present in all vertebrates. During evolution, most ERVs have accumulated mutations and/or deletions that hampered their ability to replicate and hence to harm the host. However, some ERVs have retained at least some of their open reading frames intact even after several million years. One possible reason for the selection of ERVs is that they protected the host against incoming pathogenic exogenous retroviruses. The fact that the vast majority of ERVs do not possess circulating exogenous counterparts supports this view.

Ovine pulmonary adenocarcinoma (OPA) is a transmissible lung cancer of sheep caused by a Betaretrovirus known as Jaagsiekte sheep retrovirus (JSRV). Notably, the sheep genome possesses approximately 30 ERVs highly related to JSRV (and hence referred to as enJSRVs). A specific enJSRV provirus, termed enJS56A1, acts as a transdominant restriction factor that blocks the exogenous JSRV at late stages of the replication cycle. The main determinant of this block (termed JLR for *JSRV late restriction*) has been mapped to the N-terminal region of the Gag polyprotein, more precisely to residue 21 of the matrix protein. In this study, the molecular basis of JLR was investigated by functionally characterizing the JSRV and enJS56A1 Gag polyproteins. Putative Gag trafficking signals, such as membrane binding (M) and late domains (L) were identified, although it was determined that these domains lack any involvement in JLR. enJS56A1-expression constructs bearing truncated Gags were unable to block JSRV, indicating that an intact Gag polyprotein is required for JLR. Moreover, intracellular colocalization and protein-protein interaction between JSRV and enJS56A1 Gag molecules was determined by confocal microscopy and co-immunoprecipitation, respectively. Complementation assays suggest that JSRV and enJS56A1 Gag likely co-assemble. These data are supported by the fact that deletion of the major homology region of enJS56A1 Gag allows JSRV to escape JLR. Confocal microscopy experiments indicate that JSRV must reach the pericentriolar region as part of its normal cell cycle and that JLR takes place before this stage.

This work unveils basic aspects of JSRV biology that were previously unknown. It also provides insight on the molecular basis of JLR, highlighting the dynamic evolutionary interplay between viruses and host.

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## List of Accompanying Materials

Copies of the following articles (published papers or articles in press) are included in this thesis:

Mura, M., Murcia, P., Caporale, M., Spencer, T.E., Nagashima, K., Rein, A., and Palmarini, M. (2004). Late viral interference induced by a transdominant Gag of an endogenous retrovirus. *Proceedings of the National Academy of Sciences USA*, 101: 11117-11122.

Murcia, P., Arnaud, F., and Palmarini, M. (2007). The transdominant endogenous retrovirus enJS56A1 associates with and blocks JSRV Gag intracellular trafficking. *Journal of Virology*, 81: 1762-72.

Arnaud, F., Murcia, P., and Palmarini, M. (2007). Mechanisms of late restriction induced by an endogenous retrovirus. *Journal of Virology*, 81:11441-51.

Marcelo De las Heras, Pablo Murcia, Aurora Ortín, Javier Azúa, Luis Borderías, Ramiro Álvarez, José Antonio Jiménez-Más, Antonio Marchetti and Massimo Palmarini. (2007). Jaagsiekte sheep retrovirus is not detected in human lung adenocarcinomas expressing antigens related to the Gag polyprotein of Betaretroviruses. *Cancer Letters*, 258 (1):22-30.



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## Declaration

I hereby declare that the work presented in this thesis is original and was conducted by the author under supervision except where stated.

I certify that no part of this thesis has been submitted previously for the award of a degree to any University but has been reproduced in parts in the following scientific papers:-

Mura, M., Murcia, P., Caporale, M., Spencer, T.E., Nagashima, K., Rein, A., and Palmarini, M. (2004). Late viral interference induced by a transdominant Gag of an endogenous retrovirus. *Proceedings of the National Academy of Sciences USA*, 101: 11117-11122.

Murcia, P., Arnaud, F., and Palmarini, M. (2007). The transdominant endogenous retrovirus enJS56A1 associates with and blocks JSRV Gag intracellular trafficking. *Journal of Virology*, 81: 1762-72.

Arnaud, F., Murcia, P., and Palmarini, M. (2007). Mechanisms of late restriction induced by an endogenous retrovirus. *Journal of Virology*, 81: 81:11441-51.

Marcelo De las Heras, Pablo Murcia, Aurora Ortín, Javier Azúa, Luis Borderías, Ramiro Álvarez, José Antonio Jiménez-Más, Antonio Marchetti and Massimo Palmarini. (2007). Jaagsiekte sheep retrovirus is not detected in human lung adenocarcinomas expressing antigens related to the Gag polyprotein of Betaretroviruses. *Cancer Letters*, 258 (1):22-30.

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## Definitions

<b>AIDS</b>	Acquired immune deficiency syndrome
<b>ALV</b>	Avian leukosis virus
<b>APC</b>	Antigen presenting cell
<b>APOBEC</b>	Apolipoprotein B mRNA editing enzyme
<b>AU</b>	Analytical ultracentrifugation
<b>BAC</b>	Bronchioloalveolar Carcinoma
<b>BaEV</b>	Baboon endogenous retrovirus
<b>BLV</b>	Bovine leukaemia virus
<b>CA</b>	Capsid
<b>CD</b>	Circular dichroism
<b>CH</b>	Cystein-Histidine
<b>CMV</b>	Cytomegalovirus
<b>CoIP</b>	Coimmunoprecipitation
<b>CTE</b>	Constitutive transport element
<b>CTRS</b>	Cytoplasmic targeting/retention signal
<b>CypA</b>	Cyclophilin A
<b>DNA</b>	Deoxyribonucleic acid
<b>DTT</b>	Dithiothreitol
<b>dUTPase</b>	deoxyuridine triphosphatase
<b>EIAV</b>	Equine infectious anemia virus
<b>EM</b>	Electron microscopy
<b>enJS56A1</b>	Endogenous Jaagsiekte sheep retrovirus 56A1
<b>enJSRV</b>	Endogenous Jaagsiekte sheep retrovirus
<b>ENTV</b>	Enzootic nasal tumour virus
<b>env</b>	Envelope
<b>ERV</b>	Endogenous retrovirus
<b>ESCRT</b>	Endosomal complex required for transport
<b>FeLV</b>	Feline leukaemia virus
<b>FIV</b>	Feline immunodeficiency virus
<b>FV</b>	Friend leukaemia virus
<b>gag</b>	Group-specific antigen

<b>GaLV</b>	Gibbon ape leukaemia virus
<b>GFP</b>	Green fluorescent protein
<b>HA</b>	Hemagglutinin
<b>HAART</b>	Highly active antiretroviral therapy
<b>HERV-K</b>	Human endogenous retrovirus-K
<b>HERV-L</b>	Human endogenous retrovirus-L
<b>HERV-W</b>	Human endogenous retrovirus-W
<b>HFV</b>	Human foamy virus
<b>HNF-3</b>	Hepatocyte nuclear factor-3
<b>HPLC</b>	High pressure liquid chromatography
<b>hpt</b>	Hours post-transfection
<b>HTLV-1</b>	Human T-cell leukaemia virus 1
<b>HTLV-2</b>	Human T-cell leukaemia virus 2
<b>I domain</b>	Interaction domain
<b>ICAPS</b>	Intracytoplasmic type-A particles
<b>ICTV</b>	International Committee on Taxonomy of Viruses
<b>IL-2</b>	Interleukin 2
<b>IN</b>	Integrase
<b>IP</b>	Immunoprecipitation
<b>IRES</b>	Internal ribosome entry sites
<b>ISD</b>	Internal scaffold domain
<b>JLR</b>	JSRV late restriction
<b>JSRV</b>	Jaagsiekte sheep retrovirus
<b>L domain</b>	Late domain
<b>LINE</b>	Long interspersed elements
<b>LTR</b>	Long terminal repeat
<b>M domain</b>	Membrane binding domain
<b>MA</b>	Matrix
<b>MCF</b>	Mink cell focus forming viruses
<b>MHR</b>	Major homology region
<b>MITE</b>	Miniature inverted-repeat transposable element
<b>MLV</b>	Murine leukaemia virus
<b>MMTV</b>	Mouse mammary tumour virus

<b>Mo MSV</b>	Moloney murine sarcoma virus
<b>M-PMV</b>	Mason-Pfizer monkey virus
<b>MTOC</b>	Microtubule organizing center
<b>MuERV-L</b>	Murine endogenous retrovirus-like gene
<b>MVV</b>	Maedi-Visna virus
<b>MWCO</b>	Molecular weight cut off
<b>NC</b>	Nucleocapsid
<b>Nef</b>	Negative factor
<b>NIH</b>	National Institute of Health
<b>NMR</b>	Nuclear Magnetic Resonance
<b>oLE</b>	Ovine Lumen Epithelial
<b>OPA</b>	Ovine Pulmonary Adenocarcinoma
<b>ORF</b>	Open reading frame
<b>oST</b>	Ovine Stroma
<b>pbs</b>	Primer binding site
<b>PBS</b>	Phosphate buffer saline
<b>PCR</b>	Polymerase chain reaction
<b>PERV</b>	Pig endogenous retrovirus
<b>PIC</b>	Preintegration complex
<b>PM</b>	Plasma membrane
<b>pol</b>	Polymerase
<b>PPT</b>	Polypurine tract
<b>RAV-0</b>	Rous-associated virus
<b>RCSB</b>	Research Collaborative for Structural Bioinformatics
<b>RER</b>	Rough endoplasmic reticulum
<b>RNA</b>	Ribonucleic acid
<b>RNAse H</b>	Ribonuclease H
<b>RP-HPLC</b>	Reverse-phase high pressure liquid chromatography
<b>RRE</b>	Rev-responsive element
<b>RSV</b>	Rous sarcoma virus
<b>RT</b>	Reverse transcriptase
<b>SA</b>	Splice acceptor
<b>Sag</b>	Superantigen

<b>SD</b>	Splice donor
<b>SDS-PAGE</b>	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
<b>SeMet</b>	Seleno-methionine
<b>SERV</b>	Simian endogenous retrovirus
<b>SINE</b>	Short interspersed elements
<b>siRNA</b>	Small interfering ribonucleic acid
<b>SSV</b>	Simian sarcoma virus
<b>SU</b>	Surface
<b>TBS-T</b>	Tris Buffer Saline-Tween
<b>TE</b>	Transposable element
<b>TM</b>	Transmembrane
<b>TNE</b>	Tris-Sodium-EDTA
<b>TRIM-5<math>\alpha</math></b>	Tripartite motif-5 $\alpha$
<b>U3</b>	Unique 3
<b>U5</b>	Unique 5
<b>Ub</b>	Ubiquitin
<b>Vif</b>	Virion infectivity factor
<b>Vpr</b>	Viral protein R
<b>VPS</b>	Vacuolar protein sorting
<b>Vpx</b>	Viral protein X
<b>VR</b>	Variable region
<b>WDSV</b>	Walleye dermal sarcoma virus
<b>ZAP</b>	Zinc-finger antiviral protein

# Chapter 1

## General Introduction

### ***Summary***

This chapter will provide the theoretical framework to the studies detailed in this thesis. It starts with a concise history of retrovirology followed by an overview on the biology of this family of viruses with specific emphasis on endogenous retroviruses. Since a whole book could be written about the history of retroviruses, only seminal works and landmark papers will be referenced. Along the same line, I will briefly describe the taxonomy, viral structure and life cycle of retroviruses. The second part of the chapter will focus on Jaagsiekte sheep retrovirus (JSRV) and the related endogenous sheep Betaretroviruses. Finally, since the primary focus of this thesis is the interaction between two Betaretroviruses, the reader should be aware that whenever possible I will use members of this genus as examples.

### ***Brief history of retrovirology***

It may sound presumptuous to say that we owe what we are today to retroviruses. However, since approximately 8% of our genome is constituted by retroviral DNA (Gifford and Tristem 2003; Weiss 2006), that statement is not far from the truth. Retroviruses have evolved with us for millions of years, and in doing so they co-shaped our genome, modified our phenotype and in recent times they have even changed our customs and practices. However, from a human perspective, the history of this family of viruses started only around a hundred years ago and can be divided in three periods that reflect the dynamics of the evolving paradigms in the field. During the first or “oncogenic period”, the driving force for researchers was the study of viruses as causative agents of cancer. It was followed by the “reverse-transcriptase and AIDS period”, characterized by the discovery of the reverse-transcriptase that challenged the central dogma of molecular biology, and the AIDS epidemics that preceded the discovery of HIV. Finally, our current time or “post-genomic period” in which



systematic sequencing of diverse genomes has unveiled the fossil imprints of past retroviral infections, allowing us to study viral and host coevolution, as well as the dynamics of their interaction.

The earliest report on the infectious nature of retroviral diseases dates back to 1904, with studies on equine infectious anemia (EIAV) by Henri Vallée and Henri Carré (Vallée and Carré 1904). However, what is here regarded as the initial period of retrovirology started with the experimental transmission of the first oncogenic retroviruses. In 1908 two Danish veterinary surgeons, Vilhelm Ellerman and Olaf Bang, who were studying erythroleukaemia in chickens, succeeded in transmitting the disease from one chick to another by means of cell-free filtrates (Ellerman and Bang 1908). Three years later, Peyton Rous published his seminal work on the propagation of a solid tumour (sarcoma) of the chicken using a similar approach (Rous 1911). Because in those days leukaemia was not regarded as cancer and chickens were not considered a relevant animal model for the study of human diseases, none of those landmark studies had the impact they deserved. With time and subsequent discoveries those studies received the importance they should have initially had and we now regard the group of agents discovered by Ellerman and Bang as avian leukosis virus (ALV) (Vogt 1997). Peyton Rous on the other hand was awarded a Nobel Prize in 1966 and the avian sarcoma virus bears his name (Rous sarcoma virus, RSV). Until the discovery of HIV, RSV had arguably been the most informative retrovirus, based on the number of research articles published on it.

A new impetus was brought to the field with the development of inbred strains of mice that displayed a high incidence of cancer. As the occurrence of the tumours could not be explained solely by genetic factors, an "extra-chromosomal influence" was claimed to be involved. In 1936, John Bittner performed foster nursing experiments with suckling mice and showed that the "extra-chromosomal influence"--which we now know as Mouse Mammary Tumour Virus (MMTV) - was transmitted from mothers with high-incidence of mammary tumours to litters with low incidence of tumours by maternal milk. Quoting his cautious statement in the original article "the incidence of mammary gland tumours in mice may be affected by nursing" (Bittner 1936).

It took fifteen more years to provide convincing evidence on the viral aetiology of mouse tumours. Ludwig Gross, working on leukaemia of inbred mice at the Bronx Veterans Hospital, showed in 1951 that mice from a low-incidence strain developed tumours when inoculated within the first 12 hours of life with supernatants prepared from leukaemic tissues of AKR mice (Gross 1951). Discovery of other strains of murine leukaemia virus soon followed, as well as several other tumour viruses that affected different species. At the same time, the development of cell culture techniques together with the invention of the electron microscope resulted in significant advances in virology, from which retrovirology also benefited.

The decade from 1960 was a particularly troublesome period for those researchers working on retroviruses. Diverse lines of investigation gave rise to theories that did not fit with the current paradigms of the time, or were simply against them. For example, the observation that progeny cells derived from infected cultures displayed an "infected" phenotype in the absence of infection was puzzling, as was the occurrence of endogenous viral genomes in healthy animals, and/or the presence of intermediate DNA forms of RNA tumour viruses.

Howard Temin, who was working on RSV replication, postulated the DNA provirus hypothesis, which followed the same line of reasoning of André Lwoff's prophage theory. The latter claimed that the bacteriophage –a DNA virus that infects bacteria– inserts its genome within the host's genome. This integration leads to a non-infectious form called the "prophage" that can be later reactivated back to its original lytic form. Analogously, Temin (as well as other researchers working on Simian Virus 40 in Renato Dulbecco's laboratory) coined the term "provirus" when referring to the integrated state of RSV during the early stages of infection. When Temin and Rubin first proposed the term "provirus", which was a suitable hypothesis for a DNA virus, the nature of the RSV genome was at the time unknown (Marcum 2002). Therefore, the publication of a paper where the genome of RSV was shown to be RNA (Crawford 1960) brought a scientific crisis to the field, because Temin's hypothesis implicated the synthesis of DNA using RNA as a template, something simply unacceptable at that time. However, he unequivocally proved the requirement of DNA synthesis for the replication of RSV by showing that this virus was sensitive to Actinomycin D (an

inhibitor of DNA synthesis) while Newcastle disease virus, an RNA virus which does not display a DNA intermediate, was unaffected by the same drug (Temin 1963).

At the same time, endogenous retroviruses were showing traces of their existence: while studying ALV infection in chickens, Dougherty and Di Stefano were puzzled by the observation of positive serology for “group-specific antigen” (this is where the term Gag comes from) of uninfected animals (Dougherty and Di Stefano 1966). A further study by Payne and Chubb showed that this “chick antigen” was identical to the RSV group-specific antigen and moreover, it was inherited in a Mendelian fashion (Payne and Chubb 1968). But again the current paradigms of the time were against the existence of endogenous RNA tumour genomes in healthy animals. In a review about the discovery of endogenous retroviruses, Robin Weiss describes the rejection of a manuscript where he postulated the presence of a novel endogenous envelope that complemented the defective Bryan strain of RSV in normal chicken embryo cells (Weiss 2006). However, he was later vindicated by the subsequent acceptance of his work and the publication of Hanafusa’s paper showing a cell-associated factor, coined “chick-associated helper factor” (Hanafusa, Miyamoto et al. 1970), which was in agreement with Weiss’ results. Moreover, other investigators strengthened that concept describing the hereditary nature of MMTV in mice (Bentvelzen, Daams et al. 1970).

In June of 1970, the most important discovery of retrovirology was achieved: two papers from different laboratories published side by side in *Nature* announced the isolation of an RNA-dependent DNA polymerase, called reverse transcriptase (RT) from MLV and RSV (Baltimore 1970; Temin and Mizutani 1970). The consequences of this discovery are hard to summarize. First, reverse transcription provided the theoretical framework that allowed researchers to answer the riddles that had been puzzling them in the previous decade. Second, it successfully challenged the central dogma of molecular biology without contradicting it. And third, it marked the origin of a biotechnological tool that boosted incredibly the advancement of science.

Studies on RSV again in 1976 proved formative to the scientific community when Harold Varmus’ laboratory showed by hybridisation techniques that the viral genomic sequence responsible for neoplastic transformation (later characterized as the *src* gene)

was present in normal chicken genomic DNA and in the genome of other avian species such as quail, turkey, duck and the more evolutionarily distant emu (Stehelin, Varmus et al. 1976). Thus, the genetic nature of cancer was revealed and the term “oncogene” was coined. *Src* was the first oncogene and the first tyrosine kinase discovered.

The discovery of a vast number of animal retroviruses encouraged researchers to look for human retroviruses associated with high-incidence neoplasias such as leukaemia, breast cancer, prostate and lung tumours. American president Richard Nixon promoted cancer research through the National Cancer Act, which was known as the “War on cancer”. However, until the mid-seventies efforts had been fruitless and plagued with cross-contaminations with animal retroviruses. Work in Robert Gallo’s laboratory paved the way with the discovery of interleukin-2 (IL-2), a T-cell growth factor that allowed the long-term culture of primary T-lymphocytes (reviewed in Gallo, 2005). This technology, together with sensitive RT assays made it possible to identify a retrovirus associated with a T-cell malignancy: human T-cell leukaemia virus 1 (HTLV-1) (Poiesz, Ruscetti et al. 1980). HTLV-1 was found to be the causative agent of adult T-cell leukaemia, and tropical spastic paraparesis (TSP).

By the time HTLV-1 and -2 were being discovered, an outbreak of an unknown illness was detected in the US. The disease was mainly observed among gay men from California and New York City, and common symptoms were the presence of Kaposi’s sarcoma and opportunistic infections due to a severe immunosuppression. The “AIDS era” had started and nothing was going to be the same. Despite the immense controversy about the actual discoverers of HIV, it was Luc Montaigner’s group in Paris who were the first to isolate the virus (Barre-Sinoussi, Chermann et al. 1983; Gallo 2005), and Robert Gallo’s laboratory who linked HIV with AIDS (Gallo, Salahuddin et al. 1984; Popovic, Sarngadharan et al. 1984; Sarngadharan, Popovic et al. 1984; Schupbach, Popovic et al. 1984). A detailed account about the discovery of HIV including original transcripts, memos, pictures, and recordings is freely accessible at the following NIH website: <http://aidshistory.nih.gov/home.html>. Since its discovery, HIV has probably been the most studied virus in science history and despite the efforts of some of the brightest minds in the scientific community, neither a protective vaccine nor a curative treatment have been developed yet. Current therapies with antiretroviral

drugs have kept AIDS patients alive for a number of years now, but undesired side effects are common. Moreover, the emergence of HIV/AIDS unveiled the limitations of our society: antiretroviral drugs do not reach underdeveloped countries due to their high cost and preventions campaigns have proved inefficient. Therefore, 65% of infected people live in sub-Saharan Africa (an updated incidence rate can be obtained at the World Health Organization HIV/AIDS website: <http://www.who.int/hiv/en/>).

The inherent ability of retroviruses to integrate into the host's genome has been used to develop retroviral vectors capable of expressing specific proteins in transduced cells (Miller 1997). The development of this technology started in the early eighties when the first packaging cell lines were created and retroviral-mediated gene transfer was achieved in somatic stem cells (Baum, Schambach et al. 2006). Commercial retroviral vectors are nowadays widely available and used in the laboratory. The RSV-derived RCAS system constitutes an example that has been extensively applied for *in vitro*, *ex-vivo* and *in vivo* studies (Orsulic 2002). Furthermore, germ line stem cells can be transduced to generate transgenic animals (Nagano, Brinster et al. 2001) and intense research is currently being carried out to develop retroviral vectors for human gene therapy (Baum, Schambach et al. 2006). Although promising, this approach has recently suffered a serious drawback when two out of ten patients that received a therapeutic MLV-based retroviral vector in a clinical trial to correct an X-linked severe combined immunodeficiency developed leukaemia, most likely caused by insertional mutagenesis in proximity of the LMO2 proto-oncogene promoter (Haccin-Bey-Abina, Von Kalle et al. 2003). Recent studies on integration patterns by different retroviruses suggest that the use of lentiviral vectors could achieve better results (see below).

The post-genomic era started at the beginning of the twenty-first century with the publication of the first integrated draft sequence of the human genome (Lander, Linton et al. 2001; Venter, Adams et al. 2001), although smaller genomes had been sequenced before. Since then, rapid progress followed, and genomes from diverse organisms such as fruit flies, dogs, chimps and yeast have been sequenced. It is obvious that the benefits obtained from the generation of such a vast amount of data boosted the advance of all areas of science, not just retrovirology. We can now look for genes that provide susceptibility or resistance for viral infections in a given species, determine their

distribution in different populations or breeds, and address their evolutionary importance in related species. The same applies for endogenous retroviruses and transposable elements: although we have known of their existence for many years, we are currently able to easily identify *in silico* previously uncharacterized loci. Such approach can be used as a starting point for further *in vitro* and *in vivo* studies by means of the vast variety of molecular biology techniques that are presently available.

Throughout millions of years, germ line infections by exogenous retroviruses led to further accumulation of endogenous retroviral sequences within the genome of every species where they have been sought. Therefore, although it may sound presumptuous to say that we owe what we are to retroviruses, it may actually be true.

### ***Viral taxonomy***

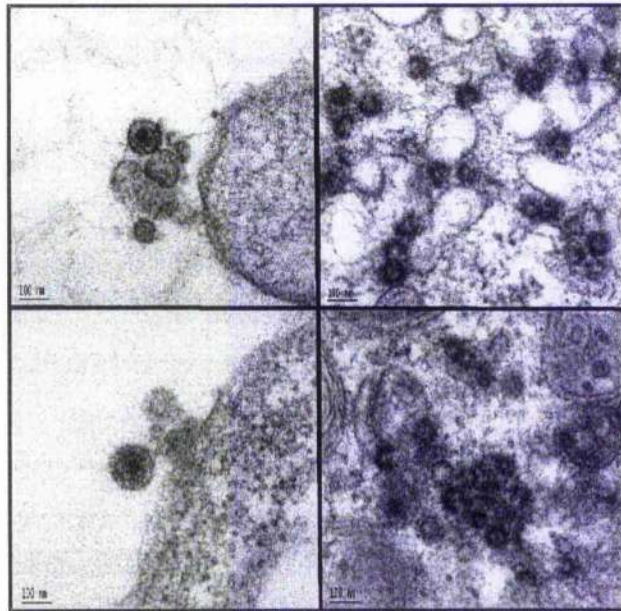
Retroviruses are enveloped viruses with a diameter of 80-120 nm. The viral genome within the particle consists of two copies of linear, single-stranded RNA of positive polarity with a size of 7-12 Kb (Vogt 1997). Retroviruses are classified as “simple” when they encode the minimum canonical genes *gag*, *pol* and *env* (see below), or “complex” if they bear other genes, which are regarded as “accessory”. Retroviruses display a unique replication strategy: after entering their target cell, they reverse-transcribe their RNA genome into DNA and further integrate into the genome of the host cell. The integrated viral DNA genome, termed provirus, is transcribed by the cellular RNA polymerases and the resulting messenger RNAs (mRNAs) will in turn be translated into viral proteins and also provide the viral genomes that will be assembled within progeny virions (Vogt 1997). The family *Retroviridae* has been recently reclassified by the International Committee on Taxonomy of Viruses (ICTV) and is currently divided in two subfamilies, *orthoretrovirinae* and *spumaretrovirinae*, and further divided in seven genera that are listed in Table 1.1 (ICTVdB 2006).

Subfamily	Genera	Example	Genomic organization
Orthoretrovirinae	Alpharetrovirus	Rous sarcoma virus	Simple
	Betaretrovirus	Jaagsiekte sheep retrovirus	Simple
	Gammaretrovirus	Murine leukaemia viruses	Simple
	Deltaretrovirus	Bovine leukaemia virus	Complex
	Epsilonretrovirus	Walleye dermal sarcoma virus	Complex
	Lentivirus	Human immunodeficiency virus type 1	Complex
Spumavirinae	Spumavirus	Human foamy virus	Complex

**Table 1. Taxonomy of retroviruses.**

The *Alpharetrovirus* genus comprises a group of viruses whose members have been identified only in birds and were previously regarded as avian type-C retroviruses (ICTVdB 2006). Avian leukosis virus (ALV) is the prototype of the genus, and distinct isolates are classified according to envelope specificities (Vogt 1997). *Betaretroviruses* comprise the former mammalian type-B and type-D viruses (see below). Well studied members of this genus are Mason-Pfizer monkey virus (M-PMV), Mouse mammary tumour virus (MMTV) and Jaagsiekte sheep retrovirus (JSRV). *Gammaretroviruses* are simple retroviruses that infect more than one vertebrate class (Gifford and Tristem 2003). Some examples are feline leukaemia virus (FeLV), murine leukaemia virus (MLV), gibbon ape leukaemia virus (GaLV), and bird reticuloendotheliosis viruses that affect ducks, chickens, turkeys, geese and pheasants. *Epsilonretroviruses* are complex retroviruses described in fish that include Walleye dermal sarcoma virus (WDSV). *Spumaretroviruses* also bear a complex genome and despite their broad distribution

amongst mammals, no disease has been associated with them. Members of the genus include feline, bovine, and chimpanzee foamy viruses (Gifford and Tristem 2003). Finally, *Deltaretroviruses* and *Lentiviruses* comprise two different genera with common features: they bear a complex genome encoding *trans*-acting regulatory proteins and produce slow chronic diseases. Remarkably, no endogenous counterparts of the former genera have been identified yet (Gifford and Tristem 2003), whereas the discovery of the first endogenous Lentivirus has recently been reported (Katzourakis, Tristem et al. 2007).



**Figure 1. Electron micrographs of retroviral particles.**

Extracellular (left) or intracellular (right) JSRV viral particles, where phenotypical differences between mature and immature virions can be observed (see text). Pictures are courtesy of Massimo Palmarini, Alan Rein and Kunio Nagashima.

Originally, retroviruses were classified according to the morphology they displayed by electron microscopy. This gave rise to A, B, C, or D-type viruses. For example, A-type particles possess an electron-lucent centre surrounded by one or two electron-dense rings. The translucence of the core is typical of immature particles, while mature retroviruses display a compact opaque core due to rearrangements in the structural proteins that will be described later. Both type-B and type-D viruses assemble in the cytoplasm (Figure 1), but while the former displays an eccentrically positioned round

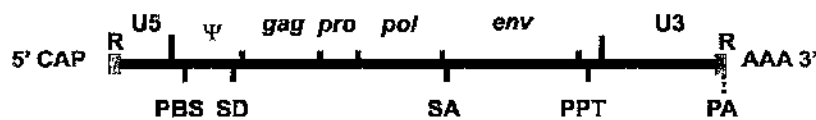


core, the latter possesses a cylindrical core. Type-C retroviruses assemble at the plasma membrane and have a round core located in central position. Spumaviruses on the other hand, have a unique morphology, with conspicuous spikes protruding from the surface and an uncondensed core located in the centre of the viral particle. Although the new classification has been adopted for a number of years now, the old one is still broadly used, in particular when describing the site of assembly.

### ***Genomic organization of retroviruses***

Retroviruses are functionally diploids as the viral genome comprises in general two identical copies of linear single-stranded RNA of positive polarity. The dimer linkage structure (DLS) is a self-complementary region located at the 5' end of the RNAs that holds the two molecules together forming a kissing loop. The viral genome shares many common features with cellular messenger RNAs since they are both synthesized using the same cellular transcriptional machinery (i.e. they are capped at the 5' end and polyadenylated at the 3' end). Viral genes are flanked by non-coding sequences that play specific roles in reverse transcription and will constitute the promoter that drives the expression of the proviral DNA. A schematic organization of the retroviral RNA genome is depicted in Figure 2.

At both ends of the RNA molecule; downstream of the cap at the 5' end and upstream of the poly(A) signal at the 3' end, lie two short repeated regions termed R. There are also two unique sequences: U5, located after the R sequence at the 5' extreme, and U3, at the 3' end just upstream of the R region. Both unique regions bear the *att* sites that are required for integration. An 18-nucleotide sequence termed primer binding site (pbs) is located downstream of U5. The pbs anneals with a host transfer RNA (tRNA) to prime the synthesis of viral DNA during reverse transcription (see below). Signals for encapsidation of the viral RNA into the viral particles lie after the pbs and comprise the Psi element ( $\Psi$ ). The genomic organization of the RNA genome is slightly different to that of the proviral DNA, which will be described later. The subtle changes that take place during reverse transcription have functional implications for the expression of the viral genome, which is then part of the host genome.



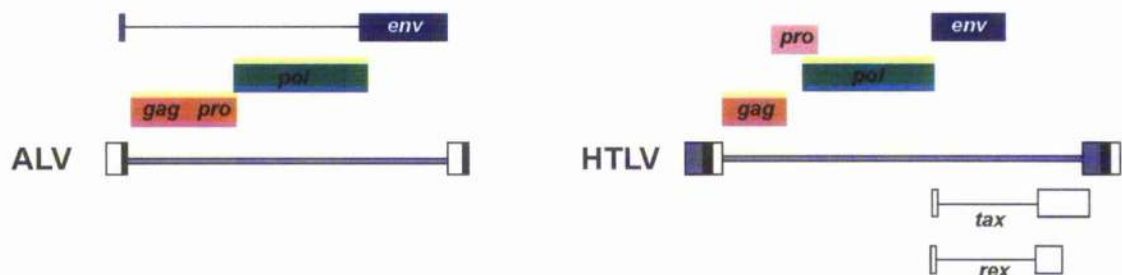
**Figure 2. Schematic organization of a prototypical retroviral RNA genome.**

The RNA molecule is shown as a straight line, displaying a 5' cap followed by the first repetitive sequence (R); a unique sequence U5; the primer binding site (PBS); the packaging signal  $\Psi$ ; the splice donor (SD) and acceptor (SA); the viral genes *gag*, *pro* and *pol* and *env*; the polypurine tract (PPT); the unique sequence U3, is followed by the second repetitive sequence R and a polyadenylation signal (PA). Genomic sequences are not drawn to scale.

All replication-competent retroviruses bear a minimum of four genes. These open reading frames (ORFs), termed *gag*, *pro*, *pol* and *env*, take up most of the space of the genome and follow that invariable order. Simple retroviruses (like JSRV) possess only these canonical genes. The *gag* gene encodes a structural polyprotein that after assembly is cleaved into at least three polypeptides: matrix (MA), capsid (CA) and nucleocapsid (NC). This cleavage is performed during maturation by the viral protease encoded by *pro*. The changes produced by this cleavage are reflected in the phenotypical differences observed by electron microscopy between immature and mature viral particles (see Figure 1). The gene products of *pol* are the viral polymerase (an RNA-dependent DNA polymerase with associated RNase H activity) and the integrase (IN), which mediate the replication of the viral genome. Finally, *env* encodes the envelope glycoprotein, which derives from the synthesis of a spliced sub-genomic mRNA. The envelope is composed by the surface (SU) domain that interacts with the cellular receptor and by the transmembrane (TM) domain that fixes the Env to the cell membrane. In the case of JSRV, *env* possesses also oncogenic properties, as will be discussed later.

Retroviruses that possess a small array of accessory genes in addition to the canonical *gag*, *pro*, *pol* and *env* are regarded as “complex” (Figure 3). These regulatory genes

coordinate viral gene expression or have other roles during the replication cycle. Examples of complex retroviruses are human immunodeficiency virus-1 (HIV-1), bovine leukaemia virus (BLV) and human T-cell leukaemia virus (HTLV) (Goff 2001). Other retroviruses carry host-derived genes whose expression leads to cell transformation, and hence they are referred to as oncogenes. In general they are defective because of deletions on genes required for viral replication, and hence require the presence of a non-defective or 'helper' virus to replicate. Some examples are Moloney murine sarcoma virus (Mo MSV), and simian sarcoma virus (SSV). It should be noted that some strains of Rous sarcoma virus (RSV) are replication competent despite bearing oncogenes (Vogt 1997).



**Figure 3. Genetic organization of a simple (left) and a complex (right) retrovirus (see text).**

ALV: avian leukaemia virus, HTLV: human T-cell leukaemia virus. Rectangles represent the open reading frame for the gene indicated. Horizontal line linking two segments in ALV *env* represents splicing out of the indicated segment. Adapted from Vogt (1997).

### ***Viral proteins and virion structure***

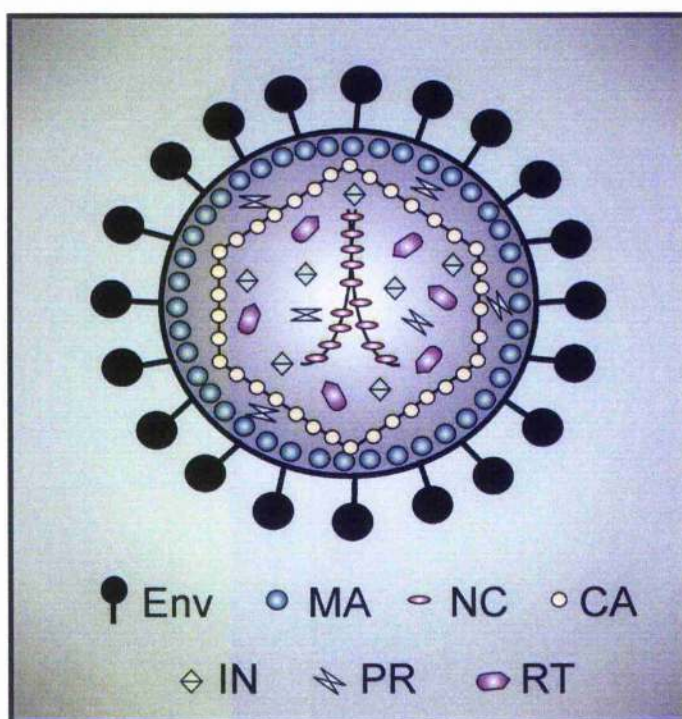
Throughout the years, different studies on the structure of retroviruses have produced a vast body of information that allows us to know important aspects of the retroviral virion such as size, density, and the approximate proportion of lipids, proteins, and nucleic acids (Vogt 1997). Moreover, electron microscopy studies played a fundamental role in the early days of retrovirology, when retroviruses were classified according to their morphological features. However, the tridimensional arrangement that all the viral components adopt to constitute a retroviral particle is currently ignored. We must therefore rely on models for retroviral structure. Several proteins that form the virion

have been solved either by crystallography and/or nuclear magnetic resonance (NMR) (Vogt 1997; Amarasinghe, De Guzman et al. 2000; Klein, Johnson et al. 2000; Sarafianos, Das et al. 2001) and the resulting structures provided a great support in building up such models.

The structural basis of the retroviral particle is provided by the Gag polyprotein and the polypeptides derived from it. The innermost part of the particle is constituted by the genomic RNA tightly associated with the nucleocapsid protein (Figure 4). This complex is surrounded by the capsid protein, which in turn is encircled by another Gag-derived protein, the matrix. The viral envelope originates from the cellular membrane, surrounds the matrix and contains the viral glycoproteins. These are believed to form trimers with a single-spanning transmembrane domain (TM) bound to an extra-virion surface domain (SU) by means of non-covalent interactions, or in some cases, by disulfide bonds (Vogt 1997; Goff 2001). Together with the structural proteins, the viral enzymes such as reverse-transcriptase, integrase, and protease are also co-packaged within the virion. Some accessory proteins are found in high amounts within the viral particle whereas others are either absent or found in small quantities. In the case of HIV-1, while Vpr and Vpu are incorporated into virions at high levels, Vpx seems to be absent, and Vif and Nef can only be detected in small amounts (Freed and Martin 2001). Finally, cellular proteins are also incorporated into virions, giving the host a chance to introduce restriction factors that can act at the early stages of the following round of infection. The APOBEC family of mammalian polynucleotide cytidine deaminases constitutes an excellent example (Mangeat, Turelli et al. 2003; Okeoma, Lovsin et al. 2007) that will be described later in this chapter.

All the different building blocks that constitute a viral particle must be synthesized in a coordinate fashion and targeted to a specific site within the cell in order to assemble into viral particles. By synthesizing polyproteins, retroviruses simplify this issue because they can make multiple proteins from a single ORF simultaneously, transport them to a specific cellular compartment using one trafficking signal (therefore requiring less transporters), and more importantly, they are delivered at the required ratio. In addition, since viruses are arguably living (or at least dynamic) entities, the function of the polyprotein as a whole, or of its individual components, can be regulated by proteolysis

(i.e. exposing or hiding specific domains). As was mentioned above, all the structural proteins are derived from Gag, which is indeed synthesized as a polyprotein, always following the same invariable order: NH<sub>2</sub>-MA-CA-NC-COOH (Figure 5). This constant order matches the spatial distribution of the constituting polypeptides within the viral particle. Several retroviruses bear other proteins derived from Gag, usually located between MA and CA, or after NC (Figure 5). As will be shown further, this is also the case for JSRV.



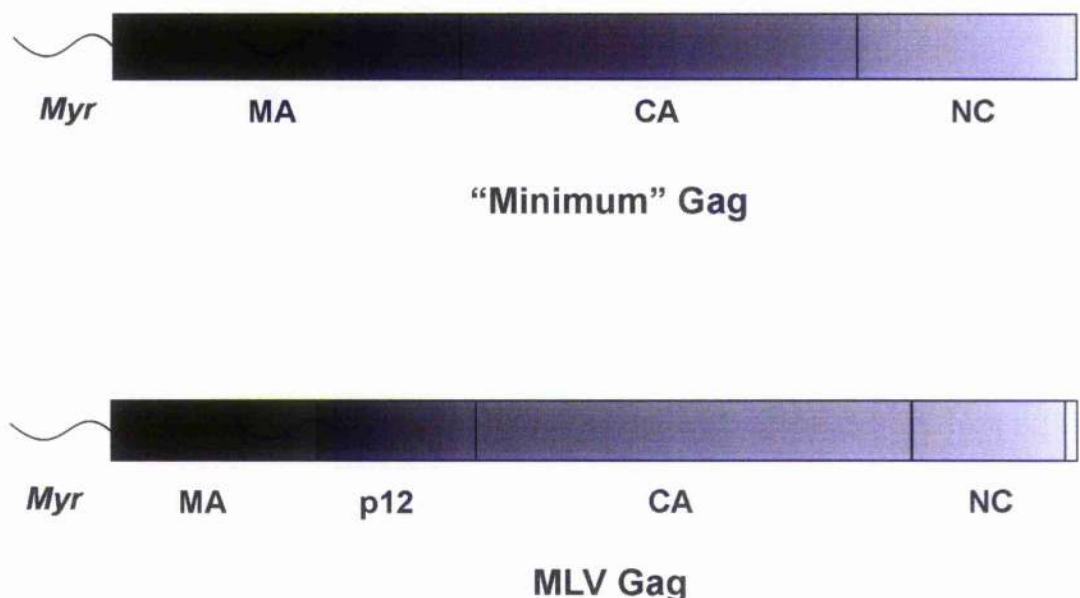
**Figure 4. Schematic representation of a generic retroviral particle.**

Structural features of a given retrovirus are indicated: Surface envelope protein (SU), transmembrane envelope protein (TM), capsid (CA), matrix (MA), nucleocapsid (NC), polymerase (Pol), protease (Pro), integrase (IN), and viral RNA. (Courtesy of Mariana Varela. Adapted from *Retroviruses*, Cold Spring Harbor Laboratory Press).

The presence of short spacer peptides has also been described (Henderson, Bowers et al. 1992; Tobin, Sowder et al. 1994).



The MA is the most external of the Gag polypeptides, as faces and associates with the lipid envelope. In several (but not all) retroviruses, addition of a myristate together with the presence of a basic stretch of amino acids constitutes the membrane (M) domain that targets Gag to the plasma membrane and provides the chemical requirements for this association (Henderson, Krutzsch et al. 1983; Swanstrom and Wills 1997). The importance of this interaction is underscored by the fact that assembly is prevented in certain retroviruses, such as MLV, when myristoylation is abrogated (Rein, McClure et al. 1986). In some retroviruses such as MLV, Gag can be subject to glycosylation (Edwards and Fan 1979), and this post-translational modification seems to play an important role in viral infectivity, as well as in budding and release (Low, Datta et al. 2007). Moreover, because the MA associates with the envelope glycoproteins in a seemingly non-specific fashion, a well known feature of retroviruses is their ability to form pseudotypes (Vogt 1997).



**Figure 5. Schematic representation of the Gag polypeptide**

Above: Cartoon of a minimal Gag polypeptide. Below: cartoon of MLV Gag. MLV Gag is depicted to show an example of a Gag polypeptide bearing other polypeptides besides MA-CA-NC). Adapted from Vogt (1997).

Within the viral particle, the capsid protein limits inward with MA and outward with the NC-RNA complex. In a given Gag polyprotein, CA determines the size and morphology of the virion, as well as the other Gag molecules that will coassemble with that particular Gag (Ako-Adjei, Johnson et al. 2005). Furthermore, CA possesses a highly conserved sequence termed the “major homology region” (MHR) (Vogt 1997). Deletion of this 20 residue-long sequence can abrogate assembly of M-PMV, RSV, and HIV-1 (Strambio-de-Castilla and Hunter 1992; Craven, Leure-duPree et al. 1995; Provitera, Goff et al. 2001).

The nucleocapsid protein is complexed with the viral genomic RNA within the virion constituting the inner core of the retroviral particle. To associate with the viral genome, NC must have an intrinsic affinity for RNA and this is achieved by the presence of basic residues and one or two Cys-His motifs bearing the consensus sequence CX<sub>2</sub>CX<sub>4</sub>HX<sub>4</sub>C. As mentioned above, the viral RNA displays a packaging signal  $\Psi$  that is recognized by the NC protein (Berkowitz, Ohagen et al. 1995) and thus helps to discriminate between molecules of viral and cellular origin, which is of great aid since the latter are more abundant. Within the nucleocapsid there is also a domain responsible for the interaction between Gag molecules, termed the interaction domain (I). This domain is important during assembly and does not display a characteristic sequence, although basic residues have been implicated as its primary components (Swanstrom and Wills 1997; Bowzard, Bennett et al. 1998). Furthermore, RNA has been proposed as a structural component of the virion, acting as a scaffold for Gag-Gag interactions (Muriaux, Mirro et al. 2001). This role can be performed either by viral or cellular RNA, and it has been supported by the observation that HIV-1 MA can also bind RNA, featuring a redundant role with NC (Ott, Coren et al. 2005). Consequently, MA-RNA binding is required for particle production in the absence of NC, (Ott, Coren et al. 2005).

The presence of other domains besides MA, CA, and NC in some retroviral Gags reflects its variable molecular organization. These domains are usually located between MA and CA or between CA and NC, and in certain cases at the C-terminus of NC. In M-PMV, Gag processing releases two proteins that lie between MA and CA: pp24/16 and p12, and a short polypeptide downstream from NC, p4. pp24/16 displays two short amino acid sequences required for viral budding known as late (L) domains (Gottwein,

Bodem et al. 2003). p12 in turn, seems to constitute an “internal scaffold domain” (ISD) that modulates assembly efficiency (Sakalian and Rapp 2006). p6, a short polypeptide present in HIV-1 Gag, represents a further example: it lies downstream of NC and bears the late domains required for viral budding (Henderson, Bowers et al. 1992; Martin-Serrano and Bieniasz 2003).

Besides the structural proteins, all retroviruses possess three enzymes derived from *pol* and *pro*: reverse transcriptase (RT), integrase (IN), and protease (PR). They also originate from polyprotein precursors such as Gag-Pro or Gag-Pro-Pol, but the number incorporated within viral particles is much smaller than Gag alone. Other non-structural proteins incorporated in virions are some products of accessory genes and host derived proteins, both of which have been mentioned before.

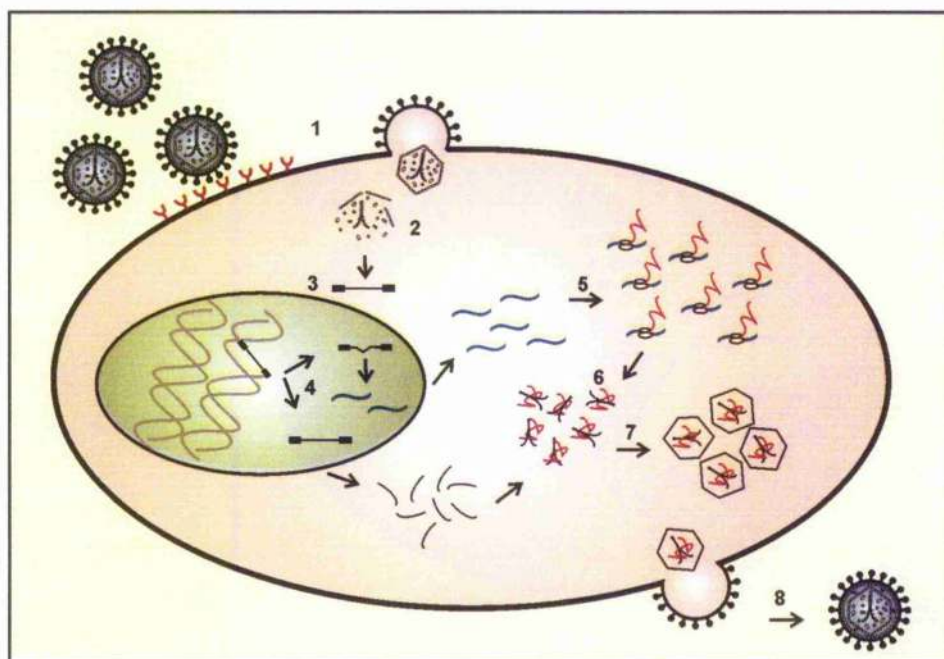
The most external layer of the viral particle is the envelope, a lipid bilayer derived from the cellular plasma membrane adorned with a variable number of protruding glycoproteins (Vogt 1997). The obvious function of the envelope is to interact with cellular receptors to promote viral entry (Wyatt and Sodroski 1998). However, other less conspicuous and very interesting roles have been described both *in vitro* and *in vivo*, such as intercellular fusion, syncytium formation in placental development (Mi, Lee et al. 2000; Rote, Chakrabarti et al. 2004; Dunlap, Palmarini et al. 2006) and cell transformation (Maeda, Palmarini et al. 2001; Caporale, Cousens et al. 2006).

### ***Overview of the retroviral cycle***

The replication cycle of retroviruses can be summarized as follows: free extracellular virions attach to the host target cells, bind to their cognate receptor (and in some cases coreceptor), and fuse their membranes with the plasma membrane. Internalized particles uncoat and reverse transcription of the RNA genome rapidly starts. Double stranded viral DNA is transported to the nucleus for further integration into the host's genome, and once the provirus is established, precursor polyproteins are synthesized using the cellular transcriptional and translational machinery. Viral RNA that will constitute the genome of the progeny virions is then packaged into assembling particles, which in turn will either bud out of the productively infected cell and release new progeny of free



extracellular viral particles, or infect target cells through a virological synapsis (Figure 6).



**Figure 6. Schematic representation of a simplified retroviral cycle.**

Each stage of the cycle is numbered. 1: viral attachment and entry, 2: uncoating and reverse transcription, 3: entry of the pre-integration complex into the nucleus and integration, 4: transcription of the provirus, 5: synthesis of viral proteins, 6: assembly, 7: targeting of immature progeny virions to the plasma membrane, 8: viral budding and maturation. See text for further details. Figure kindly provided by Mariana Varela.

### **Viral attachment and entry**

The first step of the retroviral cycle comprises a complex series of events that result in the entry of the viral particle into a target cell. The earliest stage is viral attachment, which is characterized by the specific binding of the envelope glycoproteins to membrane receptors and defines the first order of tropism (Figure 6, step 1). To date, many receptors and coreceptors for retroviral entry have been identified, such as HYAL2 for JSRV (Rai, Duh et al. 2001) and CD134 for feline immunodeficiency virus (FIV) (Shimojima, Miyazawa et al. 2004). It is thought that the SU protein recognizes the cell receptor and TM mediates membrane fusion (Goff 2001). Based on receptor specificity, retroviruses can be classified in four categories: ecotropic, xenotropic,

amphotropic, and polytropic. This classification criterion was originally applied to endogenous MLVs (Stoye and Coffin 1987) but it is used by some authors also for other retroviruses. The so-called ecotropic viruses can infect only cells of the species where they originate. Non-ecotropic endogenous MLVs are classified as xenotropic and polytropic. The former can not infect cells of laboratory mice but can infect a wide range of cells from different species (Levy 1973); whereas the latter can replicate in both murine and non-murine cells. Amphotropic MLVs are exogenous MLVs that can infect mouse and non-mouse cells (Boeke and Stoye 1997; Hunter 1997). Amphotropic and polytropic MLVs use different cell receptors (Rein and Schultz 1984). After infection, the envelope proteins expressed by the provirus bind to their cognate receptors intracellularly, diminishing the number of available molecules at the plasma membrane, and consequently blocking the entry of any virus that requires such receptor.

## **Reverse transcription**

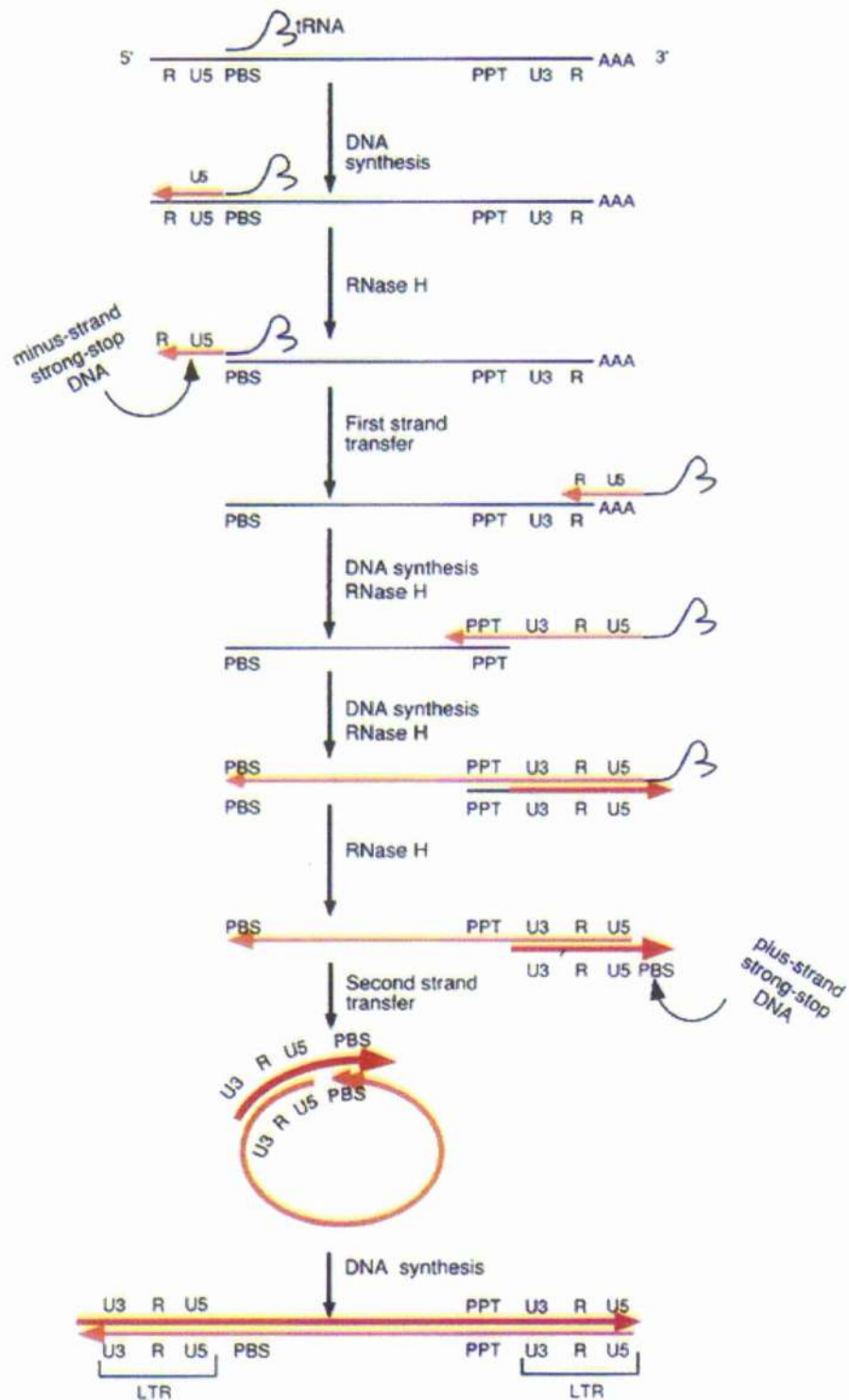
After internalization, the incoming virus uncoats (Figure 6, step 2). Mature Gag is required for this step (Goff 2001), as well as host factors, an example being Cyclophilin A (CypA), a cellular protein incorporated in HIV-1 virions that is necessary to allow replication in non-human primate cells (Sokoloskaja and Luban 2006). A more detailed description on the function of CypA will be given below where restriction factors will be covered. Reverse transcription, the hallmark of the retroviral cycle, is a highly complex reaction and has been thoroughly studied. In members of the subfamily *Orthoretrovirinae*, reverse transcription starts after uncoating and requires at least RT, IN, NC, and of course, the viral RNA. For foamy viruses (*Spumavirinae*), reverse transcription is a late event during the viral replication cycle (Moebs, Enssle et al. 1997). Briefly, a tRNA anneals with the primer binding sequence located near U5, and the polymerase synthesizes the minus-strand strong-stop sequence using the viral RNA as a template. This step is followed by the first “jump”: as the RNA of the formed heteroduplex is degraded by the RNase H activity of Pol, the minus-strand strong-stop sequence can now anneal with the 3' R of the (remaining) viral RNA, to further proceed with the completion of the long minus-strand sequence that ends close to the pbs. Synthesis of this DNA strand is coupled with the degradation of the RNA template, with the exception of the polypurine tract (PPT), a short RNA sequence which in some

retroviruses (i.e. spumaviruses) is duplicated (Kupicic, Tobaly-Tapiero et al. 1988). The PPT will prime the initiation of the plus-strand DNA sequence. At this stage, RT will use DNA as a template, extending the nascent complementary chain towards the 5' end of the minus-strand DNA, and stopping at the level of the pbs of the tRNA that primed the first step of the reaction and is still bound to the minus-strand DNA. After removal of the tRNA, the second jump occurs, and now the two exposed pbs regions of both chains anneal forming a circular structure that allows the completion of the synthesis of both strands. At the end of this final elongation step, a linear double-stranded DNA molecule, named provirus, is formed (Goff 2001). As a consequence of reverse transcription, the proviral DNA displays two long terminal repeats (LTRs) that arose after duplication of U3 and U5. The whole process of reverse transcription is depicted in Figure 7.

## Integration

The following step of the retroviral cycle after reverse transcription is the integration of the provirus into the host's genome (Figure 6, step 3). This process, which puzzled researchers for many years, has profound implications in retroviral biology. First, a cell is infected by a retrovirus it is persistently infected and hence becomes a permanent source of virus. Second, infection is heritable since the viral genome is transmitted during mitosis from infected cells to daughter cells like a host gene following Mendelian rules. Third, integration is mutagenic *per se* and this characteristic accounts for the oncogenic nature of some retroviruses. Fourth, endogenous retroviruses can originate from germline infections, with potential evolutionary consequences for the host, and/or the exogenous virus from which they derive.

Following reverse transcription, the viral DNA that is contained in a nucleoprotein complex termed the preintegration complex (PIC) must gain access to the nucleus to further integrate into the host's genome. To achieve this, simple retroviruses just wait until the nuclear membrane breaks down during mitosis, hence they can only infect dividing cells. In contrast, Lentiviruses have means to pass through the intact nuclear membrane as they successfully infect non-dividing cells (Goff 2001). Foamy viruses instead remain as assembled capsids in the vicinity of the microtubule-organizing centre



**Figure 7. Scheme of reverse transcription.**

RNA is represented as a black line, minus-strand DNA as a light line, and plus-strand DNA as a dark line. Process is described in the text. From *Retroviruses* (Cold Spring Harbor Laboratory Press, with permission).

(MTOC) until the infected cell is stimulated to divide. Upon cell activation, viral disassembly takes place and infection proceeds (Lehmann-Che, Renault et al. 2007).

The viral integrase has a preponderant role in the whole integration process (Lewinski and Bushman 2005). Integration starts with the removal of the two terminal nucleotides located at the 3' end of the linear DNA (3' end processing). The resulting 3' OH ends attack the phosphodiester bonds of the host DNA, forming a new linkage between the extremes of the viral DNA and the host DNA, and originating gaps at the sites of this bond. Filling in these gaps gives rise to short duplications that flank the proviral DNA that now lies neatly integrated within the host genome (Brown 1997). The mechanism that actually determines the sites of integration is currently unknown, but genome-wide studies suggest that active chromatin is mainly targeted (Lewinski and Bushman 2005). For MLV, a *Gammaretrovirus* adapted to use as gene delivery vector in the aforementioned gene therapy trials, regions near transcription start sites and associated CpG islands are preferred targets for integration (Wu, Li et al. 2003; Lewinski, Yamashita et al. 2006). In contrast, Lentiviruses show an increased frequency of integration within transcription units (Schroder, Shinn et al. 2002). Recent work indicates that the main viral determinant in the selection of the integration site is IN, although Gag seems to play an auxiliary role (Lewinski, Yamashita et al. 2006). It has recently been suggested that integration profiles can be predicted based on phylogenetic analysis of the integrase protein (Derse, Crise et al. 2007). Understanding the molecular basis of this targeting is essential for the development of safe gene delivery therapies with retroviral vectors.

From a 'retroviral point of view' integration is a massive achievement, as viral genes will share the same status as host genes, and will therefore be expressed by the cellular machinery (Brown 1997; Goff 2001).

### **Expression of viral genes**

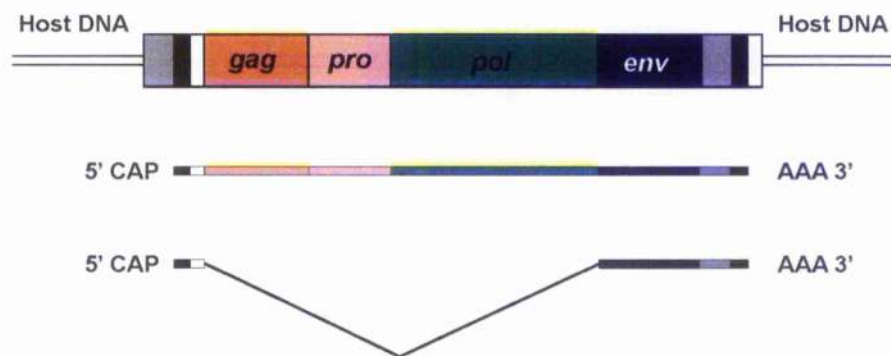
The cellular polymerase II system synthesizes the viral RNA as a long primary transcript that is further processed (Figure 6, step 4), and in some cases spliced before nuclear export. Viral RNAs are subject to capping and polyadenylation and the signals

for such processes have been described above. Both LTRs possess the necessary information to function as promoters in order to drive the expression of viral genes, but in general the 5' LTR is dominant. The efficiency of transcription initiation is influenced by the presence or absence of cellular transcription factors (determined by the cell lineage), the physiological state of the cell, and the positional location of the provirus (Rabson and Graves 1997; Goff 2001). Unspliced RNAs serve as genomes of progeny virus or to synthesize Gag and Gag-Pol proteins, whereas spliced molecules are either translated into envelope glycoproteins or, for complex retroviruses, accessory proteins (Figure 8). Some retroviruses such as MLV display two distinct populations of unspliced viral RNA that are either translated or packaged into virions (Butsch and Boris-Lawrie 2002). Furthermore, a recent study suggests that the Gag polyprotein of HIV-1 can modulate its own translation in a bimodal fashion by binding to its own packaging signal and thus obstructing the translation initiation complex (Anderson and Lever 2006).

LTRs possess core transcriptional elements such as TATA and CCAT boxes that are recognized by transcription factors such as TFIIIB and CEBP (Ryden and Beemon 1989; McGee-Estrada and Fan 2006). Besides, positive and negative regulatory factors can bind regulatory elements located in the LTR. For example, the glucocorticoid receptor binds to the hormone responsive element located in the U3 of MMTV (Payvar, Firestone et al. 1982) and enhances its expression, in opposition to the CCAAT displacement protein (CDP) that binds to the negative regulatory element (NRE) and repress transcription (Zhu, Gregg et al. 2000). For complex retroviruses, accessory proteins activate transcription *in trans*, as the Tax protein encoded by HTLV-1 does. Since transcription termination apparently cannot be controlled by retroviruses, the exact end of the viral transcript is set at a posttranscriptional stage. The long primary transcript is cleaved and polyadenylated, giving rise to an RNA molecule that ends at the 3' LTR, precisely at the boundary between R and U5 (Rabson and Graves 1997). However, a small proportion of mRNAs can be read-through and thus incorporate cellular sequences, this being a feasible mechanism for the acquisition of proto-oncogenes (Herman and Coffin 1987).



Because in normal cells only fully processed mRNAs can be exported from the nucleus, the unspliced, full-length viral transcripts that will be either packaged within newly formed virions or serve for synthesis of Gag and/or Gag-Pol, must find a way to get out of the nucleus and into the cytoplasm. Simple retroviruses achieve this by means of *cis*-acting elements that bind to cellular proteins responsible for mediating nuclear export, as is the case of the constitutive export element (CTE) found near the 3' end of M-PMV (Bray, Prasad et al. 1994; Ernst, Bray et al. 1997). In complex retroviruses, RNA export relies on accessory genes encoding *trans*-acting proteins that interact with *cis*-acting sequences. For examples HIV-1 and -2, encode the Rev protein that facilitates RNA export by binding the Rev responsive element (RRE), a highly structured segment of RNA located at the 3' end of the viral genome (Freed and Martin 2001).



**Figure 8. Transcription of retroviral genes.**

Top: proviral structure of a simple retrovirus. Middle: Full length genomic RNA that can be either incorporated in newly formed viral particles or serve as mRNA for the synthesis of Gag and/or Gag-Pol. Bottom: subgenomic transcript from which the envelope glycoprotein is translated.

Synthesis of viral proteins and viral assembly are highly dynamic processes in which the Gag polyprotein has a central role as all the structural proteins derive from it (Figure 6, step 5). Moreover, the viral enzymes (Pro, RT, and IN) are synthesized as fusion proteins with Gag when the stop codon of the latter is strategically by-passed. Gag and Gag-Pro-Pol are all translated by free ribosomes and directed to the site of assembly through trafficking signals located in Gag. In contrast, the envelope glycoprotein is

translated through the rough endoplasmic reticulum (RER) and transported via the Golgi apparatus towards the plasma membrane (Swanstrom and Wills 1997).

Some retroviruses such as JSRV, M-PMV and MMTV assemble within the cytoplasm of the infected cell, whereas others (the so-called C-type viruses) assemble at the plasma membrane. Despite the existence of these two morphogenetic pathways, the mechanistic nature of the assembly process itself must not be very different since a single amino acid substitution within Gag transforms M-PMV to a type-C retrovirus (Rhee and Hunter 1990). After assembly and upon budding, the viral protease cleaves Gag into its constituent polypeptides, and this maturation process accounts for the dramatic phenotypic changes observed between immature and mature viral particles by electron microscopy.

Although viral RNAs are capped, ribosome scanning must be an arduous task due to the high degree of secondary structure observed in the leader of retroviral RNA. Therefore, cap-dependent and independent mechanisms for translation initiation have been proposed (Yilmaz, Bolinger et al. 2006). The latter strategy is supported by reports on the presence of internal ribosome entry sites (IRES) in some murine (Berlioz and Darlix 1995; Berlioz, Torrent et al. 1995) and avian (Balvay, Lastra et al. 2007) retroviruses.

Since a viral particle needs many more structural (MA, CA, and NC) than catalytic molecules (Pro and Pol) it is not surprising that the number of Gag molecules is larger than those of Gag-Pro-Pol. The way retroviruses arrange this ratio is another example of their resourcefulness: to bypass the termination codon that determines the end of Gag, some retroviruses such as MoMLV, misread it as a sense codon and incorporate Glutamine instead (Yoshinaka, Katoh et al. 1985). This mechanism is regulated by *cis*-acting sequences with defined secondary structure downstream from the stop codon (Swanstrom and Wills 1997). Another way to bypass the termination codon is by frameshift suppression, a strategy employed by HIV-1, bovine leukaemia virus (BLV) and other retroviruses (Jacks, Madhani et al. 1988) where *gag* and *pol*, or *gag* and *pro* overlap in the -1 direction. Therefore, the translational machinery slips backwards one base to continue adding residues in the alternative frame. This frameshift is also regulated by a heptanucleotide sequence that acts in concert with a pseudoknot formed



by the RNA secondary structure downstream from the termination codon (Balvay, Lastra et al. 2007). In Betaretroviruses, *gag*, *pro*, and *pol* are all in different frames and thus consecutive frameshifts are required to make Gag-Pro and Gag-Pro-Pol. Spumaviruses synthesize Pro-Pol from a sub-genomic mRNA that is separated from Gag (Yu, Baldwin et al. 1996). Post and co-translational modifications are common among retroviral Gag proteins. Examples include myristoylation, acetylation, ubiquitination, glycosylation, phosphorylation and proteolytical cleavage and will be described below in more detail. The envelope glycoproteins are expressed from a spliced RNA molecule, different from the long viral transcript that gives rise to Gag and/or Gag-Pro-Pol. SU and TM are also derived from a polyprotein, but in contrast with Gag, they are synthesized at the RER since Env bears a hydrophobic signal peptide. The Env protein is glycosylated in specific Asparagine residues located within canonical sequences. Further, the protein is folded and oligomerized before being exported to the Golgi apparatus, where cellular furin proteases cleave it into SU and TM (Swanstrom and Wills 1997).

### **Viral assembly**

After the synthesis of viral proteins, all the components that will constitute the virion have to come together to a specific location within the cell to assemble into viral particles that will subsequently bud out of the infected cell (Figure 6, step 6). Assembly is a tremendous task, as requires the direction of all the virion components to the site of assembly, where they have to recognize each other and make specific contacts to give rise to a highly-ordered structure of a certain size and shape. Further, the newly assembled –yet immature-particle has to pass through and pinch off the cellular membrane. Finally, the virion must undergo maturation to be fully infectious; this exquisite process being finely regulated. Strikingly, most of these functions are carried out by a single molecule: Gag. Despite the lack of sequence similarities among Gag proteins from different genera of retroviruses, they all share functional domains that play key roles during assembly. Three of them have been hitherto well characterized: a membrane-binding domain (M), an interaction domain (I), and a late assembly (L) domain (Swanstrom and Wills 1997; Goff 2001; Demirov and Freed 2004).

M domains are bipartite motifs responsible for directing and binding Gag to the plasma membrane. In general, they include a myristate group covalently linked to the N-terminal Gag plus a group of basic residues located in the MA protein, both of which provide a marked affinity for the negatively charged cytosolic face of the lipid bilayer (Swanstrom and Wills 1997). Studies on HIV-1 have shown that the composition of the lipid bilayer plays an important role in membrane targeting, although the molecular mechanisms underlying this process are poorly understood (Ono, Ablan et al. 2004; Ono, Waheed et al. 2006). Blocking myristoylation of MLV Gag abrogates viral assembly (Rein, McClure et al. 1986) whereas in M-PMV viral particles can still assemble but are unable to reach the membrane for further release (Rhee and Hunter 1987). A “myristoyl switch” model, in which the myristate group displays either an exposed or a buried form to allow reversible binding of Gag to the plasma membrane has been proposed and further supported by structural biology studies (Paillart and Gottlinger 1999; Tang, Loeliger et al. 2004).

Viral assembly requires Gag multimerization to form the structural skeleton of the virion, and these tight interactions have been proposed to take place through I domains. They have been mapped to the NC protein in many retroviruses such as RSV, HIV, MLV and human foamy virus (HFV) (Swanstrom and Wills 1997; Bowzard, Bennett et al. 1998). Unlike L domains (see below), I domains lack conserved motifs that allow their identification. However, since I-mutants display lower densities they can be identified by mutational analysis followed by isopycnic gradient centrifugation. Although located in NC, zinc fingers are not related to I domain activity. Moreover, two independent studies on RSV using different approaches showed that addition or deletion of basic residues within NC can substitute or abrogate Gag-Gag interactions, respectively (Bowzard, Bennett et al. 1998; Lee and Linial 2004).

As mentioned above, RNA is a structural element of the virion. To be packaged, the viral RNA has to interact with Gag, where Cys-His (CH) boxes present in NC, as well as basic residues located within NC and MA likely play different roles in RNA binding (Lee and Linial 2004; Muriaux, Costes et al. 2004; Ott, Coren et al. 2005). Viral RNA is recognized for packaging through the  $\Psi$  sequence located near the 5' end of the RNA molecule (Goff 2001; Muriaux, Mirro et al. 2001). Deletion of  $\Psi$  leads to the

incorporation of cellular RNA, predominantly ribosomal RNA (Muriaux, Mirro et al. 2001; Muriaux, Mirro et al. 2002). Another RNA species incorporated within the virion is tRNA, which will serve to prime reverse transcription.

### **Viral budding**

Retroviruses are enveloped viruses. The viral envelope is acquired from the plasma membrane during budding in a process that resembles membrane fission during cytokinesis (Carlton and Martin-Serrano 2007). Late domains are short, linear modular motifs that are required for virus-cell separation (Swanstrom and Wills 1997; Demirov and Freed 2004). Typically, L domain mutants are unable to pinch off the cell, and remain tethered to the plasma membrane (Swanstrom and Wills 1997). L domains have been identified not only in retroviruses but also in non-related viruses such as Ebola virus and vesicular stomatitis virus (Demirov and Freed 2004). Three L domain consensus sequences have been identified so far: P(T/S)AP, PPxY, and YPxL. There is no preferential use of specific late domains by different genera, as HIV-1 (Lentivirus), M-PMV (Betaretrovirus), and HTLV-1 (Deltaretrovirus) all possess a P(T/S)AP motif. Moreover, in several cases there are two tandem L domains separated by a couple of amino acid residues within the same Gag (i.e., HTLV-1, M-PMV) (Gottwein, Bodem et al. 2003; Heidecker, Lloyd et al. 2004). Electron microscopy studies using single and double M-PMV L-mutants showed that L domains are not redundant, but play different and cooperative roles during viral release (Gottwein, Bodem et al. 2003). The location of the L domain is variable: they can lie within the MA (HTLV-1), between the MA and CA (RSV, M-PMV, and MLV), or within the short peptides that lie at the C-terminus of NC (HIV-1, and EIAV) (Demirov and Freed 2004). This "geographical" diversity displayed by L domains allowed Lesley Parent and co-workers to predict and show that L domains could act in a position-independent fashion, suggestive of a docking site function for host proteins (see below). They also showed the remarkable feature that L domains can be functionally exchanged between distantly related viruses like RSV and HIV, or RSV and EIAV (Parent, Bennett et al. 1995). However, some L domains display functional context-dependence when inserted in different viruses (Demirov and Freed 2004).

Interestingly, ubiquitination of Gag proteins is essential for retroviral budding since ubiquitin-depleted cells exhibited accumulation of RSV viral particles at the plasma membrane (Patnaik, Chau et al. 2000). Ubiquitin (Ub) is a 76 residue-long protein that can be covalently linked to the side chain of lysine residues in a three-step reaction mediated by the ubiquitin-activating enzymes E1, E2, and E3. The fate of the tagged protein depends on the number of Ub molecules added to it: while polyubiquitination marks a protein for proteasomal degradation, monoubiquitination of a membrane cargo protein promotes receptor-mediated endocytosis into endosomes (Lodish, Berk et al. 2003). Because retroviral budding has a topology that resembles that of vesicles budding into the interior of endosomes, Gag ubiquitination may serve as a mechanism that allows retroviruses to recruit host proteins used in endosomal budding for their own advantage. In agreement with this hypothesis, it has been shown that both processes require a common set of proteins, and moreover, each late domain motif binds to a distinct protein that recruits endosomal sorting complexes required for transport (ESCRT): P(T/S)AP binds to Tsg101 (Tumour susceptibility gene 101) (Garrus, von Schwedler et al. 2001; VerPlank, Bouamr et al. 2001), PPxY binds a Nedd4-related E3 ubiquitin ligase (Neuronal precursor cell-expressed developmentally downregulated 4) (Kikonyogo, Bouamr et al. 2001; Yasuda, Hunter et al. 2002), and the YPxL motif interacts with Alix (ALG-2 interacting protein X) (Martin-Serrano, Yarovoy et al. 2003; Strack, Calistri et al. 2003). The access of a common multivesicular endosomal pathway is supported by the observation that a dominant negative VPS4 (required at the last steps of this pathway) inhibits viral budding regardless of the L domain used (Martin-Serrano, Zang et al. 2003).

The viral envelope derives from cellular membranes and the presence of Env glycoproteins is crucial to form an infectious particle. For a type-C virus like HIV-1, lipid rafts act as a platform for assembly and budding (Ono and Freed 2001; Bukrinskaya 2004), and this has also been described for other enveloped viruses (Bukrinskaya 2004). Since type-B/D retroviruses assemble in the cytoplasm, they likely obtain their envelopes in a different way, as has been shown for M-PMV, where its Gag protein interacts with Env in the pericentriolar region of the cell (Sfakianos and Hunter 2003). Foamy viruses also assemble in the pericentriolar region. However, Gag-Env

interactions have been observed at the *trans*-Golgi network, suggesting that this is the intracellular compartment where envelopment takes place (Yu, Eastman et al. 2006).

## **Maturation**

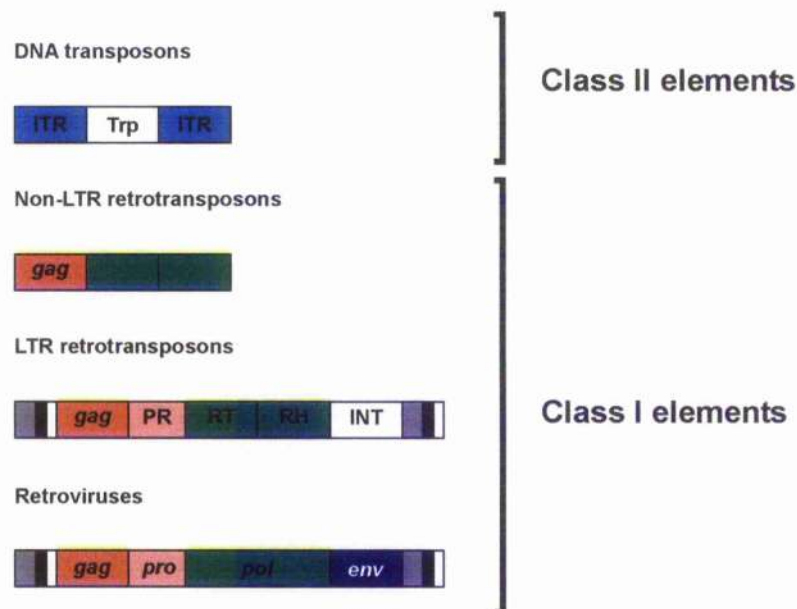
Another critical late event in the retroviral cycle is viral maturation, a step required for further infectivity of progeny virions (Figure 6, step 8). Although immature particles can be competent for budding, a cleaved Gag is necessary to initiate reverse transcription in a subsequent infection (Goff 2001). The central player of this process is the viral protease, an aspartyl protease that functions as a dimer (Goff 2001; Bukrinskaya 2004). Activation of the protease, as well as the sites and sequential order of cleavages is strictly regulated (Bukrinskaya 2004). This proteolytic processing is responsible for the morphological changes observed between immature and mature virions by electron microscopy. Moreover, cleavage of the TM glycoprotein by the viral protease has been shown to enhance the fusion activity of this protein in MuLVs (Goff 2001) and M-PMV (Brody, Rhee et al. 1994).

## ***Endogenous retroviruses***

### **Biology of endogenous retroviruses**

Endogenous retroviruses (ERVs) can originate from germ line proviral insertions or retrotransposition. From the host's perspective, ERVs can be regarded as mobile sequences of DNA within the genome, or, in other words, transposable elements (TEs). TEs are sequences of DNA that move around cellular genomes, contributing to genetic diversity in different organisms (Biemont and Vieira 2006). They are divided in two classes (Figure 9): class I, also regarded as retrotransposons, which display an RNA intermediate, and class II or DNA transposons, which mobilize through a DNA intermediate. The latter include the ubiquitously distributed miniature inverted-repeat transposable elements (MITEs). Retrotransposons on the other hand, can be further subclassified in LTR and non-LTR retrotransposons depending on the presence or absence of long terminal repeats (Dewannieux and Heidmann 2005). Endogenous retroviruses fall into the former category together with members of the *Metaviridae* and

the *Pseudoviridae*. Among the non-LTR retroelements we can find the long and short interspersed elements (LINEs and SINEs, respectively) (Biemont and Vieira 2006).



**Figure 9. Nomenclature and structure of transposable elements (see text).**

ITR: Inverted terminal repeats; Trp: transposase; PR: protease; RT: reverse transcriptase; RH: ribonuclease H; INT: integrase. Adapted from Biemont and Vieira (2006).

Integration is an obligatory step in the life cycle of retroviruses, therefore the progeny of the infected cell inherits the proviral genome and can produce virus in the absence of further rounds of infection. When this process occurs in a germ cell, the provirus will be passed on to the progeny derived from it and the offspring of the infected individual will inherit an *endogenous* virus. Like any other gene, endogenous retroviruses can become fixed in a species as a whole if they succeed in the evolutionary game played by the strict rules of natural selection.

Since endogenous retroviruses are vertically transmitted they are subject to different selective pressures than their exogenous counterparts. While a provirus that severely affects its host will be condemned to extinction, a 'beneficial' endogenous virus will likely be positively selected. However, the relationship between an endogenous retrovirus and its host is not a black and white situation where good proviruses persist

and pathogenic ones become extinct. There are grey areas in which deleterious effects and benefits achieve a long-lasting balance. A further possible outcome for ERVs is the accumulation of irreversible mutations that affect the replication capacity of the provirus and lead to an unrecognizable sequence of junk DNA as a consequence of a long decay process (Gifford and Tristem 2003). Beneficial endogenous proviruses can confer resistance to exogenous retroviruses and the studies described in this thesis support this view. An example of this kind is the presence of a *gag* ORF encoded by the *Fv1* provirus in mice that will be described below (Best, Le Tissier et al. 1996). Moreover, there are examples of endogenous proviruses that have become “domesticated” to the extent of regulating placental morphogenesis in sheep and humans (Gifford and Tristem 2003; Dunlap, Palmarini et al. 2006), or the tissue-specific expression of amylase in primates, including humans (Samuelson, Phillips et al. 1996). In contrast with the examples described above, ERVs have also been associated with disease. Activation of endogenous retroviruses can lead to virally-induced tumours, as has been shown with certain inbred strains of mice that were developed and selected for having a high incidence of mammary tumours. For example, the proviral loci *Mtv1* and *Mtv2* have been implicated in the development of mammary adenocarcinoma of GR and C3H mice (Michalides, van Nie et al. 1981; Michalides, Verstraeten et al. 1985; Callahan and Smith 2000).

Endogenous retroviruses constitute a significant portion of our genome and have been found in every vertebrate species where they have been sought (Stoye 2001; Weiss 2006), even in extinct species such as the woolly mammoth (Gifford and Tristem 2003). ERVs were initially identified by means of Southern blot hybridization and later by specific polymerase chain reaction (PCR) assays. Nowadays the search for endogenous retroviruses has been greatly facilitated by the availability of complete genome sequence data combined with powerful computer-based programs. Proviral expression is controlled in an LTR-dependent fashion, but other factors such as its position within the chromosome, or DNA methylation are likely to play a role in this process (Boeke and Stoye 1997). With some exceptions, ERVs are usually replication-defective and non-pathogenic, even though they can retain a certain level of activity if their essential regulatory sequences are preserved and the proteins necessary for replication are provided in *trans*, either by other ERVs or by exogenous retroviruses (Boeke and Stoye

1997; Gifford and Tristem 2003). Moreover, if this complementation mechanism is followed by recombination, it can lead to full restoration of the replication ability of the provirus, as may have happened with the baboon endogenous retrovirus (BaEV), a Gammaretrovirus that bears a betaretroviral *env* gene (Gifford and Tristem 2003). Curiously, it seems that BaEV was subsequently transmitted to cats horizontally, and gave rise to RD114, an endogenous virus of cats (Weiss 2006).

Endogenous retroviruses are also used as markers to perform phylogenetic studies based on the ERVs distribution in modern hosts (Gifford and Tristem 2003; Coffin 2004; Weiss 2006). Because the chance for an independent integration event to occur at the exact same site within a genome is negligible (Coffin 2004), species who share an integration site must have a common ancestor (Boeke and Stoye 1997). Moreover, since the sequence of the LTRs is identical at the time of integration, the age of a provirus within a certain lineage can be calculated just by comparing the mutations accumulated between the two LTRs (Coffin 2004).

### **ERVs distribution**

Endogenous retroviruses related to most retroviral genera have been identified (Gifford and Tristem 2003). Remarkably, and with the controversial exception of spumaviruses, all but one (an endogenous Lentivirus) of the ERVs described so far derive from simple retroviruses (Weiss 2006; Katzourakis, Tristem et al. 2007), although this perception may change in the near future as more genomes of different animal species are sequenced. ERVs can be divided in two categories: *ancient* and *recent*, depending on whether integration took place before or after speciation, respectively (Coffin 2004). Therefore, while ancient ERVs can be found in all vertebrates, recent proviruses are only present in few species. Germline infection with subsequent generation of ERVs is an ongoing and dynamic process. At the present time, koalas are undergoing endogenization of the koala retrovirus (KoRV) (Tarlinton, Meers et al. 2006), and the sheep genome is seemingly being invaded by a Betaretrovirus highly related to JSRV (Arnaud, Caporale et al. 2007).



Alpharetrovirus-related ERVs have been found in the genome of birds. For example, the genome of the domestic chicken (*Gallus gallus*) exhibits various families of ERVs related to ALV (Boyce-Jacino, O'Donoghue et al. 1992). They can act as "helper viruses" of replication-defective exogenous ALVs by providing essential functions *in trans*. The ALVE family, closely related to exogenous ALV sequences, can originate RAV-0, a replication competent retrovirus (Vogt and Friis 1971). Betaretroviruses display a wide distribution of ERVs in mammals, some of which possess exogenous counterparts like JSRV (Hecht, Stedman et al. 1996). Examples of endogenous Betaretroviruses that lack exogenous counterparts are simian endogenous retrovirus (SERV) in baboons (van der Kuyl, Mang et al. 1997), and *Trichosurus vulpecula* endogenous retrovirus type D (T<sub>v</sub>ERV[D]) in possums (Baillie and Wilkins 2001). Within this category, human endogenous retrovirus K (HERV-K) is a particularly interesting example. Although thought to be replication-deficient, HERV-K may have replicated no longer than 1 million years ago (Hughes and Coffin 2004; Lee and Bieniasz 2007). Recently, two independent laboratories reconstructed HERV-K (Dewannieux, Harper et al. 2006; Lee and Bieniasz 2007) using *in silico* data to build a consensus sequence. Notably, one of these studies showed *in vitro* that human infectious retroviruses could arise from recombination events among different HERV-K loci (Dewannieux, Harper et al. 2006).

Gammaretrovirus-related ERVs have been thoroughly studied, in particular MLV-related ERVs in inbred mice (Boeke and Stoye 1997). As stated above, they were classified according to their host-range, and one singular class was that of xenotropic MLVs that cannot infect murine cells since they do not bear the appropriate receptor. But to become endogenous, these xenotropic MLVs must have once been able to find a cognate receptor. A post-endogenization mutation in the genes that encode either the receptor or the envelope is a possible explanation. RD114, a feline endogenous retrovirus, constitutes an interesting example of a "xenotropic" virus: RD114 is generally non-infectious in feline cells (Fischinger, Peebles et al. 1973; Livingston and Todaro 1973) and it was hence regarded as a xenotropic virus (Livingston and Todaro 1973). However, this view has proved wrong as cat cells possess a functional receptor for RD114 (Fischinger, Nomura et al. 1975), and it has been shown that RD114 can replicate in a feline embryonic glial cell line with high titres (Haapala, Robey et al.

1985). Subsequent studies indicated that non-permissive cat cells support one cycle of infection without further spread of the virus (Dunn, Yuan et al. 1993). Endogenous Gammaretroviruses have been also found in pigs (Patience, Switzer et al. 2001), a potential organ donor species for human xenotransplants. Because pig endogenous retroviruses (PERVs) can infect human cells *in vitro* (Patience, Takeuchi et al. 1997), there is a great concern about the pathogenic potential of such ERVs (Patience, Switzer et al. 2001; Gifford and Tristem 2003; Weiss 2006). Another important issue concerning ERVs and human therapies regards the use of retroviral vectors for gene delivery, because endogenous proviral genomes present in packaging cell lines can contaminate therapeutic vectors. Moreover, replication-competent viruses can arise as a product of recombination between host-derived ERVs and gene-delivery retroviral vectors.

### **Interactions between exogenous and endogenous retroviruses**

Coding regions derived from ERVs have been kept within our genomes for millions of years, therefore one would be tempted to think that, as a whole, they must provide (or have provided at one point during evolution) a beneficial effect to their host. This seems to be the case since ERVs have been conclusively associated with disease only in a few cases. Expression of endogenous retroviruses can modify the outcome of exogenous retroviral infections in either positive or detrimental ways. For instance, endogenous envelopes can block the receptors needed by exogenous retroviruses to enter the cell, a phenomenon regarded as receptor interference (depicted in step 1 of Figure 14). This is the case of *Fv4*, an endogenous provirus expressing a mutated *env* that provides resistance to Friend leukaemia virus (FV) in mice (Gardner, Rasheed et al. 1980; O'Brien, Berman et al. 1983). Similar examples have been described in chicken and cats (Boeke and Stoye 1997).

But receptor blockade is not the only mechanism by which endogenous retroviruses can protect the host against viral infection. Another retrovirus-derived gene product, *Fv1*, which is present in different allelic forms in Balb/c (*Fv1<sup>b</sup>*) and NIH/swiss (*FV1<sup>n</sup>*) mice, determines susceptibility to particular murine leukaemia viruses (Lilly 1970). While the so-called N-tropic viruses can infect NIH/swiss but not Balb/c mice, B-tropic viruses

display the exact opposite host range of infection. Crosses carrying the two alleles are resistant to both virus variants. However, there are MLV viruses referred to as NB-tropic, which are not restricted by either *Fv1* allele. Although the underpinning mechanisms involved in *Fv1*-mediated block are not yet understood, it is clear that the viral determinants for this restriction lie in CA, more precisely in residue 110 (DesGroseillers and Jolicoeur 1983; Ou, Boone et al. 1983; Kozak and Chakraborti 1996). Moreover, the blockade is dominant in cell fusion experiments, saturable, and takes place after reverse transcription and before integration (see Figure 14) (Boeke and Stoye 1997; Goff 2004). Isolation and cloning of *Fv1* (Best, Le Tissier et al. 1996) showed that its gene product is a Gag-like protein that shares similarities with other endogenous retroviruses such as HERV-L and MuERV-L (murine endogenous retrovirus-like gene) (Goff 2004).

Endogenous retroviruses can modulate the immune response to either related or unrelated viruses and other infectious agents such as bacteria (Bhadra, Lozano et al. 2006). As aforementioned, MMTV displays both endogenous and exogenous counterparts. Exogenous MMTV is transmitted through milk to newborn pups, where antigen-presenting cells (APC), like B-lymphocytes and dendritic cells are the primary target of infection (Held, Shakhov et al. 1993; Held, Waanders et al. 1993). Infected APCs express *sag* (superantigen), a viral encoded gene responsible for unleashing a T-cell response whose end result is the proliferation of MMTV susceptible cells, rendering circulating lymphocytes as reservoirs of infection (Held, Waanders et al. 1993; Waanders, Shakhov et al. 1993). By the time infected cells transmit MMTV to dividing mammary cells, Sag-reactive T-cells have undergone gradual deletion or anergy (Boeke and Stoye 1997; Bhadra, Lozano et al. 2006). Laboratory mice possess between 2 and 8 endogenous replication-defective MMTVs referred to as *Mtvs* (Kozak, Peters et al. 1987), most of which encode functional *sag* genes. Transgenic expression of MMTV *sag* prevents exogenous MMTV infection in mice (Golovkina, Chervonsky et al. 1992; Golovkina, Prescott et al. 1993; Boeke and Stoye 1997). However, the notion that endogenous MMTVs have beneficial effects (Boeke and Stoye 1997) has recently been challenged: Balb/*Mtv*-null mice (mice lacking *Mtv* genes) are resistant to mammary tumour development and leukaemia by two different MMTV strains. Moreover, these knock-out mice display an increased resistance to lethal doses of *Vibrio cholerae*

(Bhadra, Lozano et al. 2006). In chickens, expression of ERVs correlates with reduction in neutralizing antibodies titre upon challenge with ALV, likely as a result of immunotolerance induced by shared antigens between endogenous and exogenous viruses. On the other hand, birds expressing ERVs are less likely to be affected by wasting syndrome (Boeke and Stoye 1997), a major economic disease in the poultry industry associated with ALVs and REV (Rosenberg and Jolicoeur 1997), characterized by anemia, poor growth, and immunosuppression (Mussman and Twiehaus 1971).

Alternatively, ERVs constitute a genetic source for recombination with exogenous viruses. If cells expressing ERVs are infected by an exogenous retrovirus, RNA from the endogenous provirus could be co-packaged within the progeny virion, possibly leading to further recombination and generation of viruses with modified biological characteristics, as described above with regard to BaEV (Benveniste, Lieber et al. 1974; Benveniste and Todaro 1974). Besides, co-packaging and further recombination pose a practical threat with regard to the cell lines used to develop retroviral vectors.

### **Endogenous retroviruses and disease**

As mentioned above, ERVs generally do not cause disease (otherwise they would be counterselected during evolution). However, studies on inbred mouse strains selected for their high incidence of tumours have shown that ERVs expression can lead to disease even in the absence of exogenous viruses. This has been well documented in spontaneous lymphoma and mammary adenocarcinoma of AKR mice, which display a high incidence of thymic lymphoma likely resulting from viral insertional activation of proto-oncogenes. Remarkably, the oncogenic agent is not just an activated ERV, but rather recombinant viruses termed mink cell focus forming viruses (MCF). MCF viruses are the end product of a consistent series of recombination events involving at least three different ERVs (Stoye, Moroni et al. 1991). Moreover, the proviral loci *Mtv1* and *Mtv2* have been implicated in development of mammary tumours in the GR strain of mice in the absence of exogenous MMTV. Apparently, these proviruses release infectious virus when expressed in the lactating glands, causing mammary cancer in a similar fashion to their exogenous counterparts, as novel insertions near the same genes have been observed in both type of tumours (Boeke and Stoye 1997).

## Biological roles played by endogenous retroviruses

There is mounting evidence supporting the view that some ERVs have been maintained throughout evolution not just because they protect the host against exogenous viral infections, but also, because they provided a different kind of selective advantage. As aforementioned, these genetically domesticated endogenous retroviruses have been shown to participate in placental morphogenesis of humans and sheep, as well as in tissue-specific expression of amylase in the salivary gland of certain primates (Ting, Rosenberg et al. 1992).

Fusion of the cytotrophoblast cells gives rise to the syncytiotrophoblast layer of the human placenta (Midgley, Pierce et al. 1963). This layer is located between the foetal and maternal compartments, and is of literally vital importance for the development of the foetus (Aplin 1991). Curiously, the highly fusogenic envelope glycoprotein derived from IHERV-W, termed syncytin, is highly expressed in the placenta (Mi, Lee et al. 2000). Moreover, blockade of *envW* expression in primary cultures of human villous cytotrophoblast results in decrease of cell fusion and differentiation (de Parseval and Heidmann 2005) suggesting that these molecules are involved in placental development. Unfortunately, due to the impossibility of experimentally testing this hypothesis *in vivo*, the actual role of syncytin in gestation remains obscure. However, recent studies in sheep underscore the importance of endogenous Envs in placental morphogenesis (see below), but these results should be cautiously interpreted due to cross-species physiological differences. Syncytin has also been suggested to play some role on maternal immunotolerance to the foetus, but this remains speculative (Knerr, Huppertz et al. 2004).

In humans, the amylase gene family comprises a cluster of five genes located on chromosome 1. Expression of two of these genes takes place in the pancreas (pancreatic amylase), while the other three genes are expressed in the salivary gland (salivary amylase). All five human amylase genes are associated with insertions of two TEs: a  $\gamma$ -actin pseudogene and an endogenous virus. Studies using transgenic mice have shown that the amylase-associated provirus bears transcriptional control elements specifically active in the parotid gland, suggesting that salivary specificity is due to the retroviral

insertion. However, further studies with New and Old World monkeys (which lack the provirus) disputed this result since these species display a high level of amylase in the saliva (Ting, Rosenberg et al. 1992; Samuelson, Phillips et al. 1996).

## ***Jaagsiekte sheep retrovirus***

### **History**

Jaagsiekte sheep retrovirus is the causative agent of ovine pulmonary adenocarcinoma (OPA) (Palmarini, Sharp et al. 1999), a transmissible lung tumour of sheep that is widely distributed around the world (York and Querat 2003). Because of the development of pulmonary tumours, affected animals display signs of respiratory distress that include nasal discharge that becomes evident when the rear limbs are lifted (Garcia-Goti, Gonzalez et al. 2000; De las Heras, Gonzalez et al. 2003). This is why in the earliest descriptions of the disease (dating from the nineteenth century in South Africa) farmers coined the term *Jaagsiekte* to point out that their sheep appeared as if they had been chased (in Afrikaans *jag* means chase and *ziekte* sickness) (Tustin 1969). Transmissibility of the disease to in-contact sheep was shown in the late 1920s (DE Kock 1929), and suspicions of a retroviral aetiology arose in the seventies with the observation of retrovirus-like particles by EM from adenomatous lung samples (Perk, Michalides et al. 1974). Soon after that, OPA was experimentally transmitted to sheep inoculated with particles bearing reverse transcriptase activity (Martin, Scott et al. 1976; Verwoerd, Williamson et al. 1980), and the incubation period was observed to be inversely proportional to the RT activity of the inoculum (Verwoerd, Williamson et al. 1980). Despite these promising results, isolation of a virus associated with the disease proved to be a formidable challenge. The first problem encountered was (and still is) the impossibility of growing the virus in cell culture. Second, because the main source of virus was lung fluid from affected sheep, purification protocols were tedious and yielded a highly contaminated “purified” sample. Such contaminants made the development of useful reagents (i.e. antibodies) extremely difficult (York and Querat 2003). Adding more confusion to the picture was the fact that many affected animals were also infected with Maedi-Visna virus (MVV), a Lentivirus that also causes a respiratory disease. JSRV and MVV could finally be discriminated by using antisera

directed against the capsid proteins of MPMV and MMTV, which cross-react with JSRV but not with MVV (Sharp and Herring 1983). The presence of endogenous JSRVs (enJSRVs) in normal and tumour tissues made it difficult to associate JSRV with OPA as capsid proteins were immunodetected not only in affected tissues but also in normal ones (York and Querat 2003). Molecular studies on JSRV started in 1991 when York and coworkers identified its genome from virus present in lung fluid from an OPA-affected sheep in South Africa (York, Vigne et al. 1991). A year later these workers published the complete genome sequence of what we now know as JSRV-SA (South Africa), and also provided evidence for the presence of endogenous JSRVs (enJSRVs) in genomic DNA from normal sheep and goats (York, Vigne et al. 1992). The finding of enJSRVs in the sheep genome raised doubts about the exogenous virus as the cause of OPA. There was a possibility that oncogenesis was caused by activation of enJSRVs, or alternatively, tumorigenesis could lead to activation of enJSRVs. A hint on this issue was given by the development of reagents and techniques to discriminate between endogenous and exogenous JSRV (Bai, Zhu et al. 1996; Palmarini, Cousens et al. 1996) together with the finding that the exogenous form was detected consistently only in tumour tissue (Palmarini, Cousens et al. 1996). Conclusive evidence of JSRV as the etiological agent of OPA was provided when an infectious molecular clone termed JSRV<sub>21</sub> was developed, and virus produced *in vitro* was sufficient to reproduce the disease in inoculated animals (Palmarini, Sharp et al. 1999).

### **Ovine pulmonary adenocarcinoma (OPA)**

As aforementioned, animals affected with OPA display signs of respiratory distress due to the development of lung tumours. Although it is assumed that OPA tumours originate from type II pneumocytes and Clara cells (Nisbet, Mackay et al. 1971; Perk, Hod et al. 1971; Hod, Herz et al. 1977; Payne and Verwoerd 1984), the cell type from which the tumours derive has not been unequivocally determined. It is possible that a common precursor of type II pneumocytes and Clara cells is the origin of the neoplasm (Caporale, Centorame et al. 2005). OPA is endemic in many countries including the UK. However, only a few animals show clinical signs of disease during their commercial lifespan in naturally infected flocks. This is likely because infection of target cells is not a common event in infected animals (Caporale, Centorame et al.

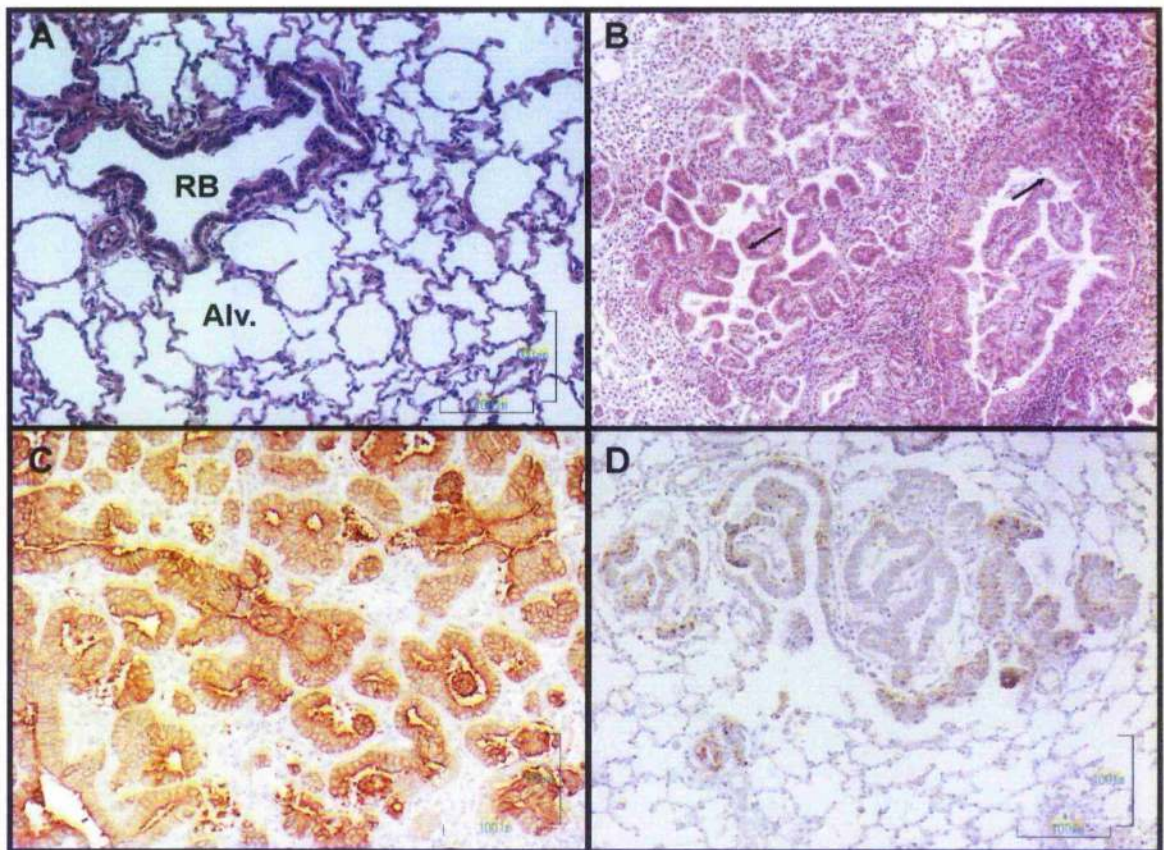
2005). Remarkably, OPA affected animals do not display circulating antibodies against JSRV (Ortin, Minguijon et al. 1998). Macroscopically, OPA lungs display neoplastic lesions that range from small nodules to massive tumours involving vast pulmonary areas (Justin 1969; Wandera 1971). Histologically, the disease is characterized by the presence of several papillary foci of epithelial proliferation in alveolar and bronchiolar regions (Figure 10 B). Usually, the architecture of the alveoli is not disrupted although columnar cells replace the normally flat pneumocytes (Wandera 1971; Gonzalez 1989). JSRV structural proteins can be readily detected in tumour cells by immunohistochemical techniques using antibodies raised against either the matrix, the capsid, or the surface antigen (Figure 10 C and D) (Palmarini, Dewar et al. 1995; Salvatori, Gonzalez et al. 2004; Caporale, Centorame et al. 2005).

OPA has been proposed as an animal model for the study of human bronchioloalveolar carcinoma (BAC) as both diseases share many clinical and histological features (Palmarini and Fan 2001; Mornex, Thivolet et al. 2003). Furthermore, and based on these similarities, a link between JSRV (or a JSRV-related Betaretrovirus/es) and human BAC has been sought using either PCR-based or immunohistochemical methods, without conclusive results (De las Heras, Barsky et al. 2000; Morozov, Lagaye et al. 2004).

### **JSRV genomic organization and molecular pathogenesis**

JSRV belongs to the Betaretrovirus genus and is phylogenetically related to M-PMV and MMTV. JSRV was originally classified as a type-B/D retrovirus, because the *gag*, *pro*, and *pol* genes were closely related to M-PMV, whereas the *env* gene was related to MMTV (York, Vigne et al. 1992; York and Querat 2003). A schematic diagram of the JSRV genetic structure is shown in Figure 11. In recent years, a third JSRV isolate, JSRV<sub>JS7</sub>, was cloned from a cell line developed from a tumour of an OPA-affected





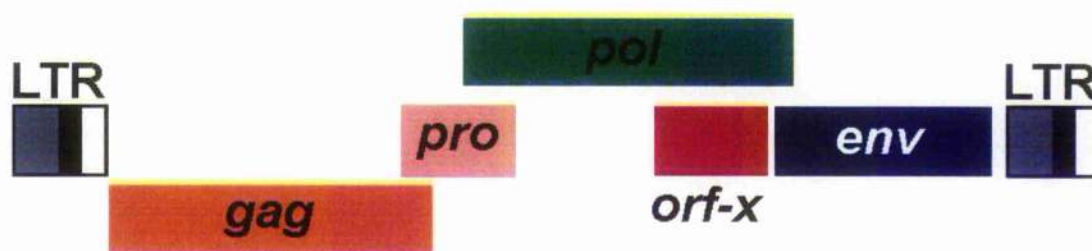
**Figure 10. Histological features of normal and OPA-affected lungs (see text).**

A: section of sheep lung stained with haematoxylin and eosin (H/E). RB: Respiratory bronchiole; Alv: alveoli. B: Section of an OPA-affected lung. Arrows indicate neoplastic alveoli. C: Immunolabeling of an OPA affected lung section with anti-SU. Tumour cells exhibit positive brown staining. Slides were counterstained with haematoxylin. D: Immunolabeling of an OPA affected lung section with anti-MA. Tumour cells exhibit positive brown staining. Slides were counterstained with haematoxylin. Pictures are courtesy of Marcelo de Las Heras.

sheep (DeMartini, Bishop et al. 2001). Of the three JSRV isolates studied so far (JSRV<sub>21</sub>, JSRV-SA and JSRV<sub>JS7</sub>), JSRV<sub>21</sub> (the first molecular clone) is probably the most thoroughly studied. However, it should be noted that the sequence homology among them is remarkably high, over 93% identical (Palmarini and Fan 2003). The genome of JSRV is 7.5 Kb long and bears the classical retroviral genes *gag*, *pro*, *pol* and *env* plus an additional open reading frame of unidentified function known as *orf-x*, which overlaps *pol* (Palmarini, Sharp et al. 1999). The U3 of JSRV<sub>21</sub> is 266 base pairs (bp) long, possesses a TATA box, putative enhancer motifs, and a polyA signal,



whereas the R and U5 regions are 13 bp and 115 bp long, respectively (Palmarini and Fan 2003). Whether *orf-x* represents an accessory gene has been the subject of debate, since its level of conservation in all three isolates contrasts with the fact that JSRV<sub>21</sub> *orf-x* mutants are not affected in their ability to infect and transform cells, as well as to induce tumours *in vivo* (Palmarini and Fan 2003; Cousens, Maeda et al. 2007). However, transcripts bearing an in-frame spliced *orf-x* have been detected in either transfected or infected cells, as well as tumour cells (Palmarini, Murgia et al. 2002).



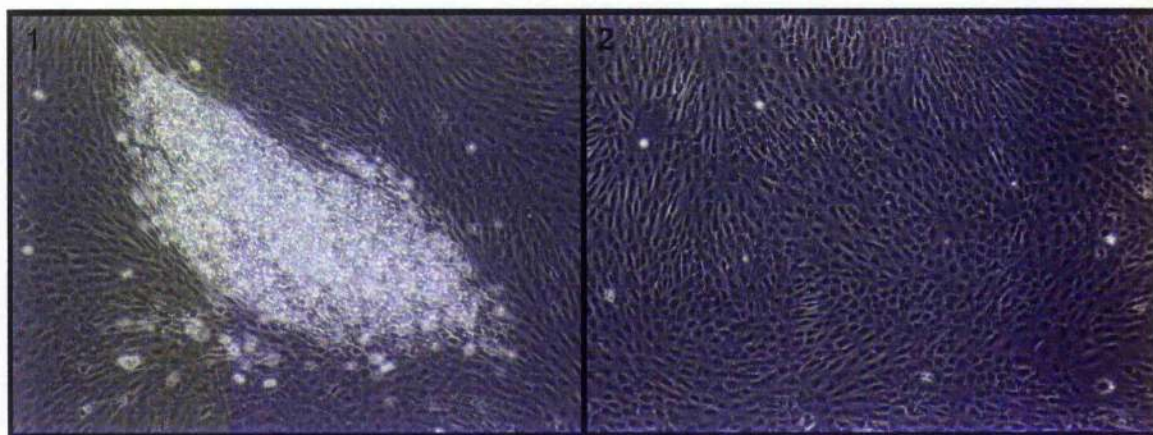
**Figure 11. Genetic structure of Jaagsiekte sheep retrovirus.**

JSRV is represented using standard retroviral notation. The names of the open reading frames and the regulatory sequences are indicated.

A detailed characterization of JSRV Gag has been lacking and will be provided in this thesis. As described for other Betaretroviruses, *pro* and *pol* are encoded in different ORFs (Palmarini, Sharp et al. 1999). The *pro* gene likely encodes a deoxyuridine triphosphatase (dUTPase) and a protease, and *pol* expresses the reverse transcriptase and the integrase. The envelope glycoproteins are derived from a single-spliced, 2.4 Kb-long transcript (Palmarini, Murgia et al. 2002). The JSRV envelope mediates viral entry into the target cells and constitutes the main determinant of viral transformation *in vitro* (Figure 12) and *in vivo* (Maeda, Palmarini et al. 2001; Caporale, Cousens et al. 2006). While the SU fragment is responsible for the interaction with Hyal2, the cellular receptor of JSRV (Rai, Duh et al. 2001), the TM subunit anchors the envelope glycoproteins to the lipid bilayer through its membrane-spanning domain. The cytoplasmic tail of the TM is the main determinant of the envelope oncogenicity apparently through the activation of the Ras/MEK/MAPK and the PI-3K/Akt-dependent

pathways (Palmarini, Maeda et al. 2001; Chow, Alberti et al. 2003; Maeda, Fu et al. 2005; Varela, Chow et al. 2006).

JSRV is able to enter and integrate within a variety of cell types. Proof of this is the detection of its genomic DNA in lymphoid tissues from experimentally infected animals (Palmarini, Holland et al. 1996) and sheep cell lines (Palmarini, Sharp et al. 1999). However, viral expression is limited *in vivo* to the transformed lung epithelium (Palmarini, Dewar et al. 1995). This selective tropism is likely due to a high tissue-specificity of the LTRs, which have been found to be active only in differentiated lung epithelial cell lines (Palmarini, Datta et al. 2000). These cell types express a transcription factor termed hepatocyte nuclear factor-3 (HNF-3) because it is also present in hepatocytes. NIH3T3 cells do not display LTR activity and do not express any isoform of HNF-3. However, they show a remarkable dose-dependent LTR activation when transiently expressing HNF-3, further supporting that view (Palmarini, Datta et al. 2000).



**Figure 12. Transformation of rodent fibroblasts by JSRV Env.**

1: Focus of transformed 208F cells transfected with plasmid DNA expressing JSRV Env. 2: Monolayer of mock-transfected cells. Pictures are courtesy of Mariana Varela.

Enzootic nasal tumour virus (ENTV) is another exogenous Betaretrovirus that causes a naturally occurring neoplasm in the respiratory tract of sheep and goats (De las Heras, Garcia de Jalon et al. 1991; De las Heras, Sharp et al. 1991; De las Heras, Garcia de Jalon et al. 1995). ENTV displays high sequence homology with JSRV, with more than

95% similarity at the amino acid level (Cousens, Minguíjon et al. 1999). Not surprisingly, the main differences observed between JSRV and ENTV lie within the U3 LTR and the 3' env region (Cousens, Minguíjon et al. 1999). Moreover, the envelope protein of ENTV induces transformation and can also use Hyal2 as a cellular receptor *in vitro* (Alberti, Murgia et al. 2002).

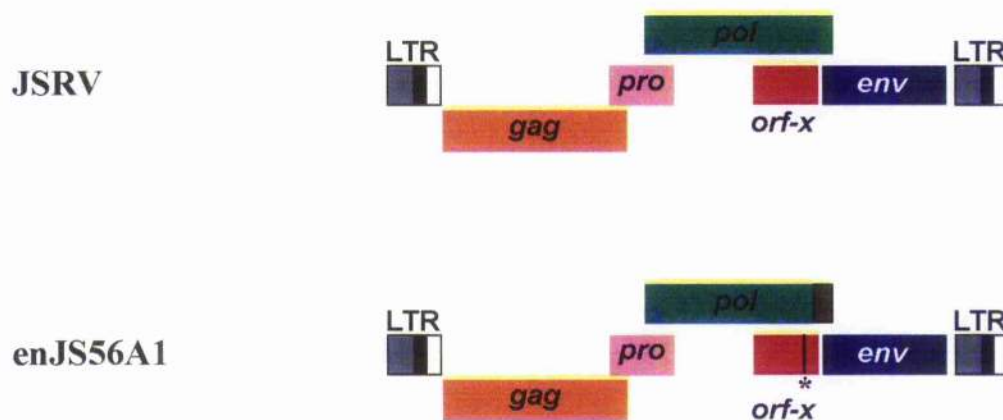
### Endogenous JSRVs

The existence of endogenous sequences in sheep and goats was realized as soon as the viral genome of JSRV was isolated (York, Vigne et al. 1992). Based on Southern blot analysis of sheep genomic DNA using probes derived from JSRV *gag*, *pol* and *env*, it was estimated that 15-20 copies of enJSRVs are present in the sheep genome (DeMartini, Carlson et al. 2003), although this number is likely to be larger (Arnaud, Caporale et al. 2007). Using probes derived from JSRV CA and SU, Hecht and coworkers studied the distribution of endogenous sequences not only within members of the order Artiodactyla but also other orders within *Mammalia* (Hecht, Stedman et al. 1996). They concluded that domestic sheep share several endogenous proviruses at similar integration sites regardless of the breed studied, and moreover, they displayed similarities in some proviral integration patterns with wild sheep. Because the probes used hybridized at highly stringent conditions with genomic DNA of other member of the genus *Capra*, endogenous retroviruses of sheep and goats seem to be closely related. However, they must have been amplified after goats and sheep diverged. Although this kind of work provides useful information to draw a general picture on the distribution of endogenous Betaretroviruses among mammals, they are just the starting point of a long series of studies since they pose new questions (i.e. are these ERVs still active? If they are, what tissues are they expressed in? What is the sequence of these ERVs? Do they provide an evolutionary trait? Are they linked with disease? Is there any of these ERVs that has not been completely endogenized yet?).

In a previous study, three intact endogenous Betaretroviruses highly related to JSRV have been cloned and sequenced from an OPA tumour DNA library (Palmarini, Hallwirth et al. 2000). These proviruses, termed enJS5F16, enJS56A1, and enJS9A1, display high sequence homology when compared with JSRV and ENTV, U3 being the



most divergent region. Besides that, only three other variable regions, two in Gag and one in Env, have been identified (VR1, VR2, and VR3). While enJS59A1 and enJS5F15 possess major defects in *pol*, enJS56A1 showed a -2 bp deletion at the 3' end of the IN domain. Therefore, enJS56A1 is the only one to maintain full (or nearly full) ORFs in all structural genes. The schematic genomic organization of enJS56A1 (in comparison with JSRV) is shown in Figure 13. The -2 bp deletion at the end of *pol* in enJS56A1 causes a frameshift that would render the predicted resulting protein 14 residues shorter and with 33 different amino acids at the carboxy terminus as a consequence of the ORF change. A similar plasmid to the infectious JSRV<sub>21</sub> clone was constructed, but bearing enJS56A1 instead. Surprisingly, expression of enJS56A1 did not result in viral particle release in the supernatant of transfected cells. By using JSRV/enJS56A1 chimeras, the defect in viral release was mapped to the first two thirds of Gag, where VR1 and VR2 lie (Palmarini, Hallwirth et al. 2000). Phylogenetic analysis of all the JSRV isolates, the three enJSRVs, and ENTV results in a three-branched tree. The endogenous loci and the exogenous JSRVs constitute two different branches, and the third one is formed by ENTV (Palmarini, Hallwirth et al. 2000).



**Figure 13. Genetic structure of JSRV and enJS56A1**

JSRV and enJS56A1 are represented using standard retroviral notation. The names of the open reading frames and the regulatory sequences are indicated. A premature stop codon in orf-x of enJS56A1 is indicated by a vertical bar underlined by an asterisk. A grey box at the end of *pol* in enJS56A1 indicates a different peptide sequence due to a frameshift.

While characterizing transcripts in the uterine epithelium, Thomas Spencer and coworkers found that enJSRVs were actively transcribed in the genital tract of the ewe (Spencer, Stagg et al. 1999). If compared with other tissues (i.e. lungs, kidney, bone marrow), the level of expression of enJSRVs along the genital tract is significantly notable. *In situ* hybridization and immunofluorescence studies have shown that high levels of enJSRVs expression were detected specifically in the endometrial lumen, the uterine glandular epithelium, the epithelia of the oviducts and the cervix (Palmarini, Gray et al. 2001). Moreover, the activity of the LTR of enJS59A1 displayed a 10-fold enhancement in the presence of progesterone. A further study in which foetal expression of endogenous proviruses was assessed showed that enJSRVs transcripts were detected in the cortico-medullary junctions of the thymus, the region where selection of T-cells seems to occur (Spencer, Mura et al. 2003). This observation suggests the induction of tolerance towards JSRV-related epitopes during development, and could explain the lack of humoral response observed in OPA affected animals (Ortin, Minguijon et al. 1998). On the other hand, expression of enJSRVs seems to block viral entry by receptor blockade since JSRV cannot enter a cell line derived from the endometrial luminal epithelium (oLE cells) that expresses high levels of enJSRVs. In contrast, viral entry is not affected in a cell line derived from the uterine stroma (oST) where expression of enJSRVs is not detected. Supporting this hypothesis, stable expression of the envelope protein of enJS56A1 in oST cells restored receptor blockade (Spencer, Mura et al. 2003).

Based on these data, one can speculate about the past existence of JSRV-related exogenous retrovirus(es) with genital tropism transmitted via coitus. In this scenario, the expression of an endogenous virus that blocked infection via receptor interference would have provided a great evolutionary advantage to the host and favoured the selection for exogenous viruses with a different tropism, for example for the respiratory tract through a genital-oral route of transmission.

Expression of endogenous retroviruses in the genital tract of the ewe has a crucial role in placental morphogenesis. As mentioned, it was recently shown that the envelope of enJSRVs regulate trophoblast growth and differentiation in the periimplantation conceptus *in vivo* (Dunlap, Palmarini et al. 2006). When the expression of enJSRVs *env*

was inhibited by intrauterine inoculation of morpholino antisense oligonucleotides on day 8 of pregnancy, retarded trophoctoderm outgrowth during conceptus elongation was observed, as well as inhibited differentiation of trophoblast giant binucleate cells and further pregnancy loss by day 20 (Dunlap, Palmarini et al. 2006). Although these results should not be extrapolated to the role of syncytin in human placental development, they may reflect a convergent role of ERVs in placentation among distantly-related species.

### ***Restriction factors and their role in intrinsic immunity against retroviruses***

Because viruses are obligate intracellular parasites, they must use the cellular machinery to replicate, sometimes with harmful consequences to the host. Consequently, multicellular organisms have developed a variety of defense mechanisms with different levels of complexity to avoid or clear viral infections. The fact that we study viruses stands by itself as proof of fitness and imperfection of such systems.

Besides possessing physical and chemical barriers such as the skin and secretions with antiviral activity, we are equipped with a highly sophisticated immune system that in most cases is able to recognize invading viruses and mount a strong and specific response against them. Using classical immunology terms, these responses can be classified as *innate* and *acquired*, depending on the cell types and cytokines involved. In general, mobilization of innate defense takes place within minutes to hours of infection, and requires sentinel and cytolytic cells (i.e. macrophages and natural killer cells), as well as the aforementioned cytokines and an array of serum proteins referred to as complement. Adaptive immune responses, which include custom-made activated lymphocytes plus the synthesis and release of epitope-specific antibodies, are obviously much slower, taking days to weeks to be effective (Flint, Enquist et al. 2004). In either case, they are both *responses*, implying that infection is already in progress. Indeed, even the shortest time frame required to mount an innate response may be enough to set an irreversible viral invasion.

There is a third type of immune defense that takes place at the level of the individual cell and is constitutively active against viral infection, sparing the cell the dangers of

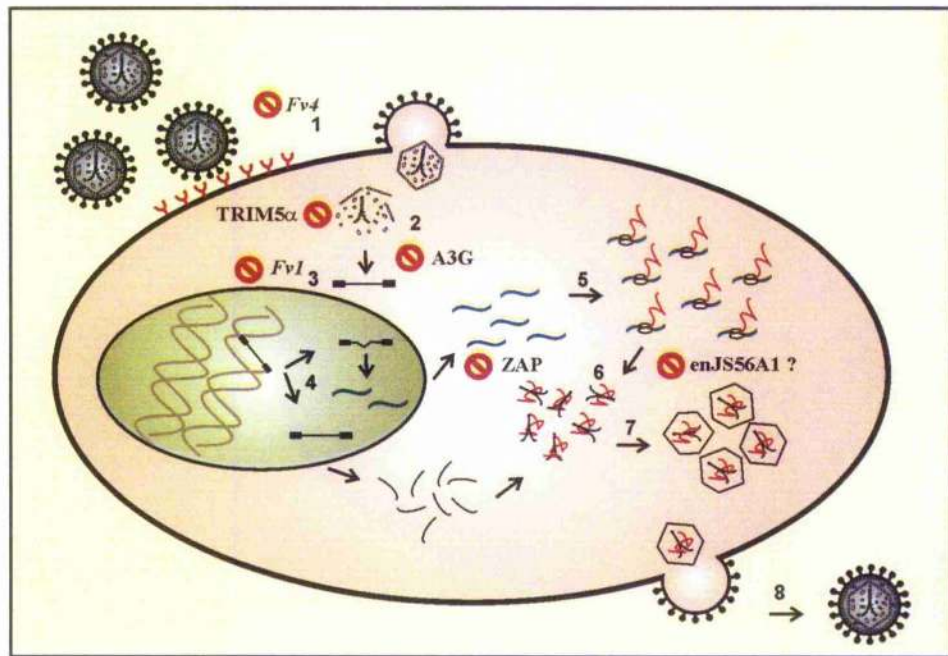
“waiting” for an immune response. It is termed *intrinsic* immunity (Bieniasz 2004), and comprises a collection of evolutionary selected genes that encode *restriction factors* capable of blocking viral replication (Goff 2004). The presence or absence of such factors in particular cells/tissues is one of the determinants of viral tropism. With regard to retroviruses, an increasing number of antiretroviral molecules have been characterized over the past few years. The list includes the previously described *Fv1* and *Fv4*; TRIM5 $\alpha$ , the APOBEC family of cytidine deaminases, and enJS56A1, that will be further described in detail (Gardner, Rasheed et al. 1980; O'Brien, Berman et al. 1983; Best, Le Tissier et al. 1996; Sheehy, Gaddis et al. 2002; Stremlau, Owens et al. 2004).

In theory, restriction factors can target any step of the retroviral cycle (Figure 14), although the majority of them have been found to act in early stages. From a cellular perspective and in particular with regard to retroviruses, an early blockade is crucial to avoid integration. Proviruses are extremely successful in persisting in the cell once integrated, since there is not an effective way to eliminate them. However, blockade of late steps is an appealing strategy to avoid spreading of infection, especially when infected cells are not recognized by the immune system, as seems to be the case in OPA. Understanding the molecular basis of intrinsic immunity is of utmost importance in order to develop effective antiretroviral therapies and to better characterize certain aspects of the retroviral replication cycle.

### **TRIM5 and Cyclophilin A**

*Fv1*, as described above, is a *gag*-like gene present in the genome of certain strains of mice whose expression blocks MLV after reverse transcription. The observation that distinct human cell lines displayed similar resistance to N-MLV infection, as the one described in murine cells bearing *Fv1*<sup>b</sup> (Towers, Bock et al. 2000; Besnier, Ylinen et al. 2003) unleashed the search for a new restriction factor. This new antiretroviral activity, termed Ref1, exhibited the very same determinant for susceptibility of Fv1 (DesGroseillers and Jolicoeur 1983; Ou, Boone et al. 1983; Kozak and Chakraborti 1996), located in residue 110 of the CA, although the block seemed to take place at an earlier step of the cycle (Figure 14), before reverse transcription. Moreover, Ref1 not





**Figure 14. Schematic representation of the retroviral cell cycle displaying the name and step blocked by different restriction factors (see text).**

only abrogated MuLV infection but also the distantly related equine infectious anemia virus (EIAV) (Towers, Collins et al. 2002) and was further observed in cells from dogs, hamsters, pigs, cows, African green monkeys and bats (Goff 2004). These findings prompted an experimental re-examination of non-human primates cells originally believed to be non-permissive for HIV-1 infection. Results obtained showed a similar effect: virus was restricted before reverse transcription in a dominant and saturable fashion, CA being the main determinant for virus susceptibility (Cowan, Hatzioannou et al. 2002; Hatzioannou, Cowan et al. 2003; Kootstra, Munk et al. 2003; Goff 2004). This restriction was referred to as Lv1, for Lentivirus restriction factor 1. The gene responsible for Lv1 activity was cloned using a cDNA library from rhesus macaque lung fibroblasts that were resistant to HIV-1 (Stremlau, Owens et al. 2004). The resulting gene encodes for TRIM5 $\alpha$ , a member of the TRIM family of proteins. When human cells express TRIM5 $\alpha$ , they acquire HIV-1 resistance and concomitantly, suppression of TRIM5 $\alpha$  expression in rhesus cells abrogated the block. Finally, although both Fv1 and TRIM5 $\alpha$  block MLV-N, and the viral determinant for these

blocks lies in the same CA residue, the two restriction factors act independently and compete for incoming virions (Passerini, Keckesova et al. 2006).

TRIM proteins possess an RBCC motif containing a RING domain plus a one or two B-boxes and a predicted coiled-coil region. The domains that follow the RBCC domain are specific for each TRIM protein, and in TRIM5 $\alpha$  comprises a SPRY domain that confers restriction specificity (Nisole, Stoye et al. 2005). It should be noted that TRIM5 $\alpha$  is not the only protein with antiretroviral activity. For example, MLV is restricted by human TRIM1, and the interferon-induced TRIM22 downregulates LTR-mediated transcription of HIV-1 (Nisole, Stoye et al. 2005).

Cyclophilin A (CypA) is a ubiquitous cytoplasmic protein that binds CA and is incorporated into HIV-1 virions (Goff 2001; Luban 2007). The presence of CypA is required in target but not in producer cells, since blocking CypA-CA interaction abrogates infection at the very same stage as TRIM5 $\alpha$  (Goff 2001; Sokolskaja and Luban 2006; Luban 2007). Therefore, it is possible that HIV-1 may have evolved means to bind CypA to protect itself from TRIM5 $\alpha$ . Cells from owl monkey, a new world primate, exhibit a potent anti-HIV-1 restriction activity that is surprisingly abrogated when shRNAs are directed against CypA. Moreover, expression of CypA does not restore viral restriction. This seemingly contradictory effect of CypA is explained by the fact that owl monkeys express a remarkable TRIM5-CypA fusion protein termed TRIMCyp (Sayah, Sokolskaja et al. 2004). TRIMCyp apparently arose through retrotransposition of CypA to the same position where the SPRY domain is usually found in TRIM5 $\alpha$  (Luban 2007).

## **APOBECs**

Cytidine deamination constitutes another antiretroviral strategy of intrinsic immunity. For many years it had been known that the HIV-1 virion infectivity factor (Vif) protein was essential to support productive viral infection in primary T-cell cultures or T-cell derived cell lines. In addition, the observations that Vif-deficient viruses could replicate efficiently in a number of cell lines termed 'permissive' but heterokaryons displayed a non-permissive phenotype, raised the hypothesis that Vif was necessary to abrogate a

host restriction factor (Bieniasz 2004; Goff 2004; Holmes, Malim et al. 2007). The protein responsible for such restriction was further identified as APOBEC3G (hA3G) using subtractive cDNA libraries of genes selectively expressed in non-permissive cells (Sheehy, Gaddis et al. 2002).

APOBECs comprise a family of polynucleotide cytidine deaminases sharing one or two deaminase motifs coordinated by a  $Zn^{2+}$  ion. The catalytic activity consists of hydrolytic deamination or 'editing' that converts cytidines to uridines (Holmes, Malim et al. 2007). During HIV-1 infection, viral reverse transcripts display a high rate of guanosine (G) to adenosine (A) mutations in the presence of hA3G, suggesting that the cytidines of the negative DNA strand are edited. Although hA3G shows the most potent antiretroviral activity, different members of the APOBEC family block wild type HIV-1 or the minus Vif version. Moreover, APOBECs from other animal species such as mouse, rat, and non-human primates can block HIV-1 replication, and conversely, other viruses can be targeted for APOBEC-mediated restriction: HIV-2, simian immunodeficiency virus (SIV) from macaques or African green monkey, EIAV, MLV, foamy viruses, and even hepatitis B virus (HBV) (Holmes, Malim et al. 2007). It has recently been shown that mouse APOBEC3 inhibits MMTV replication *in vivo* (Okeoma, Lovsin et al. 2007).

APOBEC3G is packaged into virions during viral assembly and hence acts in the early stages of the subsequent round of infection (Figure 14). To counteract this, HIV-1 evolved Vif, which acts as an adaptor protein between hA3G and an ubiquitin ligase complex, inducing APOBEC3G polyubiquitination and further proteasomal degradation (Yu, Yu et al. 2003).

There are currently two models of viral inhibition by APOBEC. The classical one in which APOBEC accounts for the viral hypermutation observed *in vivo* through cytidine deamination (Harris, Bishop et al. 2003; Mangeat, Turelli et al. 2003; Zhang, Yang et al. 2003). In this model, the resulting proviruses would be mutated to an extent of being unable to encode progeny virus. Alternatively, the U residues within the reverse transcripts could be recognized and removed by host-derived DNA repair enzymes, leading to DNA degradation. However, mutations introduced by APOBEC3G could eventually lead to an increase in viral fitness. A recently proposed editing-independent

model has emerged based on the observation that catalytically inactive hA3G proteins keep certain levels of antiviral activity (Holmes, Koning et al. 2007). Moreover, the accumulation of reverse transcripts is reduced in target cells in the presence of these deficient APOBECs. Supporting this model, recent work from Michael Malim's laboratory showed that chimeric APOBECs that retain their editing capacity are severely impaired for viral inhibition (Bishop, Holmes et al. 2006).

### **Zinc-finger antiviral protein (ZAP)**

All the restriction factors described so far in this chapter act in the early stages of the retroviral infection (i.e. before integration). Apart from enJS56A1, only one antiviral gene that acts late in the life cycle has hitherto been described. This gene encodes an antiviral protein named ZAP that bears four CCCH-type zinc fingers, an uncommon feature also present in some RNA binding proteins. Expression of ZAP results in selective loss of viral mRNA in the cytoplasm but not in the nucleus (Gao, Guo et al. 2002; Goff 2004), and the *cis*-acting sequence recognized by the antiviral protein has been mapped to the 3'LTR (Guo, Carroll et al. 2004). ZAP directly binds to target RNA through the CCCH-type motifs and promotes degradation of the target mRNA (Figure 14). Moreover, ZAP can bind the exosome, a conserved 3'-5' exoribonucleases complex that participates in gene expression regulation through degradation of mRNA in the cytoplasm. Specifically, ZAP binds hRrp46p, one of the exosome components, and the sequence responsible for this binding was mapped to ZAP residues 224-254 (Guo, Ma et al. 2007). Interestingly, ZAP also inhibits filoviruses (Muller, Moller et al. 2007) and alphaviruses (Bick, Carroll et al. 2003), although its antiretroviral activity is limited only to MLV. Based on these studies, a proposed model for viral inhibition suggests that viral RNA is bound by ZAP and further targeted to exosomal degradation (Goff 2004; Guo, Carroll et al. 2004; Guo, Ma et al. 2007).

### **enJS56A1**

enJS56A1 is a specific enJSRV locus that has already been described in this introductory chapter. enJS56A1 possesses intact ORFs for all the retroviral genes, with a minor defect at the end of *pol* that results in a shorter integrase protein (Palmarini,

Hallwirth et al. 2000). When first isolated, enJS56A1 was cloned into the same vector as the JSRV<sub>21</sub> proviral clone, resulting in pCMV<sub>2</sub>enJS56A1. This clone proved unable to release viral particles into the supernatant of transfected cells. The “defect” exhibited by enJS56A1 was overcome by replacing the first two thirds of enJS56A1 *gag* with the homologous sequence derived from JSRV (Palmarini, Hallwirth et al. 2000).

Manuela Mura, a former PhD student and postdoc in the Palmarini laboratory, carried out most of the work that resulted in the first description of the interference properties displayed by enJS56A1. She showed by western blot analysis that 293T cells transfected with pCMV<sub>2</sub>enJS56A1 not only expressed Gag proteins but also exhibited intracytoplasmic viral particles by electron microscopy, usually forming conglomerates in the perinuclear region (Mura 2004). Furthermore, when enJS56A1 was cotransfected with JSRV, the defective phenotype was dominant, as JSRV viral particles could not be detected in supernatants. In addition, enJS56A1 restriction was specific for JSRV as the former could not abrogate the release of neither M-PMV nor MLV. By constructing a series of enJS56A1/JSRV chimeras and mutants, the main determinant for enJS56A1/JSRV interference was mapped to residue 21 of the MA protein, where JSRV exhibits an Arginine (R) and enJS56A1 a Tryptophan (W). A JSRV-single mutant bearing a W in position 21 displays a dominant-negative effect upon the release of wild-type virus, mimicking the phenotype showed by enJS56A1. Moreover, substitution of W21 and C98 by R residues in enJS56A1 abrogates restriction and overcomes its budding defect. Interestingly, enJSRV's transcripts bearing W21 were amplified by RT-PCR on ovine endometrium, suggesting that enJS56A1-or enJSRVs carrying this critical residue- are expressed in the sheep genital tract (Mura 2004).

## ***Concluding remarks***

In summary, although a vast body of information has been generated since the first isolation of JSRV, several questions remain unanswered and many more arise. I would like to end this introductory chapter with a few examples: what cell type does JSRV first infect *in vivo*? Why is there such a long incubation period in the natural disease? Why are only a few animals affected within flocks in the absence of a detectable immune response? Indeed, although we assume that the immune system of sheep is tolerant to JSRV, we do not know how this is regulated. Moreover, we know the sequence of only three enJSRVs, probably less than ten per cent of those present within the sheep genome. Virus and host have been involved in an endless “arms race” for millions of years that is far from being over.

The core work of this thesis aims to understand the interplay between JSRV and enJS56A1 at the molecular level, and how the latter can avoid the spread of virus from infected cells. Understanding the mechanisms that underpin this block can provide further insights to developing novel antiretroviral strategies, as well as additional clues to enrich our knowledge on the selection forces that shape coevolution between retroviruses (either endogenous and exogenous) and their hosts.

## Chapter 2

### Materials and methods

#### *Plasmids*

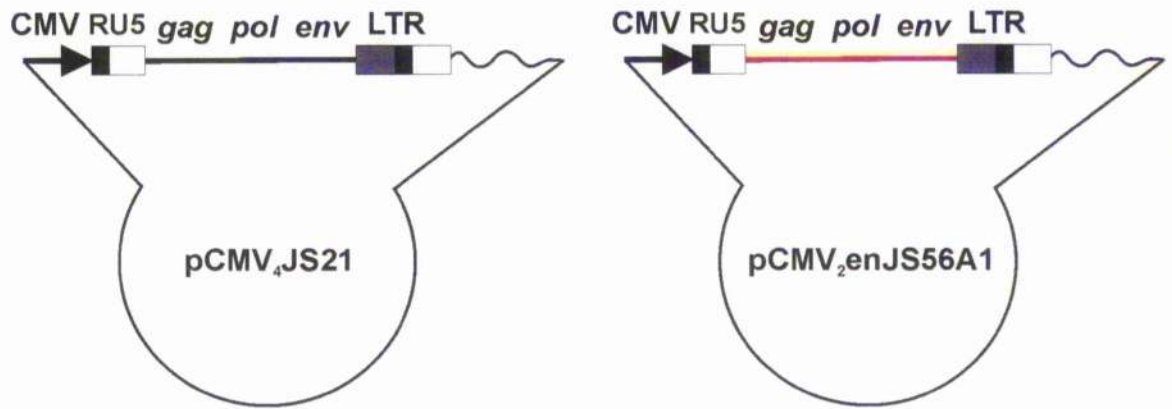
pCMV<sub>4</sub>JS21 and pCMV<sub>2</sub>en56A1 are schematically represented in Figure 15. They express the full length JSRV<sub>21</sub> clone and the enJS56A1 locus, respectively, and have been described elsewhere (Palmarini, Sharp et al. 1999; Palmarini, Hallwirth et al. 2000). pCMV<sub>4</sub>JSRV is an infectious molecular clone in which the U3 region of the 3' LTR has been replaced by the CMV promoter. pCMV<sub>4</sub>JS21 derives from pCMV<sub>2</sub>JS21, the first infectious molecular clone (Palmarini, Sharp et al. 1999). pCMV<sub>4</sub>JSRV lacks the multiple cloning site that is present at the 3' end of the LTR of pCMV<sub>2</sub>JS21, and possesses an XbaI and SalI restriction sites that have been added for cloning purposes by silent mutagenesis in Gag positions 88 and 142, respectively (Mura 2004). Infectious virus can be recovered from the supernatant of 293T cells transfected with either pCMV<sub>4</sub>JSRV or pCMV<sub>2</sub>JS21, and this method has become the standard for virus production for *in vivo* and *in vitro* studies (Palmarini, Sharp et al. 1999; Caporale, Centorame et al. 2005; Caporale, Cousens et al. 2006). JSRV and enJS56A1 Gag proteins were differentially tagged with the FLAG (DYKDDDDK) and HA (YPYDVPDYA) epitopes at the carboxy-terminal position of Gag and within the matrix domain before the variable region 1 (VR1) by overlapping PCR. pJSRVHA-MA, pJSRVFLAG-MA, penJS56A1HA-MA and penJS56A1FLAG-MA contain the HA or FLAG epitopes in the MA domain and were derived from pCMV<sub>4</sub>JS21 or from pCMV<sub>4</sub>en56A1<sub>X5</sub>. pCMV<sub>4</sub>en56A1<sub>X5</sub> was derived from p1-142xBe (Mura 2004) and possesses an XbaI and SalI restriction sites that have been added for cloning purposes by silent mutagenesis in Gag positions 88 and 142, respectively. pJSRVHA-C is derived from pCMV<sub>4</sub>JS21. In pJSRVHA-C the JSRV Gag is fused at its C-terminal with HA and is followed by the JSRV *env* gene and the 3'LTR of JSRV. penJS56A1HA-C was derived from pJSRVHA-C by replacing the JSRV *gag* with the enJS56A1 *gag* gene. pJSRVFLAG-C was derived from pCMV<sub>4</sub>JS21 by introducing the FLAG epitope at the carboxyterminal portion of Gag by overlapping PCR. penJS56A1FLAG-C contains the

enJS56A1 Gag tagged at the carboxy-terminus with the FLAG epitope and was derived from the plasmid pGePEX, a chimeric construct that possesses *gag* derived from enJS56A1 and *pol* and *env* from JSRV (Palmarini, Hallwirth et al. 2000). For cloning purposes, PCR was performed using the *Pfu* polymerase (Stratagene). Ligation reactions were done using the Rapid Ligation Kit (Roche). Plasmid DNA was produced in DH5 $\alpha$  and Top 10 strains of *E. coli* (Invitrogen), using the DNA Maxiprep kit from Invitrogen.

Mutants JSR21A, JSK19E, JSH20E, JSK19E/H20E, JSR21A, JSR21E, JSR21K, JSG22A, en56G2A, were obtained by site-directed mutagenesis using QuickChange (Stratagene) following the manufacturers' instructions. Primers for site-directed mutagenesis were designed with PrimerX, a web-based program to automate the design of mutagenic PCR primers (<http://bioinformatics.org/primerx/>). The nomenclature of the mutants indicates the virus from which they are derived (JS= JSRV; en56= enJS56A1), followed by a single letter indicating the amino acid mutated, a number representing its position in Gag and a letter indicating the amino acid residue of the resulting mutant. Multiple mutations are separated by a hyphen. Mutants JSR21W, JSR98C, en56W21R and en56W21R-C98R have been previously described (Mura 2004).

Single and double late domain (LD) mutants were obtained by site-directed mutagenesis and designated as follows: pJSALD201 carries a mutation in the proximal LD PSAP (Gag position 201-204) to AGAP, whereas in pJSALD207, the distal Late Domain PPAY is mutated to AAAY (Gag position 207-210). Double LD mutant pJSALD201-7 carries both mutations. Mutations were designed according to previous studies performed on M-PMV (Gottwein, Bodem et al. 2003).





**Figure 15.** Schematic representation of pCMV<sub>4</sub>JSRV21 and pCMV<sub>2</sub>enJS56A1

Adapted from Palmarini, Sharp et al. 1999.

Mutants penJS56A1 $\Delta$ CA-NC and penJS56A1 $\Delta$ NC were derived from pCMV<sub>2</sub>enJS56A1 by insertion of a stop codon in position 258 and 483 of Gag by site-directed mutagenesis, respectively. Deletion mutant penJS56A1 $\Delta$ NC2 was derived from pCMV<sub>2</sub>enJS56A1 by an in-frame deletion (performed by overlapping PCR) of Gag amino acid residues 490 to 548 in the nucleocapsid (NC) domain. enJS56A1 $\Delta$ NC2 has a functional protease. Mutant JSRV $\Delta$ pro is instead a full length JSRV molecular clone with a deletion in the *pro* gene resulting in a non functional protease and has been described before (Mura 2004). Truncated mutant penJS56A1 $\Delta$ MHR was obtained by overlapping PCR and derived from pCMV<sub>2</sub>en56A1 by deletion of Gag amino acid residues 403 to 422 encompassing the major homology region of the capsid (CA) domain.

Plasmids expressing either wild type or mutant Mason-Pfizer monkey virus were derived from pSARM4 (kindly provided by Eric Hunter). A myristoylation defective mutant carrying a G2A substitution (M-PMVG2A) and a type-C M-PMV carrying an R55W mutation in the cytoplasmic targeting/retention signal (CTRS) were obtained by site-directed mutagenesis and based in previous reports (Rhee and Hunter 1987; Rhee and Hunter 1990).

To express a HERV-K chimeric virus we modified pCMV<sub>2</sub>JSRV (Palmarini, Sharp et al. 1999) by replacing JSRV *gag* with HERV-K *gag* by PCR (see Figure 40A in Chapter 4). The resulting chimeric plasmid was named pCRU5-HERV-KGag. From pCRU5-HERV-KGag we derived an expression plasmid encoding a myristoylation defective chimeric virus, in which the second glycine was substituted by an alanine by site-directed mutagenesis. A further HERV-K/JSRV chimera bearing the N-terminal *gag* of HERV-K (from the initial methionine and up to 25 amino acid residues upstream of the beginning of capsid) followed by JSRV CA, NC and p4 was also constructed. We included the last 25 amino acid residues of p15 to facilitate assembly with wild type JSRV Gag (Ako-Adjei, Johnson et al. 2005). All constructs were sequenced to ensure the presence of the introduced mutations.

### ***Cell cultures, transfections, viral preparations and cell lysates***

293T, COS, and HeLa cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum, at 37°C, 5% CO<sub>2</sub>, and 95% humidity. For western blot analysis, virus was produced by transient transfection of 293T cells using the Calphos Mammalian Transfection kit (Clontech), according to the manufacturer's instructions. For confocal microscopy, cells were transfected with Lipofectamine (Invitrogen), following the manufacturer's instructions. Cell supernatants were collected at 24 and 48 hours post-transfection and viral particles were concentrated by ultracentrifugation (100000 x g for 1 hour at 4°C) on a 29% sucrose cushion. For analysis of intracellular proteins cells were lysed by standard techniques at 48 hours post-transfection. Briefly, cells were rinsed with cold phosphate buffer saline (PBS) and lysed on ice for 10 minutes with modified RIPA buffer (150mM Tris-HCl, pH7.4; 1% NP-40; 1mM EDTA; 150 mM NaCl, 1 µM PMSF; 1 mM NaF) supplemented with a cocktail of protease inhibitors (Complete, Roche) according to the manufacturer's recommendations. Cells were further snap frozen in liquid nitrogen, thawed on ice, and sonicated. Lysates were then centrifuged at 14000 x g for 20 minutes at 4°C. Supernatants were collected and protein concentration was determined by the Lowry method.

For reverse-phase high pressure liquid chromatography (RP-HPLC) separation, concentrated viral pellets from sixty 10-cm-plates were purified twice by isopycnic centrifugation on a 25 to 55% (wt/wt) linear sucrose gradient. Fractions with a buoyant density between 1.146 and 1.176 g/ml were pooled, concentrated by ultracentrifugation as above and resuspended in 8 M sequential-grade aqueous concentrate of high purity guanidine-HCl (Pierce), 2%  $\beta$ -mercaptoethanol. JSRV Gag proteins were then separated by RP-HPLC and analyzed by N-terminal protein sequencing at the NCI-SAIC AIDS Vaccine Program as previously described (Henderson, Bowers et al. 1992; Ott, Chertova et al. 1999).

### ***Western blot analysis***

SDS-PAGE and western blotting were performed on 25  $\mu$ l of concentrated viral particles and 50  $\mu$ g of protein extracts obtained from cell lysates. After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Hybond, Amersham), and blocked for 1 hour at RT with blocking buffer (5% skim milk in TBS/T [0.1% of Tween 20]). After blocking, membranes were rinsed with TBS/T three times for five minutes further incubated with the selected primary antibody. This step was performed either for one hour at RT or overnight at 4°C. JSRV Gag proteins were detected with rabbit polyclonal sera against JSRV MA, CA, and NC (Proteintech). Another antiserum raised against the first 100 N-terminal amino acid residues of the JSRV Gag was also used (Proteintech). HERV-K Gag proteins were detected with rabbit polyclonal antiserum raised against HERV-K CA (Proteintech). Gag proteins differentially tagged with either the HA or FLAG epitopes, were detected with mouse monoclonal anti-HA (Covance) or anti-FLAG (Sigma) antibodies. Primary antibodies were diluted as follows:

Anti-JSRV CA: 1:6000 in blocking buffer.

Anti-JSRV p23 (MA): 1:7500 in blocking buffer.

Anti-JSRV NC: 1:1000 in 5% BSA in TBS/T.

Anti-HERV-K Gag: 1:5000 in blocking buffer.

Anti-FLAG: 1:5000 in 5% BSA in TBS/T.

Anti-HA: 1:10000 in 5% BSA in TBS/T.

After incubation with primary antibody, membranes were rinsed three times in TBS/T for five minutes and further incubated with the appropriate peroxidase-conjugated secondary antibodies for an hour at RT. Secondary antibodies used were donkey anti-rabbit (F[ab']<sub>2</sub> fragment) from Amersham (GE), used at a working dilution of 1:40000 in blocking buffer; and goat anti-mouse (IgG [H+L]) from Jackson Research, diluted 1:10000 also in blocking buffer. Chemiluminescence was developed using ECL (Amersham, GE). If membranes were to be reblotted with a different primary antibody, they were first stripped with Restore (Pierce) for an hour at 37°C, and washed three times with TBS/T for 5 minutes. When necessary, signals were quantified by western blotting by scanning membranes and measuring chemifluorescence in a Molecular Dynamics Storm 840 imaging system using ImageQuant TL software (Molecular Dynamics). For quantification of Gag protein in viral pellets, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and chemiluminescence was developed using ECL plus (Amersham, GE).

### ***Co-immunoprecipitation assays***

Co-immunoprecipitation assays were performed in 293T cells transfected with the appropriate plasmids indicated in Chapter 3. 48 hours after transfection cells were lysed with RIPA buffer supplemented with a protease inhibitors cocktail (Complete; Roche) and 1 mM PMSF essentially as already described above. Lysates were sonicated and then centrifuged for 20 minutes at 14000 x g to remove insoluble material. For the evaluation of protein-protein interactions, 200 µg of whole cell extracts were rocked with 20 µl of protein A agarose beads (Santa Cruz Biotechnology) and primary antibody (mouse monoclonal anti-FLAG or anti-HA) at 4°C for 3 hours. After three washes with lysis buffer, beads were resuspended in SDS loading buffer, boiled for 5 minutes, subjected to SDS-PAGE and western blotting as described above.

## ***Confocal microscopy***

HeLa ( $6.5 \times 10^4$  cells/well) or COS ( $9-10 \times 10^4$  cells/well) cells were plated onto two well-chambered glass slides (Lab-Tek, Nalge Nunc International) and cultured as described above. Cells were transfected with Lipofectamine (Invitrogen) supplemented with Plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were kept with the transfection mixture (without serum or antibiotics) between 3 and 5 hours. After this time the transfection mixture was replaced by complete medium. Forty-eight hours after transfection, cells were washed with PBS and fixed on ice-cold methanol for 5 minutes at  $-20^{\circ}\text{C}$ . When cells were fixed with 3% paraformaldehyde (PFA), fixation was performed for 15 minutes at RT. After fixation, cells were processed as follows: cells fixed in PFA were quenched with 10 mM ammonium chloride and further permeabilized with PBS containing 0.1% Triton X-100 (10 minutes at RT). Quenching was not performed when cells were fixed with cold methanol. After permeabilization, slides were blocked twice for five minutes, firstly with PBS containing 0.4% fish skin gelatine and 0.2% Tween 20, and secondly with PBS containing 2.5% normal goat serum and 0.2% Tween 20. The primary antibody was diluted in PBS containing 2.5% normal goat serum and 0.2% Tween 20 and incubated for 45 minutes at  $37^{\circ}\text{C}$ . Slides were further washed with PBS containing 0.2% Tween 20 and blocked a second time as described above. Fluorescently-labelled secondary antibodies (Alexa 488 and 594 [Molecular Probes, Invitrogen]) were diluted in PBS containing 2.5% normal goat serum and 0.2% Tween 20 and incubated for 30 minutes at  $37^{\circ}\text{C}$ . Slides were washed with PBS containing 0.2% Tween 20 and mounted with Vectashield (Vector Labs) mounting medium with DAPI. Slides were analyzed with a Leica TCS SP2 confocal microscope. To detect JSRV and enJS56A1 Gag we used a rabbit polyclonal anti-MA (Proteintech) preadsorbed with HeLa cell extracts to minimize background. Serum was pre-adsorbed as follows: four 10-cm plates cultured with HeLa cells were lysed as described above, and lysis buffer was added to reach a final volume of 8 ml. Anti-JSRV-MA (2 ml) was mixed with the lysate-containing solution, rocked at  $4^{\circ}\text{C}$  for 3 hours, and further centrifuged at 14000 g for 30 minutes at  $4^{\circ}\text{C}$ . The supernatant was collected and used as primary antibody. M-PMV Gag was detected with a rabbit polyclonal raised against M-PMV capsid (Proteintech) diluted 1:1000. Anti-M-PMV CA was used without prior preadsorption. To detect HA and FLAG tagged Gags, mouse

monoclonal anti-HA (Covance) and anti-FLAG (Sigma) were diluted 1:400 and 1:100, respectively.

### ***Recombinant expression and purification of JSRV matrix protein***

The coding sequence corresponding to the N-terminal 90 amino acids of JSRV Gag (sequence AF105220) was amplified by PCR and cloned into the pET24b vector (Novagen). The insert of the resulting plasmid (pET24bJSMA) was sequenced to rule out accidental mutations introduced by PCR. Because the aim of the cloning strategy was the expression of JSRV MA with no tags that could eventually alter the expression and/or the tertiary structure of the protein, the T7 tag included in the vector was disrupted by using NheI in the cloning procedure, and a stop codon was introduced at end of the JSRV MA sequence to avoid fusion with the His-tag present at the 3' end of the multiple cloning site. Protein expression and purification of BL21 (DE3) cells transformed with pET24bJSMA was performed as follows: single colonies were cultured overnight in 40 ml of M9 Minimal Medium (47,74 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ; 22,04 mM  $\text{KH}_2\text{PO}_4$ ; 8,55 mM NaCl; 18,69 mM  $\text{NH}_4\text{Cl}$ ; 0,2% Glucose; 2 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0,1 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ; 0,001% Thiamine, 0,001%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) supplemented with Kanamycin (30  $\mu\text{g}/\text{ml}$ ). The resulting culture was further inoculated into flasks containing 960 ml of the same medium. Optical density at 600 nm was measured at different time points and cells were induced with IPTG (at a final concentration of 1 mM) when they reached a value of 0.6. After IPTG induction, cells were cultured for another 3 hours, when they were pelleted and resuspended in 50 ml of 10 mM  $\text{NaH}_2\text{PO}_4$ , pH 6.8. Resuspended cultures were sonicated and further centrifuged at 12000 x g for 30 minutes at 4°C. The supernatant was dialysed against 10 mM  $\text{NaH}_2\text{PO}_4$ , 0.5 mM DTT, pH 6.8, using Spectra/Por 7 tubing with a molecular weight cut off (MWCO) of 3500. After dialysis, the pH of the solution containing JSRV MA was adjusted to 6.8 and loaded onto an ion exchange column containing 170 ml of SP Sepharose Fast Flow resin (Amersham Biosciences Corp., Piscataway, NJ) and equilibrated with 10 mM  $\text{NaH}_2\text{PO}_4$ , 0.5 mM DTT, pH 6.8. The column was washed with equilibration buffer after the solution containing JSRVMA was loaded. After this washing step, JSRVMA was eluted with 1L of a 0-1M NaCl gradient. Fractions with a

volume of 8 ml were collected and 500- $\mu$ l aliquots were taken for further analysis by SDS-PAGE. These aliquots were precipitated for 20 minutes at  $-20^{\circ}\text{C}$  with 100% Trichloroacetic acid at a final concentration of 10% (vol/vol). The precipitated protein was pelleted ( $10000 \times g$  for five minutes) and further resuspended in 15  $\mu$ L of 0.1 N NaOH. After this last step, samples of different fractions were analysed by SDS PAGE followed by Coomassie staining, and selected fractions were subject to further purification by High Pressure Liquid Chromatography (HPLC). Prior to HPLC, the protein mixture was dialyzed against 10 mM  $\text{KH}_2\text{PO}_4$ , 10 mM KCl, pH 6.5, 0.5 mM DTT, and concentrated in a stirred cell containing a YM3 membrane. HPLC was performed using a 250 x 4.6 mm x 10  $\mu$ m column (Luna, Phenomenex) linked to a Waters 600E multisolvent delivery system. JSRV MA was eluted through a standard gradient (5 to 95% Acetonitrile). The corresponding peak was collected and dialyzed against 50 mM KCl, 50 mM  $\text{KH}_2\text{PO}_4$ , 1 mM DTT, pH 6.2, and further concentrated with a stirred cell containing a YM3 membrane.

### ***Circular dichroism spectroscopy***

A 300  $\mu$ L-aliquot containing 15  $\mu$ M JSRV matrix protein in 50 mM  $\text{KH}_2\text{PO}_4$ , 50 mM KCl, 0.5 mM DTT was loaded into a 0.1 cm quartz cuvette. Circular dichroism (CD) spectra were collected from 250 to 190 nm using a JASCO J-710 spectropolarimeter. Spectra analysis was performed using the CDSSTR algorithm available at the DICHROWEB website (Lobley, Whitmore et al. 2002; Whitmore and Wallace 2004): (<http://www.cryst.bbk.ac.uk/cdweb/html/home.html>).

### ***Analytical ultracentrifugation***

Sedimentation equilibrium experiments were performed using a Beckman Optima XL-A analytical ultracentrifuge. Three different concentrations were evaluated at three different speeds. The absorbance values (280 nm) used were 0.1, 0.25, and 0.4. When equilibrium was attained, scans were taken after 24 hours of centrifugation at the following speed values: 24000, 30000, and 40000 RPM. Sedimentation equilibrium data were analysed using the Ultrascan 6.2 software package.

***NMR spectroscopy***

NMR spectra were acquired with a Varian INOVA spectrometer at 600 MHz. Samples containing JSRV MA labelled isotopically labelled with  $^{15}\text{N}$  were assayed at different concentrations of NaCl, DTT. Various pH values and temperatures were also assayed.



## Chapter 3

### Functional characterization JSRV and enJS56A1 Gag polyproteins.

#### *Summary*

enJS56A1 is an endogenous retrovirus that blocks JSRV at a late stage of the retroviral replication cycle (Mura 2004). The molecular mechanism underpinning JSRV late restriction (hereafter termed JLR) and its precise timing are currently unknown. This section will cover the experimental work performed to answer the following question: how does enJS56A1 block JSRV release? We hypothesized two possible scenarios: (i) direct interactions between endogenous and exogenous Gag proteins result in aberrant chimeric particles that are unable to exit the cell, or (ii) enJS56A1 Gag would sequester a cellular factor required for JSRV exit.

The experimental design applied will be illustrated in detail, as well as the results obtained and the way we interpreted them. A description of materials and methods has been included in Chapter 2. It should be noted that this work constitutes a fraction of a larger project carried out with Frederick Arnaud and Manuela Mura in the Viral Pathogenesis laboratory at the Institute of Comparative Medicine of the Glasgow University Veterinary School. Therefore, their data will be mentioned and discussed to add clarity to our current understanding of JLR. However, only the experiments that I performed will be presented here. Some of our combined results have been published and the original articles are attached to this thesis (Mura, Murcia et al. 2004; Arnaud, Murcia et al. 2007; Murcia, Arnaud et al. 2007).

#### *Introduction*

It is assumed that endogenous retroviruses (ERVs) are fixed in the genome of virtually all vertebrates because they have been found in every species where they have been sought (Boeke and Stoye 1997; Stoye 2001). ERVs are thought to arise as a

consequence of germ line infections of past exogenous retroviruses (horizontally transmitted), although they can also originate by retrotransposition. Throughout evolution, the great majority of ERVs have accumulated different kind of mutations (i.e. substitutions, deletions and/or insertions) that have altered their genomic structure. As a result, the great majority of ERVs are not infectious and non-pathogenic (Gifford and Tristem 2003; Coffin 2004). Indeed, the most probable fate of any given ERV is a long decay process that results in an unidentifiable source of “junk” DNA.

ERVs that provide benefits to their hosts can escape this fate, as positive selection could avoid extinction of the ERV lineage, or even further, reverse this process. This notion is supported by the observation of transcriptionally active endogenous retroviruses that have maintained intact open reading frames despite having integrated into the genome of the host several million years ago. Amongst the different biological functions that ERVs can exert, protection of the host against infection by exogenous retroviruses has been unambiguously documented. In the previous chapter, I described that some mouse and chicken ERVs can block viral entry of exogenous retroviruses by receptor interference (Boeke and Stoye 1997). Other retroviral restriction factors block a post-entry step. This is the case of *Fv1*, a Gag-like murine gene derived from an endogenous retrovirus that restricts infection in mice by the Friend strain of murine leukaemia virus. *Fv1* acts after reverse transcription and before integration of the proviral genome (Best, Le Tissier et al. 1996; Boeke and Stoye 1997).

Ovine Betaretroviruses comprise both exogenous and endogenous members. The former include the exogenous Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumour virus of sheep and goats (ENTV) (Cousens, Minguilón et al. 1999; Palmarini, Sharp et al. 1999). Within the sheep genome there are approximately 30 endogenous retroviruses highly related to JSRV, hereafter referred to as enJSRVs (Carlson, Lyon et al. 2003; DeMartini, Carlson et al. 2003; Palmarini, Mura et al. 2004; Arnaud, Caporale et al. 2007). enJSRVs have not been associated with disease and are highly expressed in the genital tract of the ewe (Spencer, Stagg et al. 1999; Palmarini, Hallwirth et al. 2000), where they have been shown to play a crucial role in placental development as described in the introductory chapter (Dunlap, Palmarini et al. 2006). Our subject of

study is a unique restriction mechanism displayed by an endogenous Betaretrovirus of sheep that takes place at a late step of the retroviral replication cycle (Mura 2004).

To date, three enJSRV proviruses have been cloned and characterized: enJS56A1, enJS5F16, and enJS59A1 (Palmarini, Hallwirth et al. 2000). One particular locus, termed enJS56A1, possesses intact open reading frames in all its genes although a frameshift in the last portion of *pol* likely results in a non-functional viral integrase (Palmarini, Hallwirth et al. 2000). Transfection of cells with an expression plasmid for JSRV results in the release of abundant infectious virus in the culture medium (Palmarini, Sharp et al. 1999). In contrast, when a similar construct expressing enJS56A1 is transfected into cells, no virus can be detected in the supernatant although cell lysates exhibit conspicuous amounts of Gag by western blot and intracytoplasmic viral particles are observed by electron microscopy (Palmarini, Hallwirth et al. 2000; Mura 2004). Remarkably, the defect displayed by enJS56A1 cannot be rescued by complementation with JSRV. On the contrary, coexpression of these two viruses results in a defective transdominant phenotype as JSRV can no longer be detected in the medium of transfected cells (Mura 2004). Because our experimental setting is based on the transfection of cells with virus-expressing constructs, early events of the replication cycle are by-passed, limiting the time frame of JLR to the late steps of the virus replication cycle.

The main determinant of JLR has been mapped to residue 21 of the enJS56A1 Gag polyprotein (Mura 2004). Gag is the structural polyprotein of the retroviral nucleocapsid core that plays a central role in retroviral assembly and budding (Swanstrom and Wills 1997). Interestingly, the residue displayed in position 21 of JSRV Gag is an arginine (R), which is highly conserved among different members of the genus Betaretrovirus. In enJS56A1, R21 is substituted by a tryptophan (W). There are other differences in the amino-terminal portion of Gag between enJS56A1 and JSRV. Amino acid residue cysteine 98 (instead of arginine) and valine 102 (instead of leucine) are responsible for further defects in viral release, although mutants carrying these mutations are not transdominant (Mura 2004). The molecular mechanism and the exact timing of JLR are not yet understood. Nevertheless, the aforementioned observation of viral particles by electron microscopy in cells expressing enJS56A1 (or co-expressing enJS56A1 and

JSRV) suggests that JLR is dependent on a defect in Gag trafficking, possibly occurring after assembly. Based on studies performed on Mason-Pfizer Monkey Virus it is hypothesized that Betaretroviruses assemble in the cytoplasm in a pericentrosomal region and further traffic to the plasma membrane by not completely characterized mechanisms involving the recycling endosomes and the viral envelope glycoprotein (Sfakianos and Hunter 2003; Sfakianos, LaCasse et al. 2003).

In other retroviruses, a variety of determinants of Gag assembly and trafficking have been characterized to date. They include the membrane binding (M), interaction (I), and late (L) domains (Swanstrom and Wills 1997; Bowzard, Bennett et al. 1998; Demirov and Freed 2004). Whether these domains play any role in JLR has not been established yet. Most retroviruses have a bipartite M domain composed of a myristate linked to the N-terminal Gag, and a stretch of basic amino acid residues located in the N-terminal matrix (MA) domain (Swanstrom and Wills 1997). Mutation of the M-domain alters the ability of retroviral Gag to reach the cell membrane (Zhou, Parent et al. 1994; Parent, Wilson et al. 1996; Ono and Freed 1999), sometimes resulting in further abrogation of particle formation (Rein, McClure et al. 1986). JSRV mutants that possess a myristoylation defective Gag cannot release viral particles from transfected cells, nor can they interfere wild-type virus (Mura 2004). I domains have been mapped to NC and mediate Gag-Gag interactions during retroviral assembly. Virions harbouring mutated I domains display lower density when compared with their wild type counterparts (Swanstrom and Wills 1997; Bowzard, Bennett et al. 1998; Lee and Linial 2004). L domains are short amino acid sequences required during viral exit for virus-cell separation (Freed 2002; Demirov and Freed 2004). Typical L domain mutants are unable to pinch off the cell and hence remain tethered to the plasma membrane (Freed 2002).

To elucidate the molecular basis of JLR, we functionally characterized JSRV and enJS56A1 Gag polyproteins. We identified putative JSRV M and L domains and determined their lack of involvement in JLR. We show that truncation of enJS56A1 Gag polyproteins, or deletion of the major homology region (MHR) of enJS56A1 abrogates its ability to block JSRV. Furthermore, we determined the intracellular distribution of wild type JSRV, enJS56A1, and a variety of mutants by confocal

microscopy. We also provide evidence that JSRV and enJS56A1 co-localize and associate *in trans*. These results suggest that enJS56A1 blocks JSRV most likely by forming chimeric viral particles (or Gag multimers) that are unable to exit from the cell. Complementary studies performed simultaneously by Frederick Arnaud in our laboratory showed that the dominant negative phenotype displayed by enJS56A1 depends on its ability to misplace JSRV Gag localization. In a normal viral cycle, JSRV Gag concentrates in the pericentrosomal area unless enJS56A1 is co-expressed in the same cell, and the main determinant for such localization is also arginine 21 of Gag. Altogether, our results argue that enJS56A1 Gag blocks JSRV *in trans* by hampering the progression of the latter to the centrosome.

## Results

### Intracellular distribution of JSRV and enJS56A1 by confocal microscopy

Our initial experiments were aimed to determine whether enJS56A1 and JSRV exhibited differential intracellular distribution. JSRV particles are released in the supernatant of transfected cells while enJS56A1 particles remain within the cell. Thus it was reasonable to expect that cells expressing JSRV, enJS56A1, and selected mutants displayed distinct phenotypes, possibly allowing us to differentiate between an “endogenous” and an “exogenous” staining pattern by confocal microscopy. We therefore set up an immunofluorescence protocol to study virus-expressing cells by confocal microscopy (Figure 16) using a rabbit polyclonal antibody against the JSRV MA. It should be noted that this antiserum does not discriminate between JSRV and enJS56A1 Gag proteins.



**Figure 16. Confocal microscopy of HeLa cells expressing JSRV, enJS56A1, and mock-transfected.**

Gag staining is in green, nuclei are stained with DAPI (blue) or indicated with an N. Bar is approximately 10  $\mu\text{m}$ .

Cells displaying positive staining were classified into three different basic phenotypes: *diffuse*, *dispersed* and *concentrated* (Figure 17). Cells that exhibited a *diffuse* phenotype displayed uniform cytoplasmic staining usually more intense in the perinuclear region. Instead, a *dispersed* phenotype was characterized by the presence of fluorescent dots or

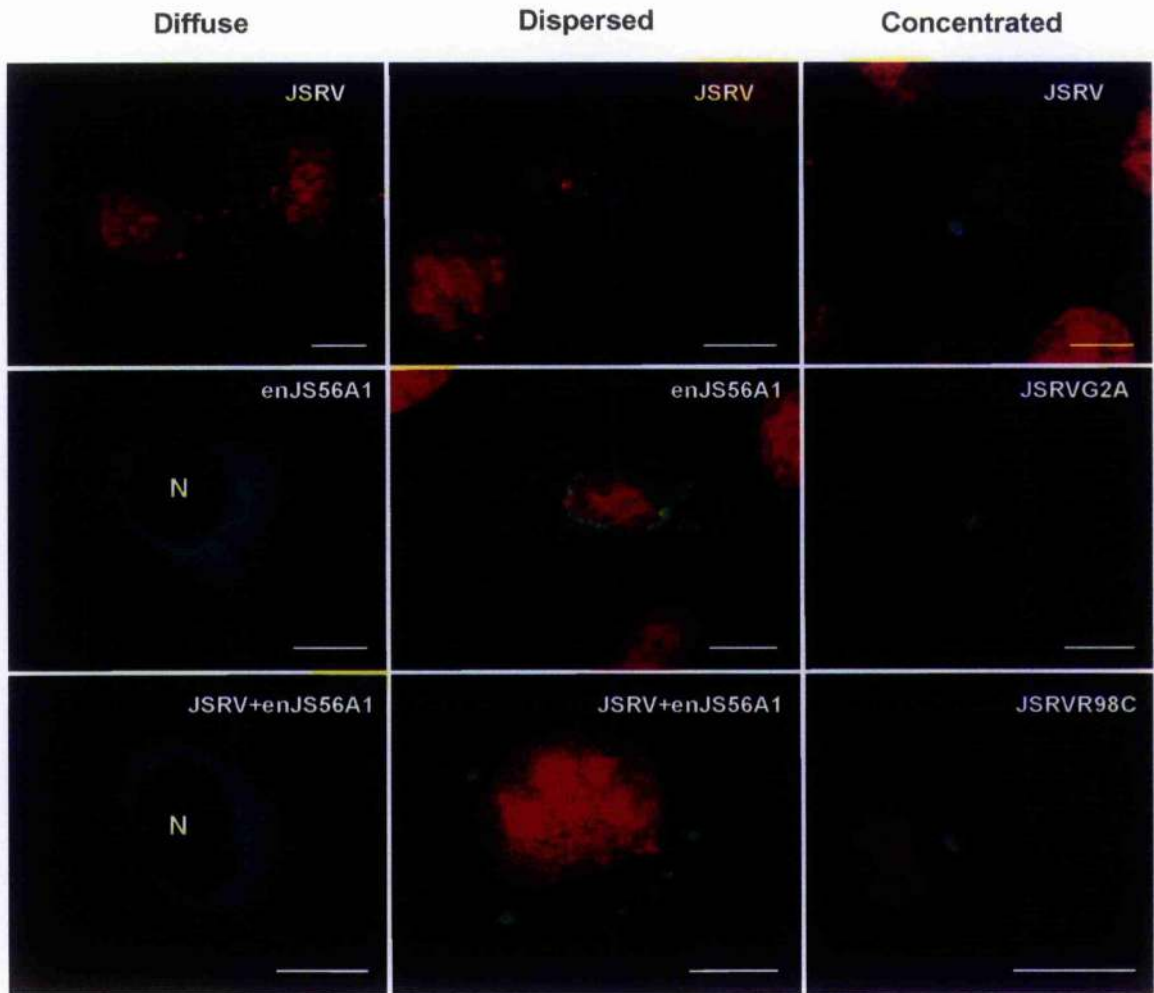
specks scattered in the cytoplasm. When cells exhibited a prominent rounded area of intense staining in the vicinity of the nucleus, they were classified as *concentrated*. The terms dispersed and concentrated are in agreement with previous descriptions of cells expressing Mason-Pfizer monkey virus, which is assembled in a pericentrosomal region of the cell (Sfakianos, LaCasse et al. 2003). Virus-expressing cells often displayed a mixed diffuse-dispersed phenotype, in which case they were classified as dispersed. Cells expressing JSRV, enJS56A1, and selected mutants were counted according to the aforementioned classification criterion. JSRV and enJS56A1 did not show significant differences in the distribution of the three phenotypes (Table 2), nor did the single mutant pJSR21W\*. Of note, cells expressing enJS56A1 always showed the highest number of positive cells and stronger fluorescent staining than JSRV. This was consistent with Gag accumulation of the former and viral release of the latter. Moreover, the fluorescent specks observed in enJS56A1-expressing cells were much bigger than the ones observed in cells expressing JSRV or any other mutant. Cells cotransfected with JSRV and enJS56A1 exhibited a high proportion of diffuse cells, in contrast with the ones expressing only enJS56A1. This was surprising, as we expected a similar phenotype to the one observed in enJS56A1-expressing cells due to the dominant-negative property of endogenous Gag (Mura 2004). Two of the single mutants assessed repeatedly showed the highest percentage of cells with a concentrated phenotype: the myristoylation-defective JSG2A, and JSR98C. Both mutants have been previously proved to be incompetent for viral exit but not transdominant over JSRV (Mura 2004).

Overall, these experiments gave us a general idea about the intracellular distribution of enJS56A1 and JSRV Gag in transfected cells. Because all the tested constructs displayed the three staining patterns that we originally defined, we could not determine a clear cut phenotype to objectively differentiate cells releasing virus versus non-releasing cells. Nevertheless, certain mutants (i.e. JSG2A, and JSR98C) showed a high proportion of cells with a concentrated phenotype when compared with the other constructs, likely reflecting a specific trafficking defect. In agreement with the results obtained by electron microscopy (Mura 2004), enJS56A1 and the defective mutants did

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\* The nomenclature of the mutants indicates the virus from which they are derived (JS= JSRV; en56= enJS56A1), followed by a single letter indicating the amino acid mutated, a number representing its position in Gag and a letter indicating the amino acid residue of the resulting mutant.

not accumulate at the plasma membrane, suggesting a post-assembly trafficking deficiency.



**Figure 17. Staining phenotypes of transfected cells.**

Confocal microscopy of HeLa cells expressing or co-expressing JSRV, enJS56A1, and selected critical mutants. Positive cells were classified in three distinct phenotypes: diffuse, dispersed, and concentrated (see text). Each column displays three examples of the phenotype indicated on the top. Transfected constructs are indicated on the top right of each photograph. Gag staining is in green and nuclei are in red or indicated with the letter N. Scale bar is approximately 10  $\mu$ m.



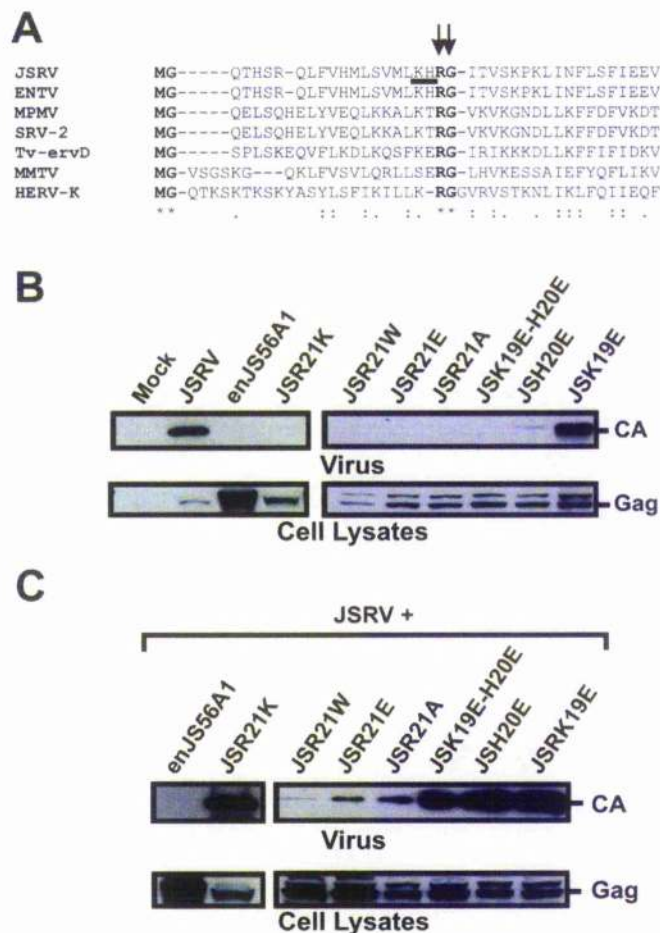
Plasmids	Diff. (%)	Disp. (%)	Conc. (%)	Total
cnJS56A1	73	22	5	717
JSRV	57	33	10	193
JSRV+cnJS56A1	91	7	2	456
JSR21W	58	37	5	93
JSG2A	50	10	40	58
JSR98C	44	14	42	117
Mock	0	0	0	0

**Table 2. Quantification of the observed Gag staining phenotypes in a representative experiment.**

Values are indicated as relative percentage of each phenotype, and the last column on the right indicates the total number of positive cells counted for each transfection.

### **JSRV viral exit is affected by residues K19, H20 and R21, but only the latter is involved in JLR**

We wanted to determine whether domains known to participate in retroviral Gag trafficking were involved in JLR. Amino acid residue in Gag W21 is the main determinant of JLR since JSRV single mutant JSR21W is defective and transdominant like the endogenous enJS56A1. Interestingly, residue R21 in Gag is highly conserved among Betaretroviruses (Figure 18A). In JSRV, R21 lies in close proximity to two other basic residues: lysine (K) 19 and histidine (H) 20 (Figure 18A). We therefore hypothesized that R21 could be part of the basic stretch of residues that constitutes the JSRV M domain. Consequently, JLR might be the result of a trans-dominant defective



**Figure 18. Mutational analysis of JSRV Gag amino acid residues 19, 20, and 21.**

(A) Alignment of the N-terminal Gag peptides from the indicated viruses, using ClustalW (Thompson, Higgins et al. 1994), showing that R21 and G22 are conserved among Betaretroviruses. Highly conserved residues are highlighted in bold. R21 and G22 are indicated with vertical arrows. K19 and H20 are underlined. Consensus symbols are displayed below the alignment: “\*” indicates identical residues in all sequences in the alignment, “:” indicates conserved substitutions, and “.” indicates semi-conserved substitutions. ENTv, enzootic nasal tumour virus; M-PMV: Mason-Pfizer monkey virus; SRV-2: simian retrovirus-2; TV-ervD: brushtail possum type-D endogenous retrovirus; MMTV: mouse mammary tumour virus; HERV-K: human endogenous retrovirus K. (B) Analysis of JSRV mutants bearing mutations in the basic region of the M domain. Viral pellets and cell lysates of cells transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted using a JSRV CA antiserum. (C) Western blot analysis of cells cotransfected with expression plasmids for wild type JSRV and indicated mutants. Viral pellets and cell lysates of cells transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted using a JSRV CA antiserum.

M phenotype displayed by enJS56A1. If this was the case, disruption of the basic domain in the JSRV MA would likely reproduce the interfering phenotype even in the presence of R21. To test this hypothesis, we derived JSRV mutants where K19, H20 (individually or in combination) and R21 were substituted by glutamic acid (E), a negatively charged amino acid residue.

When assessed by western blot using an anti JSRV CA polyclonal antiserum, viral release of JSH20E, JSK19E/H20E and JSR21E was severely impaired, while JSK19E did not display any gross defect in viral exit (Figure 18B). However, when cotransfected with JSRV, only JSR21E blocked JSRV exit in a trans-dominant fashion (Figure 18C), suggesting that JLR is not the result of a dominant-negative M domain phenotype but it is specifically dependent on Gag residue R21. We further focused on residue 21 and substituted the original R to a neutral alanine (A) and to a basic and more conservative lysine (K). Mutant JSR21A showed a transdominant phenotype as the one observed by JSR21W and JSR21E (Figs. 18B and 18C). To our surprise, mutant JSR21K did not release viral particles nor did it block JSRV release (Figs. 18B and 18C).

As previously shown, confocal microscopy of cells expressing a myristoylation-defective JSRV mutant (JSG2A) showed a relative increase in cells exhibiting a 'concentrated' perinuclear staining. Mutant JSK19E/H20E also displayed a relative increase of cells with a concentrated phenotype when compared to wild type JSRV, although not as pronounced as JSG2A (Table 3 and Figure 19). On the contrary, cells expressing JSR21W and JSR21E showed, if anything, a relative decrease in concentrated Gag staining, further supporting the hypothesis that the block induced by R21 mutations is not related to trafficking defects involving the JSRV M domain.

From the alignment shown in Figure 18, we noticed that a glycine (G) in Gag position 22 is also highly conserved among Betaretroviruses. We therefore hypothesized the R21 and G22 could be part of a previously uncharacterized functional domain required for JSRV trafficking/exit. To test this, we constructed mutant JSG22A, which was incompetent for viral exit, but did not block viral release when co-expressed with JSRV (data not shown). However, this mutant exhibited a curious phenotype: when assessed by confocal microscopy, not a single cell expressing JSG22A displayed a concentrated

phenotype in two independent experiments (Table 3 and Figure 19), suggesting that this mutation induces a different defect from the ones observed by the other constructs carrying mutations in R21 or H19/K20.

As a whole, these results indicate that the highly conserved R21 and G22 are specifically required for JSRV exit, although only the former appears to be involved in JLR. In addition, the Gag residue W21 that naturally substitutes R21 in enJS56A1 is not strictly needed to block JSRV release, since other amino acid substitutions are still transdominant (i.e. R21A and R21E). Finally, both JSRV K19 and H20 seem to contribute to JSRV exit and be part of its M domain as already characterized in other retroviruses (Swanstrom and Wills 1997).

Plasmids	Diffuse (%)	Dispersed (%)	Concentrated (%)	Total
enJS56A1	58	37	5	100
JSRV	33	57	10	100
JSG2A	16	22	62	100
JSK19E/H20E	35	46	19	100
JSR21E	38.5	56	5.5	200
JSR21K	41	43	16	300
JSG22A	48.5	51.5	0	200

**Table 3. Intracellular distribution of Gag in cells expressing JSRV, enJS56A1, and the indicated M domain mutants.**



**Figure 19. Confocal microscopy of HeLa cells expressing JSRV mutants**

JSR21K (left), JSK19/H20E (middle) and JSG22A (right). Gag staining is in green, nuclei are stained with DAPI (blue) or indicated with an N. Scale bar is approximately 10  $\mu$ m.

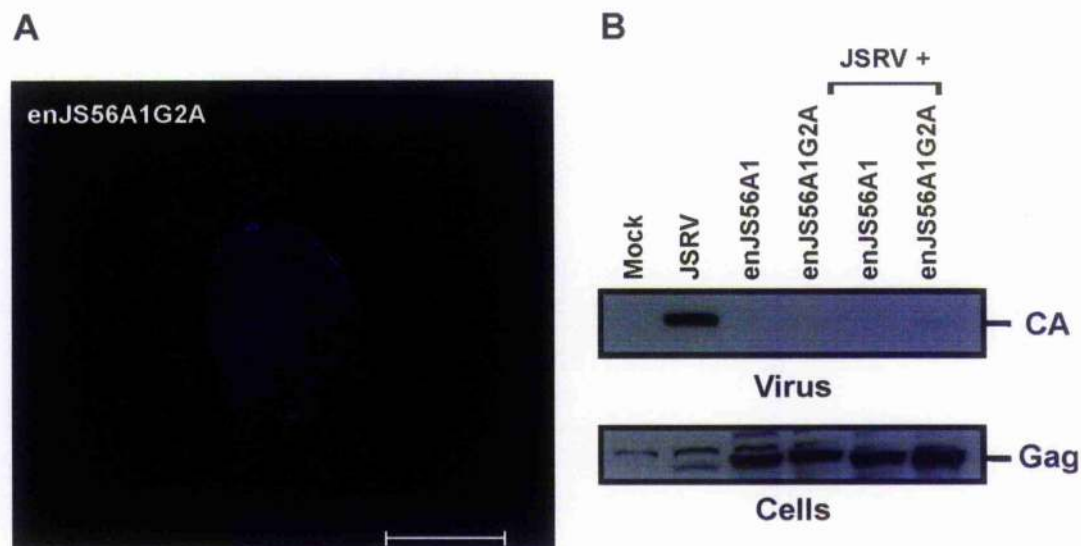
### **Myristoylation of enJS56A1 Gag is not required for JLR**

Some murine defective endogenous retroviruses bud solely from the endoplasmic reticulum membrane, resulting in immature intracisternal A-type particles (IAPs) (Swanstrom and Wills 1997; Vogt 1997). IAPs can be regarded as type-C retroviruses that are following a “wrong” course. The wild type IAP element MIA14 is a clear example of this. Addition of the membrane binding domain of the Src protein to the N terminus of MIA14 Gag results in redirection of the virions to the plasma membrane, followed by maturation and subsequent virus release (Welker, Janetzko et al. 1997).

The presence of a myristoylation signal in enJS56A1 Gag together with the observation of intracytoplasmic virus-like particles in cells expressing this endogenous virus prompted us to determine whether membrane targeting of the endogenous Gag was a functional requirement for JLR. We therefore constructed mutant enJS56A1G2A, in which the myristoylation signal was disrupted by substituting the N terminal glycine of Gag with an alanine by site-directed mutagenesis. The phenotype exhibited by this mutant in confocal microscopy was quite different from the one observed by its exogenous counterpart JSG2A: 59 out of 100 enJS56A1G2A-expressing cells counted displayed a dispersed phenotype (Figure 20). When cotransfected with JSRV,



enJS56A1G2A kept its ability to interfere with JSRV, indicating that myristoylation of enJS56A1 Gag is not required for JLR (Figure 20B).



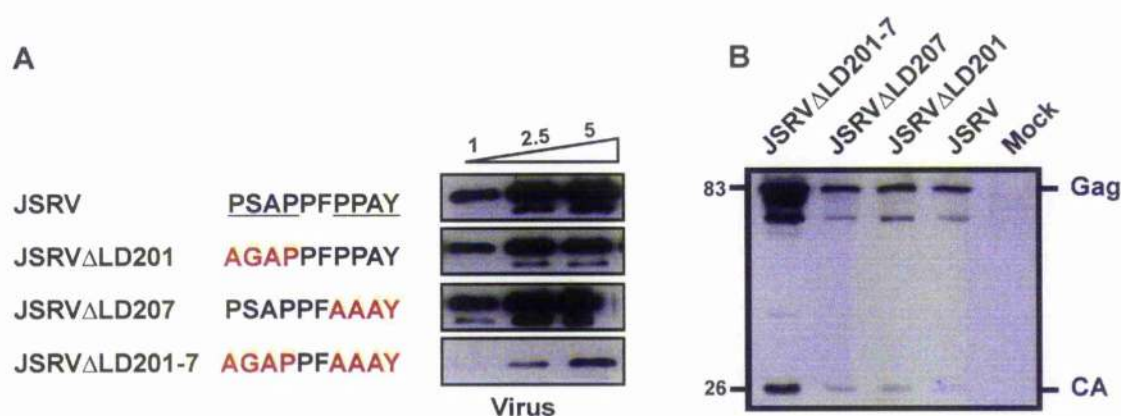
**Figure 20. Myristoylation of enJS56A1 Gag is not required for JLR.**

(A) Confocal microscopy of HeLa cells expressing the endogenous myristoylation-defective mutant enJS56A1G2A. The majority of cells expressing enJS56A1G2A displayed a dispersed pattern of staining, unlike JSG2A expressing cells that show accumulation of Gag staining in a perinuclear region. Anti-MA staining is displayed in green and nuclei in blue. The scale bar is approximately 12  $\mu$ m. (B) Viral pellets (upper panels) and cell lysates (lower panels) of cells transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted with a JSRV CA antiserum. enJS56A1G2A is defective and transdominant over wild type JSRV.

### Identification of JSRV L domains

During our functional characterization of JSRV Gag, we searched for putative L domains to experimentally rule out their involvement in JLR. We identified tandem PSAP and PPAY domains in position 201-204 and 207-210 of Gag, respectively (Figure 21A). Both the sequence and relative position of these domains in JSRV Gag are analogous to the M-PMV L domains, although the order is inverted (Yasuda and Hunter 1998; Gottwein, Bodem et al. 2003). To establish whether these sequences were *bona fide* L domains, we constructed single (JS $\Delta$ LD201 and JS $\Delta$ LD207) and double

(JS $\Delta$ LD201-7) L domains mutants (Figure 21A). Mutations were designed according to previous studies performed on M-PMV (Gottwein, Bodem et al. 2003). We further transfected these JSRV L mutants in 293T cells side by side with wild type JSRV, and compared the amount of virus produced by western blot (Figure 21A). It has been suggested with other retroviruses that defects in viral budding due to L domain mutations can be overcome by Gag overexpression (Strack, Calistri et al. 2002). Thus, our experiments have been performed using decreasing amounts of plasmid DNA expressing JSRV LD mutants to avoid artefacts due to overexpression.



**Figure 21. Identification of JSRV L domains.**

(A) Viral pellets of cells transfected with increasing amounts of the indicated plasmids were resolved by SDS-PAGE and immunoblotted with a JSRV antiserum towards MA. The names of the transfected constructs and their L-domain sequence are indicated on the left of each panel, while the amount of plasmid DNA transfected is shown on top of the upper panel. Wild type tandem L domains are underlined and mutations are highlighted in red. (B) Lysates of cell transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted with an antiserum towards JSRV CA. Cells expressing JSRV $\Delta$ LD201-7 exhibited more intense bands for both mature and immature Gag, indicative of a defect on viral exit for this virus.

Expression of single mutant JS $\Delta$ LD201 and double mutant JS $\Delta$ LD201-7 resulted in reduced virus production compared to JSRV. On the other hand, no major differences in virus production were observed between wild type JSRV and JS $\Delta$ LD207 (Figure 21A). Lysates of cells expressing double mutant JS $\Delta$ LD201-7 showed an increased amount of mature Gag by western blot (Figure 21B), consistent with a defect at late stages of the retroviral cycle.

Confocal microscopy experiments complemented the aforementioned western blot results: cells expressing either JS $\Delta$ LD201 or JS $\Delta$ LD201-7 displayed variable accumulation of Gag staining at the cell membrane (Figure 22). This characteristic L domain phenotype was more pronounced in cells expressing the double L domain mutant. In contrast, expression of JS $\Delta$ LD207 did not result in significant accumulation of Gag staining (Figure 22).



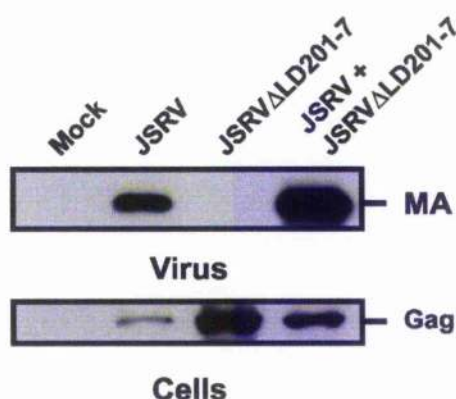
**Figure 22. Confocal microscopy of HeLa cells expressing JSRV and JSRV L domain mutants.**

Anti-JSRV MA staining is displayed in green and nuclei in blue. White arrows indicate Gag staining accumulation at the plasma membrane. Cells expressing JSRV $\Delta$ LD201-7 show a characteristic L domain staining at the cell membrane. Scale bar is approximately 10  $\mu$ m.

Based on our observations by electron (Mura 2004) and confocal microscopy, we had hypothesized that JLR was not the result of a dominant-negative L-domain phenotype. To experimentally rule out any involvement of L domains in JLR, we coexpressed JSRV with JS $\Delta$ LD201-7 and analysed virus pellets and cell lysates by western blot. We



chose this mutant because it displayed defective viral release when compared with JS $\Delta$ LD201 and JS $\Delta$ LD207. JS $\Delta$ LD201-7 was unable to block JSRV. On the contrary, it seemed to be rescued by the wild type virus based on the amount of mature Gag detected in the supernatants by western blot (Figure 23). Collectively, the data presented so far suggest that the mechanisms governing JLR appear to be unrelated to known signals/mechanisms of Gag trafficking.



**Figure 23. JLR is not due to a transdominant L domain phenotype.**

Viral pellets (upper panel) and cell lysates (lower panel) of cells transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted with a JSRV MA antiserum. JSRV $\Delta$ LD201-7 is defective for viral exit but is not transdominant over wild type JSRV.

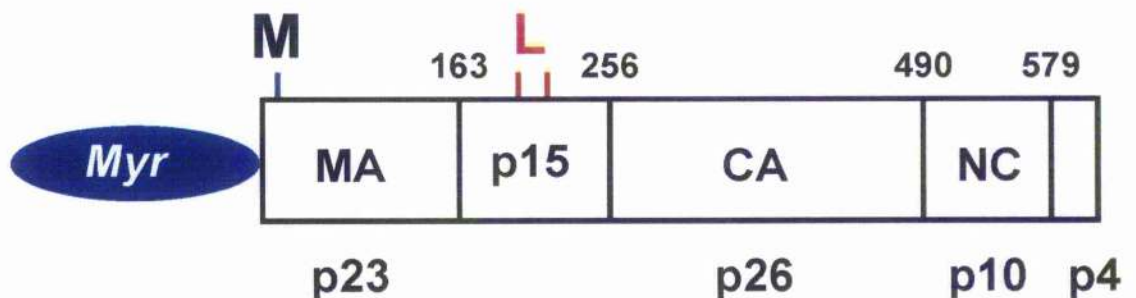
### **Truncated enJS56A1 Gag mutants do not block JSRV**

Since the main determinant of JLR is located within the N-terminal Gag, it was of great interest to determine whether truncated enJS56A1 Gag mutants could still block JSRV release. In general, retroviral Gag defective mutants are functionally complemented and further rescued by homologous wild type viruses (Muriaux, Costes et al. 2004; Lee, Boyko et al. 2007). This is not the case for enJS56A1, which is instead, defective and transdominant. As previously mentioned, one of the possible mechanisms postulated for JLR is that enJS56A1 Gag could saturate (or capture) cellular factors required for Gag

trafficking. If this was the case, enJS56A1-Gag truncated mutants could still bind these factors and interfere with JSRV exit. We therefore decided to construct enJS56A1 deletion mutants lacking entire Gag domains and test their ability to block JSRV.

We first aimed to determine the exact boundaries of the JSRV Gag mature proteins as these were unknown at the time of these studies. To determine the JSRV Gag organization, we produced a highly concentrated purified virus preparation and analysed it by reverse-phase high pressure liquid chromatography (RP-HPLC) and sequenced the N-terminal protein by Edman degradation. Sixty 10-cm plates were transfected with an expression plasmid for JSRV and supernatants containing virus were collected at 24, 48, and 72 hours to maximize viral recovery. Collected medium was clarified by centrifugation and viral pellets were obtained by further ultracentrifugation. Viral pellets were resuspended in TNE buffer, pooled together, and purified twice by isopycnic centrifugation on a 25 to 55% (wt/wt) linear sucrose gradient. Fractions with a buoyant density between 1.146 and 1.176 g/ml were pooled, concentrated by ultracentrifugation as described above and resuspended in 8M guanidine-HCl. This concentrated viral preparation was submitted to the National Cancer Institute (Frederick, MD, USA) for analysis of individual viral proteins. Briefly, viral proteins were separated by reverse-phase high pressure liquid chromatography (RP-HPLC). Each peak was individually collected and fractions were further N-terminally sequenced by Edman degradation. Results obtained suggest that JSRV Gag is cleaved into at least 5 products in the following order: MA(p23)- p15- CA (p26)- NC(p10) - p4 (Henderson and Sowder, personal communication). The sequence and predicted boundaries of the mature JSRV Gag are indicated in Figure 24. These data are in agreement with previous studies on <sup>35</sup>S-labelled JSRV virions which revealed the presence of 5 polypeptides (excluding the surface and transmembrane domain of the envelope glycoprotein) with an apparent molecular weight in a SDS-PAGE gel of 26, 23, 15, 10 and 5 kDa (Palmarini, Sharp et al. 1999). We confirmed that p26 is CA, p23 is MA, and p10 is NC by western blot analysis using a panel of specific polyclonal antisera and JSRV expression plasmids bearing HA epitopes within MA (not shown). Besides Gag, the mature surface (SU) domain of the JSRV envelope glycoprotein and cellular ubiquitin were also detected in purified viral particles (Henderson and Sowder, personal communication). The N-terminal SU sequence started at Env amino acid residue 81

MGQTHSRQLFVHMLSVMLKHRGITVSKPKLINFLSFIEEVCPWFPREGTVN  
 LETWKKVGEQIRTHYTLHGPEKVPVETLSFWTLIRDCLDFDNDELKRLGN  
 LLKQEEDPLHTPDSPSYDPPPPPPPSLKMHPSDNDDLLSSTDEAELDEEAAK  
 ↓  
 YHQEDWGFLAQEKGALTSKDELVECFKNLTIALQNAGISLPHNNTFPSAPFP  
 MA ← → p15  
 PAYTPSVMAGLDPPPGFPPPSKHMSPLQRALRQAQRLGEVVSDFSLAFPVFEN  
 ↓  
 NNQRYYESLPFKQLKELKIACSQYGPTAPFTIAMIENTLGTQALPPNDWKQTA  
 → CA  
 RACLSGGDYLLWKSEFFEQCARIADVNRQQGIQTSYEMLIGEGPYQATDTQL  
 NFLPGAYAQISNAARQAWKRLPSSSTKTEDLSKVRQGPDEPYQDFVARLLDTI  
 GKIMSDEKAGMVLAKQLAFENANSACQAALRPYRKKGDLSDFIRICADIGPS  
 ↓  
 YMQGIAMAAALQGKSIKEVLFFQQQARNKKGLQKSGNSGCFVCGQPGHRAA  
 → NC  
 VCPQKQQGPVNTPNLCPRCKKGKHWARDCRSKTDVQGNLPPVSGNWVRG  
 ↓  
 QPLAPKQCYGATLQVPKEPLQTSVEPQEAARDWTSVPPPIQY  
 → p4



**Figure 24. JSRV Gag sequence and schematic organization.**

Top: amino acid sequence of JSRV Gag. Vertical arrows indicate cleavage sites. Boundaries between the different polypeptides are indicated. Bottom: Schematic representation of JSRV Gag: the names of the Gag cleavage products are displayed inside the boxes (with the exception of p4, which is indicated below). Vertical lines indicate cleavage sites. The numbers above refer to the position of the boundaries in the mature Gag of the JSRV21 infectious

molecular clone (Palmarini, Sharp et al. 1999). The myristate is represented by a blue circle. The relative position of L and M domains is also indicated. The apparent molecular weights of MA, CA, and NC are indicated below.

(AAFWAY....) in agreement with the cleavage site of the leader peptide previously predicted (Palmarini, Sharp et al. 1999; Palmarini, Hallwirth et al. 2000).

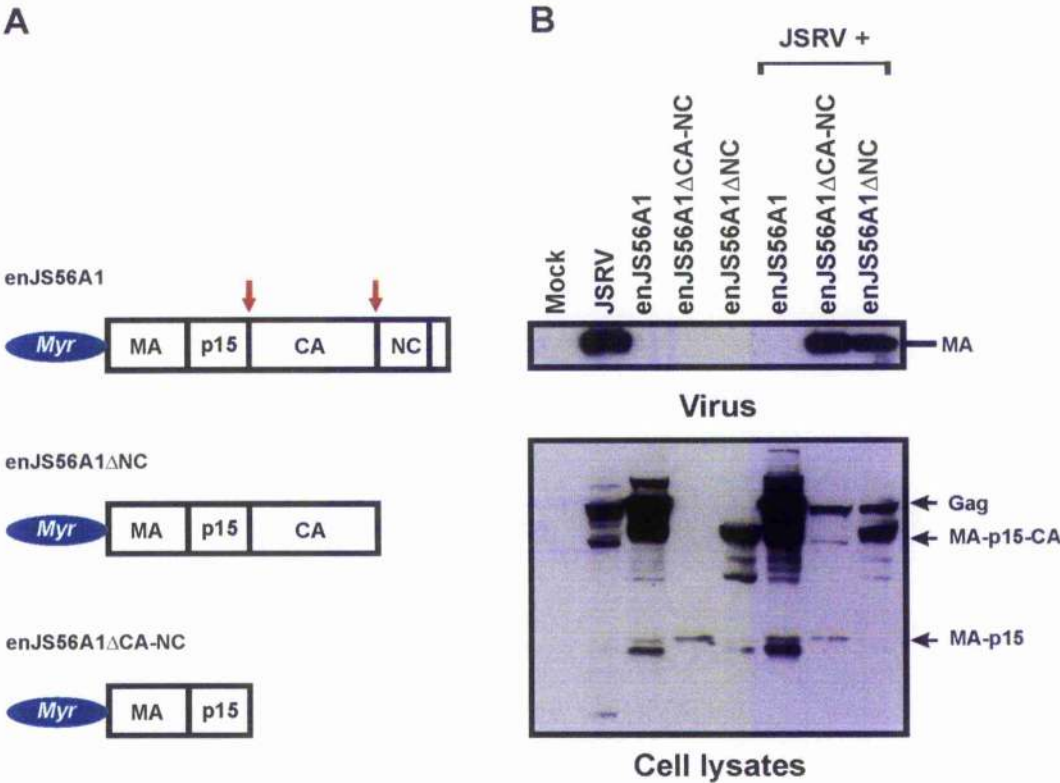
Once the boundaries of JSRV Gag were established, we introduced stop codons at the end of either p15 or CA by site-directed mutagenesis (Figure 25A). The resulting mutants lacked CA and NC (enJS56A1 $\Delta$ CA-NC), or only NC (enJS56A1 $\Delta$ NC), respectively. After transfection of 293T cells and further western blot analysis of cell lysates and viral pellets, we observed that both truncated mutants expressed Gag proteins of a lower molecular weight (Figure 25B, lower panel). When they were cotransfected with JSRV, none of them was able to block viral release (Figure 25B, upper panel), indicating that an entire enJS56A1 Gag is necessary for JLR. We noticed that expression of enJS56A1 $\Delta$ CA-NC was severely diminished, probably by nonsense mediated mRNA decay, and this low level of expression could account for its inability to restrict JSRV. In contrast, expression of enJS56A1 $\Delta$ NC was comparable to that observed with full length enJS56A1 (Figure 25B, lower panel).

### **JSRV and enJS56A1 Gag proteins colocalize**

As mentioned above, we considered the possibility that Gag-Gag interactions between JSRV and enJS56A1-derived molecules could constitute a mechanistic basis for JLR. To initially address this, we investigated whether enJS56A1 and JSRV Gag colocalize within the cell as this would be suggestive of a restriction mechanism occurring *in trans*. As stated above, our anti-JSRV MA used in confocal microscopy does not discriminate between JSRV and enJS56A1 Gag, probably because these two proteins are 95% identical (Palmarini, Hallwirth et al. 2000). Therefore, we differentially tagged JSRV and enJS56A1 Gag by fusing the FLAG and HA epitopes at the carboxy-terminal of p4 or within the MA domain (Figure 26). The biological properties of the parental viruses were not altered by the addition of the epitopes since all the tagged JSRV and



enJS56A1 constructs were competent for viral release and interference, respectively (Figure 27).

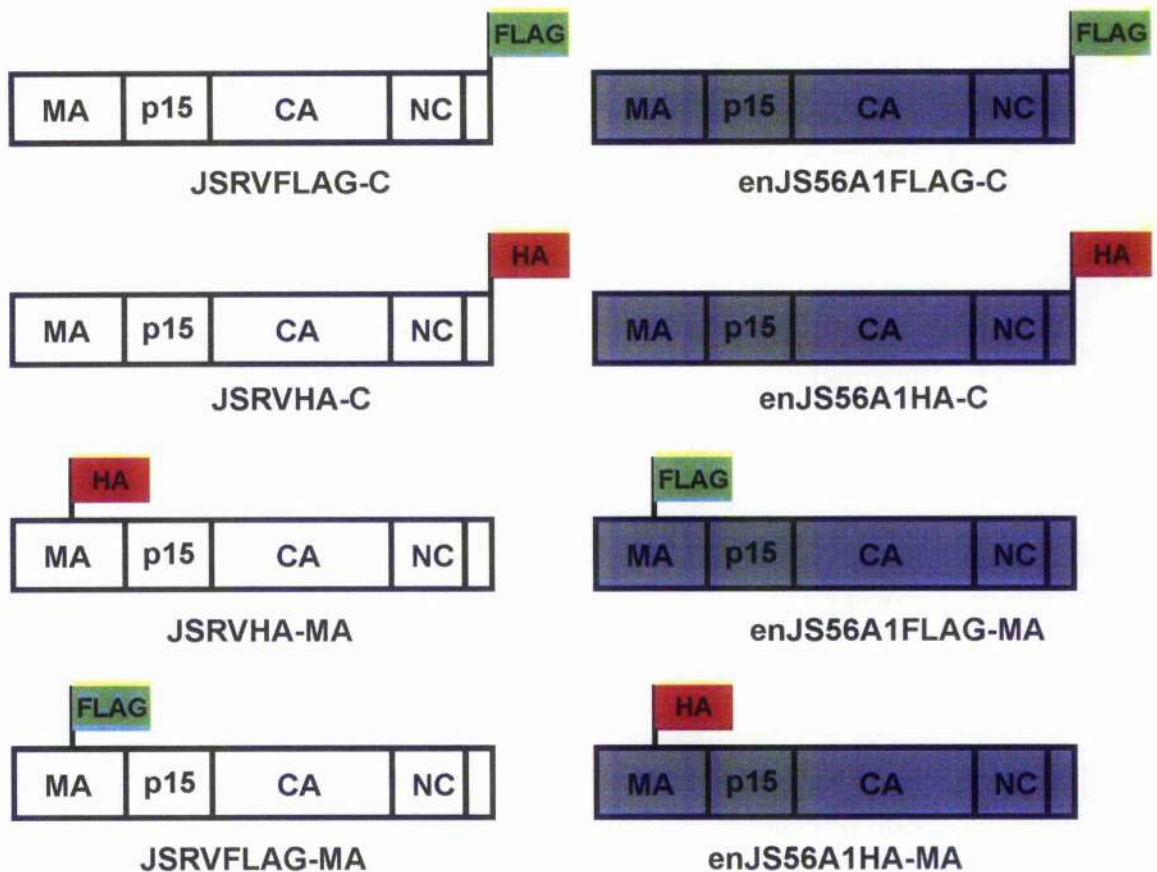


**Figure 25. Truncated enJS56A1 Gag mutants do not block JSRV exit.**

(A) Schematic representation of enJS56A1 Gag and truncated mutants. Vertical arrows indicate the position where stop codons were inserted by site-directed mutagenesis. (B) Western blot analysis of enJS56A1-deletion mutants. Viral pellets (upper panel) and cell lysates (lower panel) of cells transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted with a JSRV MA antiserum. Truncated constructs did not interfere with JSRV exit.

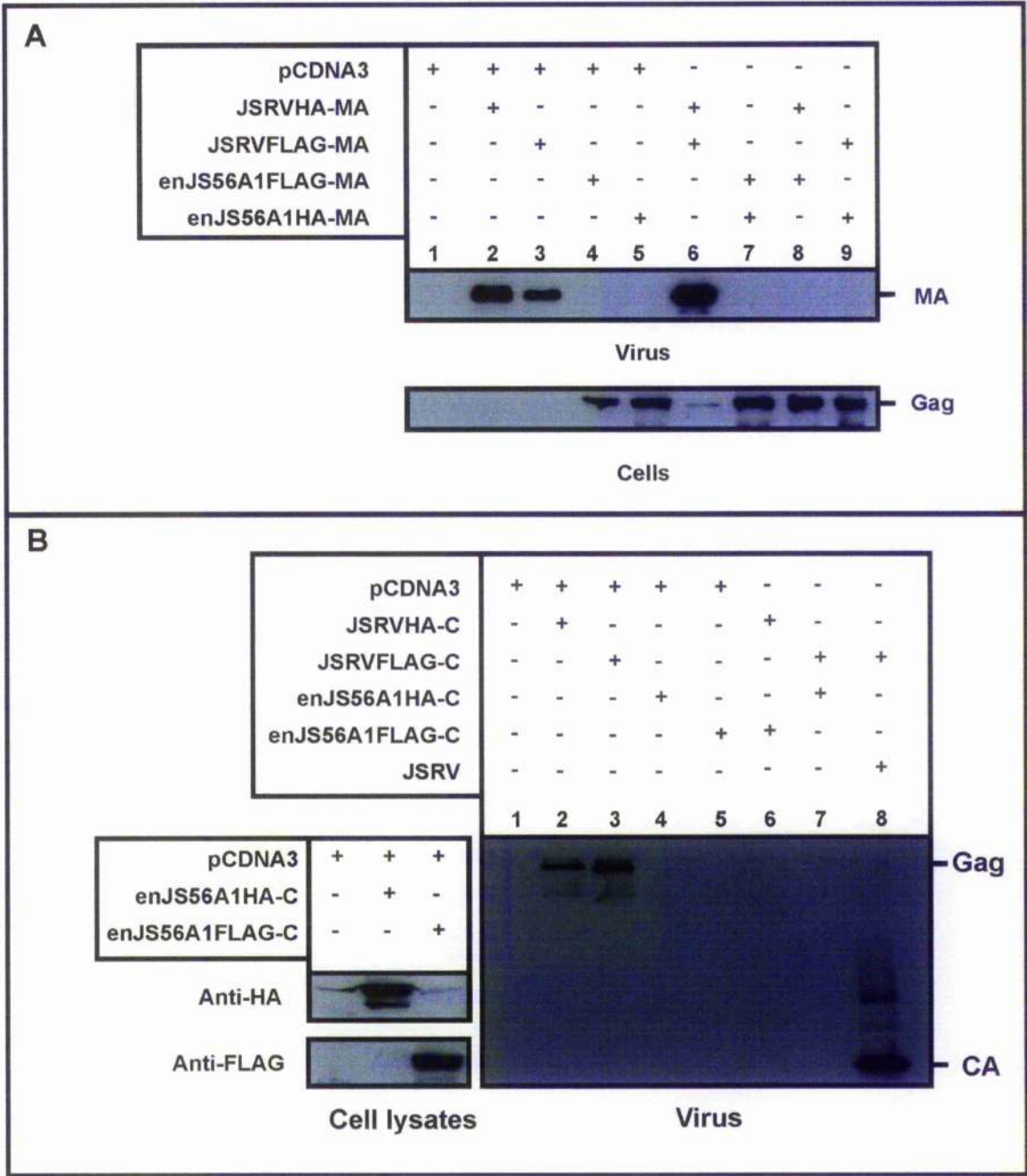
Immunostaining of labelled Gags with anti-FLAG or anti-HA was comparable to the one observed when a JSRV MA antiserum was used (not shown). To test our system for colocalization experiments, we first co-transfected HeLa cells with homologous constructs bearing different tags. As expected, they strongly colocalized regardless of the location of the epitope (Figure 28). When JSRV and enJS56A1 were co-expressed, colocalization between endogenous and exogenous virus was evident in cells displaying both dispersed and concentrated phenotype (Figure 28). We essentially obtained the

same results with all our different constructs despite the different position of the labelling epitope within Gag (i.e. at the C-terminal end or within the MA domain).



**Figure 26. Schematic representation of JSRV and enJS56A1 tagged constructs.**

Epitopes were inserted either at the C-terminal end of Gag or within MA. The FLAG and HA epitopes are indicated as green and red flags, respectively. JSRV and enJS56A1-derived constructs are drawn as white and grey boxes, respectively.



**Figure 27. Western blot analysis of plasmids expressing differentially tagged Gag proteins.**

Viral pellets of cells transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted with a JSRV CA antiserum. Gag labelling did not affect JSRV viral exit nor did it abrogate enJS56A1 ability to perform JLR. Panels A shows constructs bearing epitopes within the MA protein, and panel B displays C-terminally tagged constructs.



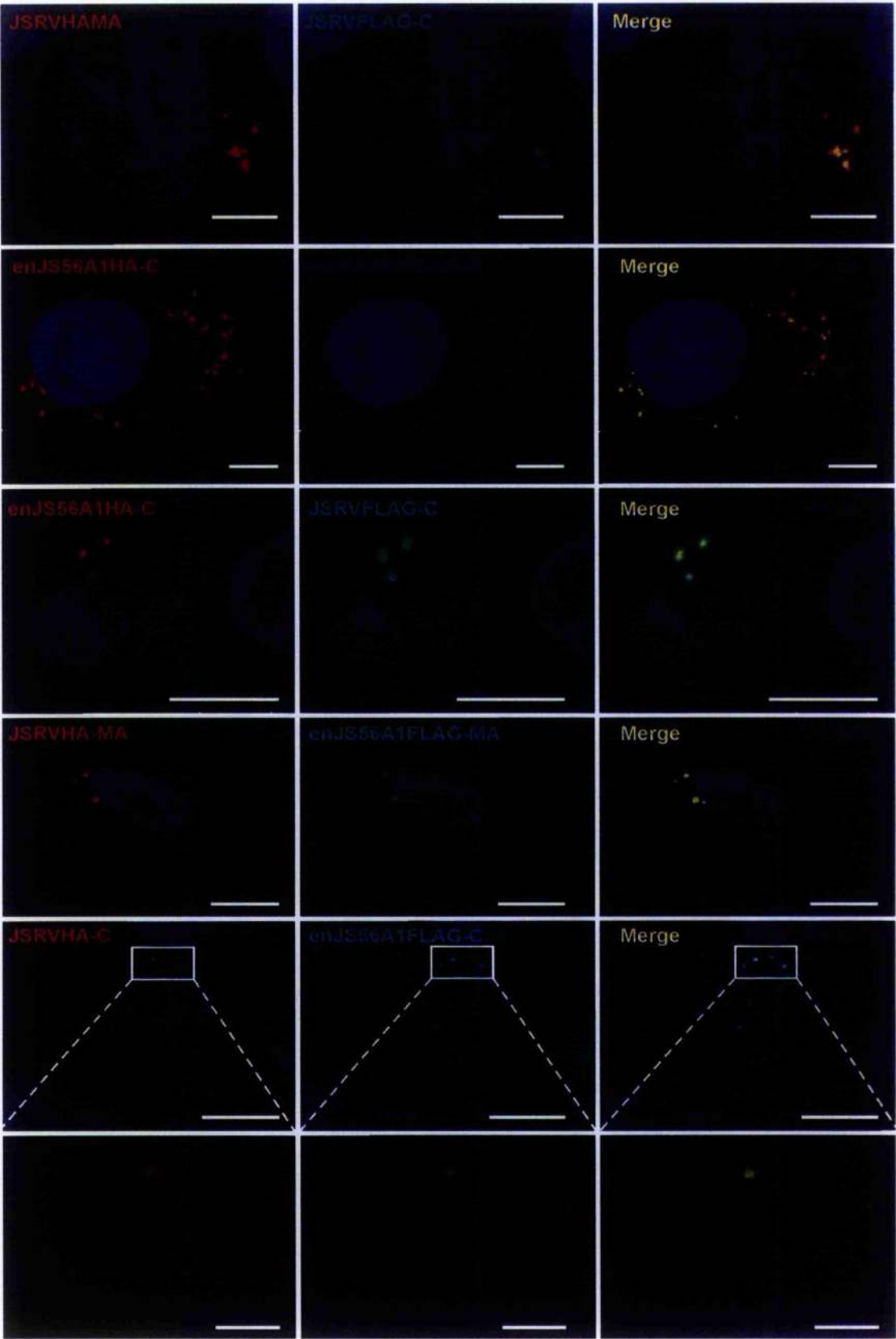


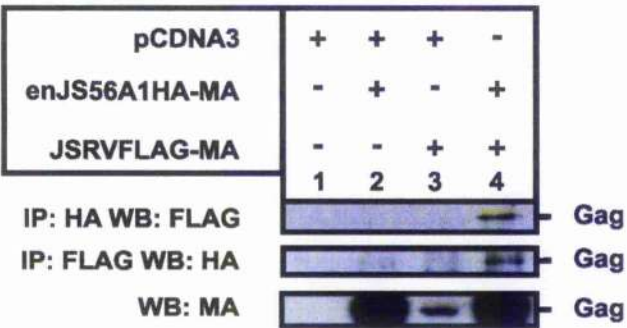
Figure 28. JSRV and enJS56A1 Gag colocalize (see legend on next page).



(From previous page). Representative images of confocal microscopy of HeLa cells co-expressing differentially tagged JSRV and enJS56A1. Strong colocalization of JSRV and enJS56A1 is observed in all cases. Both dispersed and concentrated phenotypes are represented (see text). Transfected constructs are indicated on the upper left corner of each image. FLAG staining is displayed in green, HA staining in red, and nuclei in blue. Scale bar is approximately 10  $\mu\text{m}$ , with the exception of the bottom row, where it is approximately 2  $\mu\text{m}$ . The bottom row shows a high power magnification of the area within the rectangle indicated in the pictures of the fifth row from the top.

**JSRV and enJS56A1 interact in *trans***

Results obtained by confocal microscopy using epitope-labelled viruses suggest that JLR is likely due to an interaction *in trans* between JSRV and enJS56A1 Gag. To test this, we performed co-immunoprecipitation experiments using lysates of cells coexpressing our differentially tagged JSRV and enJS56A1. As expected, JSRV and enJS56A1 co-immunoprecipitate (Figure 29) suggesting that they associate *in trans*.



**Figure 29. Association between JSRV and enJS56A1 Gag proteins.**

293T cells were co-transfected with enJS56A1HA-MA and JSRVFLAG-MA. Cell lysates were immunoprecipitated (IP) at 48 hours post-transfection and analyzed by SDS-PAGE followed by Western blotting as indicated beside each panel. Gag expression was assessed by Western blotting using a JSRV MA antiserum. enJS56A1-JSRV Gag association is evident in lysates from cells cotransfected with both viruses (lane 4). Note that the JSRV MA antiserum is polyclonal and more sensitive than monoclonal anti-HA or anti-FLAG in Western blotting.

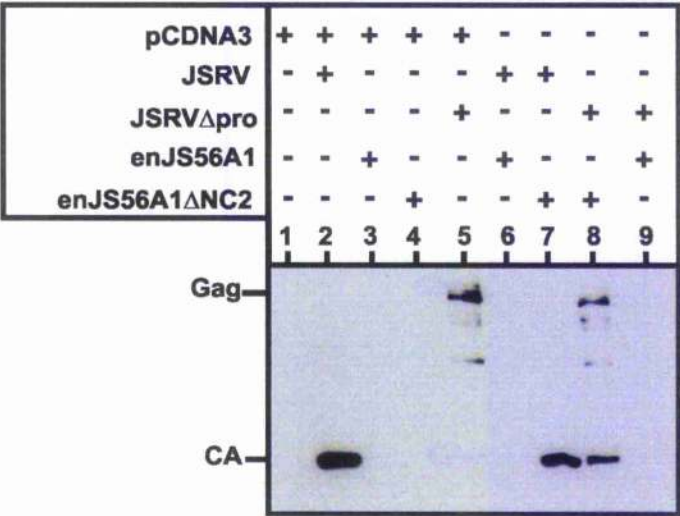
The previously described co-immunoprecipitation experiments gave us strong evidence to support the hypothesis of Gag-Gag interaction between JSRV and enJS56A1 in JLR. We therefore wanted to see whether this was reflecting a co-assembly process between endogenous and exogenous Gag proteins resulting in JSRV-enJS56A1 chimeric particles. Hence, we performed complementation assays using a JSRV virus lacking the protease (JSRV $\Delta$ pro<sup>\*</sup>), and an enJS56A1 virus bearing a major deletion in NC with an in frame intact Pro (enJS56A1 $\Delta$ NC2). When expressed in 293T cells, the former releases only immature viral particles in the supernatant. In contrast, the latter does not exit from transfected cells, nor does it block JSRV. As shown in Figure 30, co-expression of these two plasmids results in the release of viral particles bearing both mature and immature Gag. Thus, JSRV $\Delta$ pro rescues and likely co-assembles with enJS56A1 $\Delta$ NC2 since Gag cleavage can only occur when the viral protease is contained within a retroviral particle (Rabson and Graves 1997). This result suggests that enJS56A1 and JSRV can potentially co-assemble, and prompted us to wonder whether co-assembly was a required step for JLR. If this was the case, impeding enJS56A1 assembly would abrogate its capacity to block JSRV. We experimentally addressed this by making an in-frame deletion of the major homology region (MHR) of enJS56A1 Gag. This strategy was based on previous data generated on M-PMV, where it has been shown that deletion mutants lacking the MHR are unable to assemble into viral particles (Strambio-de-Castillia and Hunter 1992).

Our enJS56A1 MHR deletion mutant (enJS56A1 $\Delta$ MHR) was unable to interfere with JSRV despite its high levels of expression in cell lysates (Figure 31). These data indirectly suggest that enJS56A1-JSRV co-assembly is required for JLR, given that the retroviral MHR is required for proper assembly of viral particles.

As a whole, our results presented here together with previous observations of intracytoplasmic viral particles by electron microscopy in cells expressing enJS56A1, with or without JSRV (Mura 2004), suggest that enJS56A1 and JSRV associate *in trans* and likely co-assemble.

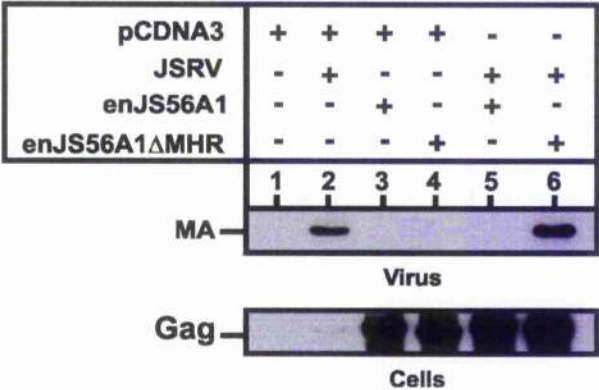
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<sup>\*</sup> Constructed by Manuela Mura.



**Figure 30.** enJS56A1 $\Delta$ NC can complement JSRV $\Delta$ Pro.

Supernatants of cells transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted with a JSRV CA antiserum. Note that JSRV $\Delta$ pro releases viral particles into the supernatant with an immature Gag (lane 5) because of the absence of a functional protease in this virus. Coexpression of JSRV $\Delta$ pro and enJS56A1 $\Delta$ NC2 (lane 8) results in the release of viral particles with both mature and immature Gag.



**Figure 31.** Deletion of the Major homology region abrogates JLR.

Supernatants (upper panel) and lysates (lower panel) of cells transfected with the indicated plasmids were resolved by SDS-PAGE and immunoblotted with a JSRV MA antiserum. enJS56A1 $\Delta$ MHR is highly expressed (lane 4, lower panel) but does not interfere with JSRV exit (lane 6, upper panel).

## Discussion

Expression of enJS56A1, an endogenous sheep Betaretrovirus, blocks the release of JSRV in a transdominant fashion at a late stage of the retroviral cycle (Mura 2004). The late timing of this block, referred to as JLR in this thesis, is unique when compared with the earlier replication steps affected by other well characterized retroviral and cellular restriction factors described in the introductory chapter (i.e. Fv1, Fv4, Trim5 $\alpha$ , APOBEC-3G, etc.)

In this section, we showed that JSRV and enJS56A1 Gag co-localize, associate *in trans*, and likely co-assemble. Furthermore, by systematic characterization of JSRV and enJS56A1 Gag polyproteins, we were able to establish other Gag requirements for JLR. With regard to the timing at which the late block occurs, we shortened the possible time frame by ruling out any involvement of L domains in JLR. Complementing these results, work done by Frederick Arnaud in our laboratory indicates that enJS56A1 displaces the intracellular localization of Gag. More specifically, his data argue that JLR is due to a block in JSRV Gag targeting to the pericentrosomal region with a mechanism independent from other previously known Gag trafficking domains (see attached papers by Murcia, Arnaud and Palmarini [Journal of Virology, 81: 1762-72], and Arnaud, Murcia and Palmarini [Journal of Virology, in press]).

We previously mapped the main determinant of JLR to amino acid residue R21 within JSRV Gag (Mura 2004), a conserved residue among Betaretroviruses that is naturally mutated into a tryptophan in enJS56A1 (Figure 18A). The main determinant of JSRV Gag centrosomal targeting is also residue R21 (see attached paper by Murcia, Arnaud and Palmarini). The naturally occurring R21W substitution observed in enJS56A1 is not strictly necessary for JLR, since JSRV single mutants carrying other amino acid residues than R in position 21 are incompetent for viral release and trans-dominant over JSRV, the only exception being the conservative mutant JSR21K.

The centrosome has been hypothesized to be the site of assembly for M-PMV (another Betaretrovirus). Once assembled, M-PMV particles traffic to the cell membrane by yet uncharacterized mechanisms that require the viral envelope and recycling endosomes

(Sfakianos and Hunter 2003; Sfakianos, LaCasse et al. 2003). Because M-PMV appears to assemble at the centrosome one could hypothesize that JLR results from altered assembly. It is reasonable to conjecture that JSRV and enJS56A1 co-assemble considering that (i) JSRV and enJS56A1 Gag co-localize and associate *in trans*; (ii) we observed viral particles within the cytoplasm of cells expressing enJS56A1 or co-expressing enJS56A1 and JSRV by EM (Mura 2004); (iii) enJS56A1 truncation mutants are defective but do not block JSRV and (iv) an enJS56A1 mutant with the MHR deleted is not transdominant. Furthermore, in a complementation assay we have shown that an enJS56A1 mutant with an in frame deletion in NC (enJS56 $\Delta$ NC2) and a functional protease co-assembles with JSRV $\Delta$ pro. Although it can be argued that enJS56 $\Delta$ NC2 is not transdominant and consequently does not completely recapitulate the enJS56A1 phenotype, it is also true that CA dictates co-assembly (Ako-Adjei, Johnson et al. 2005) and both enJS56A1 and enJS6 $\Delta$ NC2 have identical CA domains. Thus, there are indications that JSRV and enJS56A1 Gag co-assemble (at least partially) and possibly co-assembly is required for JLR.

The precise mechanisms of JLR action are not known at present. It is possible that even a minority of enJS56A1 Gag molecules alter the overall conformation of chimeric JSRV/enJS56A1 viral particles (or multimerized Gag molecules) so that they are unable to bind cellular factors that direct them to the centrosome. The nature of the association between any cellular protein assisting Gag trafficking would have to be reversible or temporary. Thus, the defect of enJS56A1 could even result from its Gag protein binding cellular factors irreversibly (or more tightly than necessary). Alternatively (or additionally) enJS56A1 Gag might actively target, in a transdominant fashion, a cellular compartment where viral particles cannot reach the cell membrane and egress from the cell.

In M-PMV a region of 18 amino acids within the MA, known as the cytoplasmic targeting-retention signal (CTRS) is responsible for directing Gag molecules to the centrosome. A single mutation in this domain (R55W), abolishes centrosomal targeting, but the resulting mutant is still able to assemble at the cell membrane like those viruses that follow the so-called C-type assembly (i.e. HIV, MLV, FeLV and others) (Rhee and Hunter 1990; Choi, Park et al. 1999). Thus, centrosomal targeting does not appear to be

absolutely necessary for betaretroviral assembly. JSRV bears a threonine at the corresponding R55 and it has not been determined whether a CTRS domain is present in this virus. The JSRV R21 might be part of an equivalent domain, but if this was the case we would expect the JSR21W mutant to display a C-type assembly followed by viral release from the cell rather than a dominant-negative phenotype.

Studies on the molecular biology of JSRV Gag were lacking despite the vast body of knowledge of this protein in other retroviruses (Freed 1998; Demirov and Freed 2004). To better understand the molecular mechanisms underpinning JLR, we identified known trafficking signals and evaluated their functionality within the context of JSRV. This approach allowed us to identify putative JSRV L and M domains and to rule out their involvement in JLR. Moreover, we determined that myristoylation of enJS56A1 Gag is not a requirement for JLR since the enJS56A1G2A mutant efficiently blocked JSRV release.

From an evolutionary perspective, the strict requirement for R21 in JSRV release suggests that the R to W mutation became fixed in the sheep genome after integration. This seems feasible since in enJS56A1 a TGG codon encodes for W while AGG encodes for R in other enJSRVs loci (Arnaud, Caporale et al. 2007). Thus, a single nucleotide substitution would have been sufficient to select a provirus with dominant-negative interfering properties. enJSRVs (including enJS56A1 or similar proviruses possessing W21 in Gag) are highly expressed in the genital tract of the ewe (Spencer, Stagg et al. 1999; Palmarini, Hallwirth et al. 2000; Palmarini, Gray et al. 2001; Mura 2004; Dunlap, Palmarini et al. 2005). enJSRVs are also able to interfere with JSRV by receptor competition (Spencer, Mura et al. 2003). Therefore, enJSRVs might have been selected in sheep because they protected the host against exogenous retroviral infection by a seemingly powerful two-step interference mechanism. However, interference with exogenous pathogenic retroviruses is not the only biological function of enJSRVs. Recently, work done in collaboration between Tom Spencer at Texas A&M and our laboratory has shown that enJSRVs are absolutely required for sheep conceptus development (Dunlap, Palmarini et al. 2006; Dunlap, Palmarini et al. 2006) further reinforcing the hypothesis that endogenous retroviruses have benefited the evolution of their host (Boeke and Stoye 1997). This unique viral block provides additional clues on

the variety of mechanisms shaping co-evolution of endogenous/exogenous retroviruses and their hosts.

## Chapter 4

### Temporal distribution of late events of JSRV/enJS56A1 cell cycle.

#### **Summary**

In the previous chapter we established that JSRV late restriction (JLR) is likely due to interactions in *trans* between enJS56A1 and JSRV Gag proteins. Moreover, Frederick Arnaud in our laboratory determined that while JSRV Gag is targeted to a pericentriolar region, enJS56A1/JSRV chimeric particles/multimers are unable to reach this area. The centrosome is thought to be the site of assembly for Betaretroviruses, hence the importance of centrosomal Gag targeting (Sfakianos, LaCasse et al. 2003). Although we have established that JLR takes place at a late stage of the retroviral cycle, its precise timing was unknown. This is what we sought to determine in this section.

#### **Introduction**

Once integrated within the host genome, proviruses express their genes to give origin to progeny virions (Goff 2001). Gag is the major structural protein of the retroviral virion, and plays a critical role in viral assembly and budding (Swanstrom and Wills 1997). This notion is supported by the fact that the sole expression of Gag is sufficient to assemble virus-like particles. As was reviewed in Chapter 1 of this thesis, several domains within Gag display various activities required to form a complete viral particle. The membrane-binding (M) domain directs Gag to the plasma membrane. The capsid (CA), interaction (I), and nucleocapsid (NC) domains are responsible for multimerization of structural proteins and packaging of genomic RNA. The late (L) domains are in charge of virus-cell separation.

An infectious virion is composed of thousands of individual elements that include structural proteins, viral enzymes, lipids and glycoproteins of the envelope, genomic RNA, and even host cell proteins, and genomic RNA. Little is known about how all



these components come together to the site of assembly and constitute a viral particle. However, it is evident that viruses have evolved efficient strategies to transform the cellular machinery of the infected cell into viral factories.

Retroviral assembly is a multistep process in which each step is spatially and temporally regulated. There are two distinct morphogenic pathways: the so called C-type and the B/D-type. Viruses classified within the former category assemble at the plasma membrane, where they are targeted by means of the aforementioned membrane-binding domain. This has been recently shown for a Lentivirus like HIV-1 (Jouvenet, Neil et al. 2006), and also for HERV-K, a Betaretrovirus (Dewannieux, Harper et al. 2006; Lee and Bieniasz 2007). In contrast, type B/D retroviruses assemble within the cytoplasm of the infected cell (Swanstrom and Wills 1997; Goff 2001). Some type B/D retroviruses, like Mason-Pfizer monkey virus (M-PMV), possess an 18-amino acid sequence within the MA domain, referred to as cytoplasmic targeting/retention signal (CTRS). The CTRS seems to constitute a dominant signal for intracytoplasmic assembly of immature capsids (Rhee and Hunter 1990). A single substitution of an arginine to alanine in the CTRS abrogates its dominant activity and allows M-PMV to follow a C-type morphogenic pathway (Rhee and Hunter 1990). Conversely, insertion of the CTRS of M-PMV into murine leukaemia virus (MLV) results in intracytoplasmic assembly of an otherwise type-C retrovirus (Choi, Park et al. 1999).

Previous studies on M-PMV suggested that the subcellular site of assembly for this virus is the pericentriolar region. M-PMV Gag centriolar targeting occurs cotranslationally via dynein-mediated transport, is determined by the CTRS, and depends on intact microtubules (Sfakianos, LaCasse et al. 2003). Once assembled, capsids traffic to the cell membrane by a process depending on the viral envelope and the recycling endocytic pathway (Sfakianos and Hunter 2003).

Manuela Mura in our laboratory has shown that a myristoylation deficient JSRV (JSG2A) mutant is defective for viral exit although not transdominant over wild type virus (Mura 2004). Cells expressing JSG2A exhibited by confocal microscopy a marked increase in the number of cells with a concentrated phenotype compared to cells expressing JSRV. Colocalization studies performed by Frederick Arnaud determined

that JSG2A Gag concentrates in the vicinity of the centrosome (see paper by Murcia, Arnaud and Palmarini attached to this thesis). The inability displayed by JSG2A to reach the plasma membrane is consistent with similar studies performed on M-PMV where it has been shown that myristoylation is required for intracellular trafficking but not for assembly of viral particles (Rhee and Hunter 1987).

In this chapter we study JSRV and enJS56A1 kinetics by confocal microscopy. We also compared the temporal intracellular distribution of JSRV and enJS56A1 Gag with that of M-PMV and HERV-K. Moreover, we determined that JSRV release is dependent on dynein transport and establish that JLR occurs before centrosomal targeting as the typically concentrated phenotype of JSG2A is lost in the presence of enJS56A1. Confocal experiments using myristoylation-defective mutants of JSRV and M-PMV suggest distinct trafficking pathways although both viruses assemble within the cytoplasm. Finally, a JSRV/HERV-K chimera bearing the matrix domain of HERV-K exhibits a similar confocal phenotype to the one observed in cells expressing HERV-K Gag, with conspicuous staining at the plasma membrane consistent with a type-C morphogenic pathway. The fact that this chimera is not interfered by enJS56A1 raises the possibility that changing the site of assembly can overcome JLR.

## **Results**

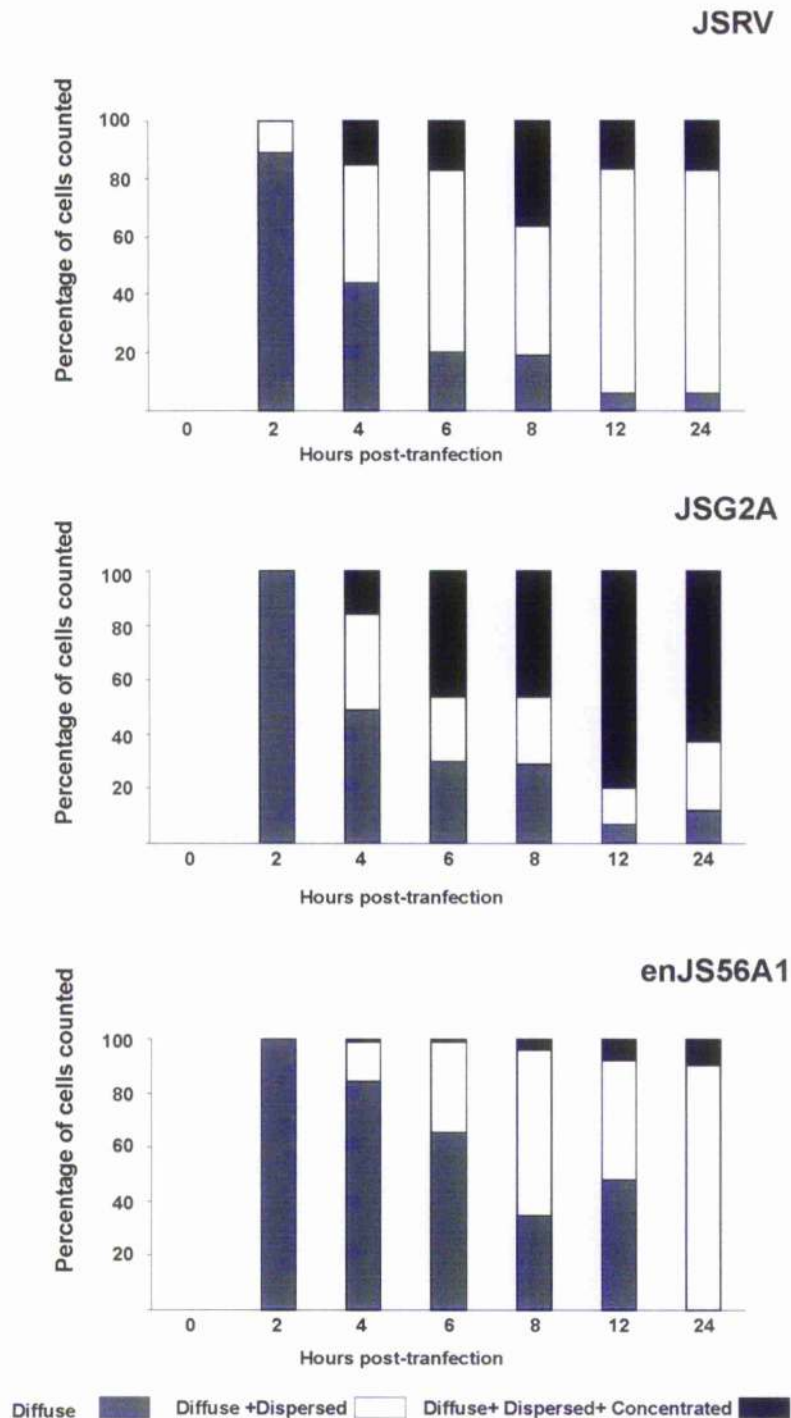
### **Temporal distribution of JSRV and enJS56A1 Gag proteins**

In the previous chapter we classified cells expressing JSRV, enJS56A1, and selected mutants in three distinct phenotypes according to their Gag staining pattern by confocal microscopy: diffuse, dispersed, and concentrated. In all cases, cells were fixed and immunostained at 24 or 48 hours post-transfection. Because the synthesis of Gag proteins and subsequent assembly of viral particles is a multistep process, we thought that what we were observing at those late time points was a mixture of different stages of the retroviral cycle. To separate these events in a temporal fashion we decided to perform confocal microscopy experiments on cells expressing JSRV, enJS56A1 and JSG2A at different intervals post-transfection. Cells were transfected with Lipofectamine and media was changed three hours after adding the transfection mixture. This stage was taken as “time zero”. We also included in these studies JSG2A, as this mutant follows a dead-end pathway that finished at the pericentriolar region (see paper by Arnaud, Murcia, and Palmarini attached to this thesis), and would therefore provide us with an accurate time frame corresponding to the time between Gag synthesis and its localization to the pericentrosomal region. The results of two independent experiments are shown in Figure 32. At two hours post-transfection (hpt) we began to detect JSRV Gag under our working conditions, when the vast majority of the few positive cells exhibited a diffuse phenotype (Figure 32, top panel). Cells with diffuse Gag staining showed a steady reduction throughout time, from almost 90% at 2 hpt to 6% at 12 and 24 hpt (Figure 32, top panel). In contrast, cells with dispersed phenotype were rare to find at 2 hours post-transfection, but progressively increased to reach their first peak at six hpt, and after a temporary decline, reached a second plateau of almost 80% at 12 and 24 hpt. Concentrated Gag staining was only observed after 4 hours post-transfection, and peaked at 8 hpt, in concomitance with a reduction of cells with dispersed staining. After 12 hpt, cells with concentrated Gag gradually declined (while the number of cells with dispersed phenotype increased, [Figure 32, top panel]).

As expected, the myristoylation-defective mutant JSG2A exhibited quite a different temporal distribution pattern (Figure 32, middle panel): although at 2 and 4 hpt the

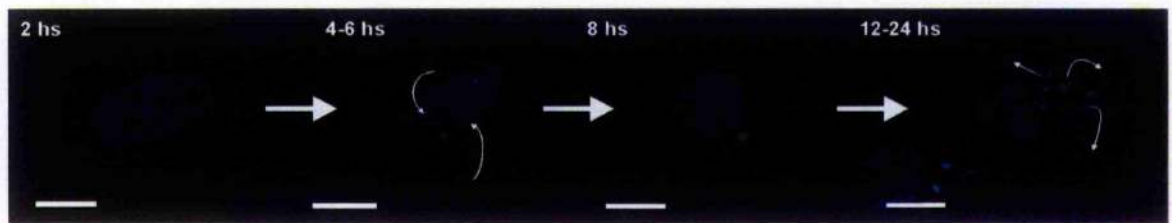
distribution of confocal phenotypes was comparable to the one observed with wild type virus, after 6 hours the number of cells with concentrated Gag staining markedly increased and constituted the preponderant phenotype. The distribution of cells expressing JSG2A with diffuse Gag staining was similar to the one displayed by JSRV expressing cells throughout the experiments. The increased proportion of JSG2A expressing cells with a concentrated phenotype corresponded to a smaller proportion (compared to cells expressing wild type JSRV) of cells with a “dispersed” phenotype, consistent with the notion that Gag accumulation in the perinuclear region is a transient step in the viral replication cycle (see below). With regard to enJS56A1, most of the positive cells observed during the timeframe of this experiment showed either a diffuse or a dispersed phenotype, as only a minor proportion displayed concentrated Gag staining (Figure 32, bottom panel). Notably, the size of the fluorescent specks was in general larger in cells expressing enJS56A1 Gag when compared with the ones exhibited in JSRV-expressing cells.

Based on these results, we built a model on the intracellular pathway followed by JSRV Gag from protein synthesis to viral exit that is depicted in Figure 33. The diffuse staining phenotype observed at early timepoints likely represents synthesis of Gag molecules at the polyribosomes (Figure 33, 2hs). At later stages, Gag proteins must assemble or multimerize, which is reflected by the appearance of characteristic discrete dots in cells with a dispersed phenotype. These newly assembled particles or Gag multimers must reach the pericentrosomal region before being targeted to the cell membrane (Figure 33, 4-6 hs). Centrosomal targeting probably encompasses an obligatory step in the JSRV replication cycle that acts as a bottleneck, since the number of cells displaying a concentrated phenotype reaches a momentary and reproducible peak (Figure 33 8 hs). After reaching the pericentrosomal region, viral particles must traffic to the plasma membrane to be released (Figure 33, 12-24 hs). This step requires an intact M domain since JSG2A cannot progress any further.



**Figure 32. Kinetics of the intracellular distribution of JSRV, JSG2A and enJS56A1.**

HeLa cells were transfected with the indicated plasmids and fixed at different times post-transfection. After immunostaining, cells were quantified according to their Gag staining phenotype. Each bar represents a specific time-point at which cells were fixed, immunostained and classified according a predefined staining criterion (see text).



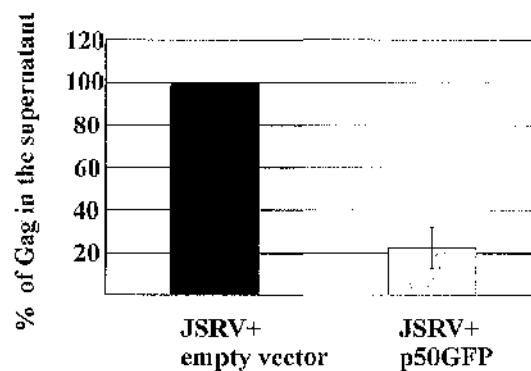
**Figure 33. Kinetics of JSRV Gag intracellular distribution.**

Model of JSRV Gag trafficking. From left to right: JSRV Gag is synthesized by the polyribosomes in the cytoplasm, exhibiting a diffuse Gag staining at 2 hours post-transfection (hpt). At 4-6 hpt, Gag molecules multimerize in discrete fluorescent dots that are directed to the pericentriolar region. Gag multimers/viral particles form a perinuclear cluster at 8 hpt, from where they are targeted to the plasma membrane for further release.

### **Dynein is required for JSRV Gag centrosomal targeting**

The cytosol is an exceptionally crowded environment where free movement is particularly restricted for molecules with a size over 20 nm (Luby-Phelps 2000). Microtubules direct intracellular organization by providing a dynamic structure in which motor proteins engage to carry their cargo to specific compartments within the cell (Dammermann, Desai et al. 2003). The polar nature of microtubules allows motor proteins to follow a unidirectional course. Given their size, intracellular Gag multimers and newly formed virions must use cellular motors to traffic within the cytoplasm (Leopold and Pfister 2006). It has been shown that M-PMV centrosomal targeting depends on the integrity of the microtubule network as well as on dynein-mediated transport (Sfakianos, LaCasse et al. 2003). We hypothesized that JSRV Gag could use the same motor proteins to reach the pericentriolar region. If this was the case, transfection of dynamin would affect both JSRV release and intracellular distribution, as its overexpression disrupts dynein-mediated transport towards the minus end of microtubules (Echeverri, Paschal et al. 1996). We cotransfected JSRV with a GFP-tagged dynamin (GFP-p50), and quantified the levels of Gag protein in western blots of viral pellets. As expected, JSRV release exhibited a drastic reduction when coexpressed with GFP-p50 (Figure 34). Moreover, in confocal experiments, the number

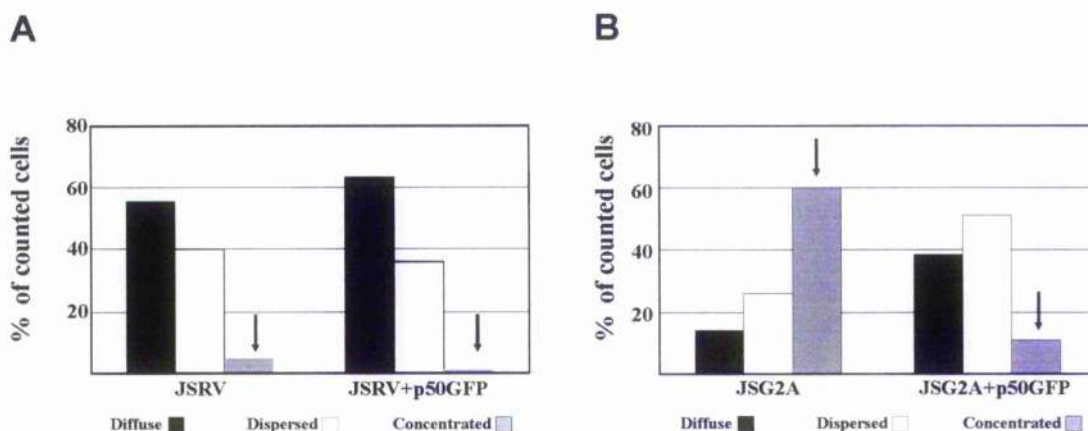
of cells with a concentrated phenotype was consistently reduced from 5% to 1% when JSRV and p50-GFP were coexpressed (Figure 35A). To further confirm this result, we repeated this experiment using JSG2A instead of wild type JSRV, as the majority of the cells expressing the former virus exhibit Gag perinuclear accumulation. The number of cells expressing JSG2A with concentrated Gag was markedly diminished in the presence of p50-GFP from 59% to 11% (Figure 35B), corroborating the results obtained with wild type virus. As a whole, these experiments indicate that dynein transport is necessary for Gag trafficking to the centrosome and that JSRV targeting towards this area is a required step during the virus cellular cycle.



**Figure 34. Overexpression of p50-GFP reduces JSRV exit.**

Viral pellets of cells transfected with the indicated plasmids were subject to SDS-PAGE and immunoblotted with a JSRV MA antiserum. The amount of JSRV Gag protein in the viral pellet was quantified by chemiluminescence.





**Figure 35. Overexpression of p50-GFP alters the intracellular distribution of JSRV Gag.**

Cells transfected with the indicated plasmids were fixed and immunostained using an anti-JSRV MA antiserum. Positive cells were classified as described in Chapter 2. (A) The percentage of cells expressing concentrated JSRV Gag staining is consistently reduced when p50-GFP is overexpressed. (B) The effect of p50-GFP is more evident if a myristoylation-defective JSRV (JSG2A, which normally exhibits a high percentage of cells with concentrated phenotype) is used. Vertical arrows show the reduction in the percentage of cells expressing a concentrated phenotype.

### JLR takes place before JSRV Gag centrosomal targeting

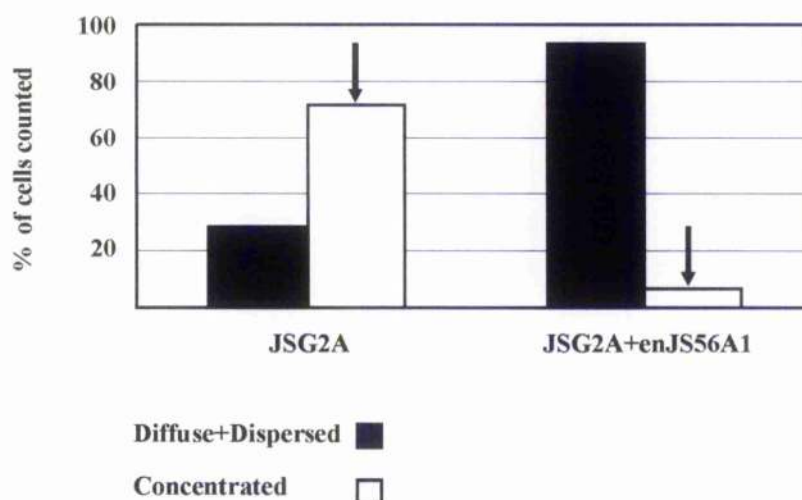
Because our data suggest that Gag centrosomal targeting is an obligatory step in the JSRV replication cycle, we can thus arbitrarily divide the post-integration viral replication events as “pre-centrosomal” or “post-centrosomal” depending on whether they take place before or after Gag reaches the centrosome. Since JSG2A accumulates in this region and cannot proceed any further, we could use it as a tool to determine the time frame in which JLR occurs. If JLR was a pre-centrosomal event, JSG2A would no longer accumulate at the pericentriolar region in the presence of enJS56A1. On the contrary, if enJS56A1 blocked JSRV after centrosomal targeting, the intracellular distribution of JSG2A would not be affected. To address this, we transiently expressed JSG2A in HeLa cells either in single transfections or in cotransfections with enJS56A1. At 24 hours post-transfection, cells were fixed, immunostained and assessed by confocal microscopy. Cells with accumulated perinuclear Gag staining were severely reduced when JSG2A was coexpressed with enJS56A1 (Figure 36). The same results



were obtained when this experiment was repeated using an HA-tagged version of JSG2A (not shown). These results suggest that JLR takes place before JSRV Gag can reach the pericentriolar region.

### Temporal distribution of M-PMV Gag proteins

As a whole, the results hitherto presented in this thesis suggest that JSRV Gag trafficking to the centrosome is an absolute requirement to complete the viral cycle. In the presence of enJS56A1, JSRV cannot reach this region because direct interactions between endogenous and exogenous Gag molecules abrogate centrosomal targeting of the latter, resulting in JLR.



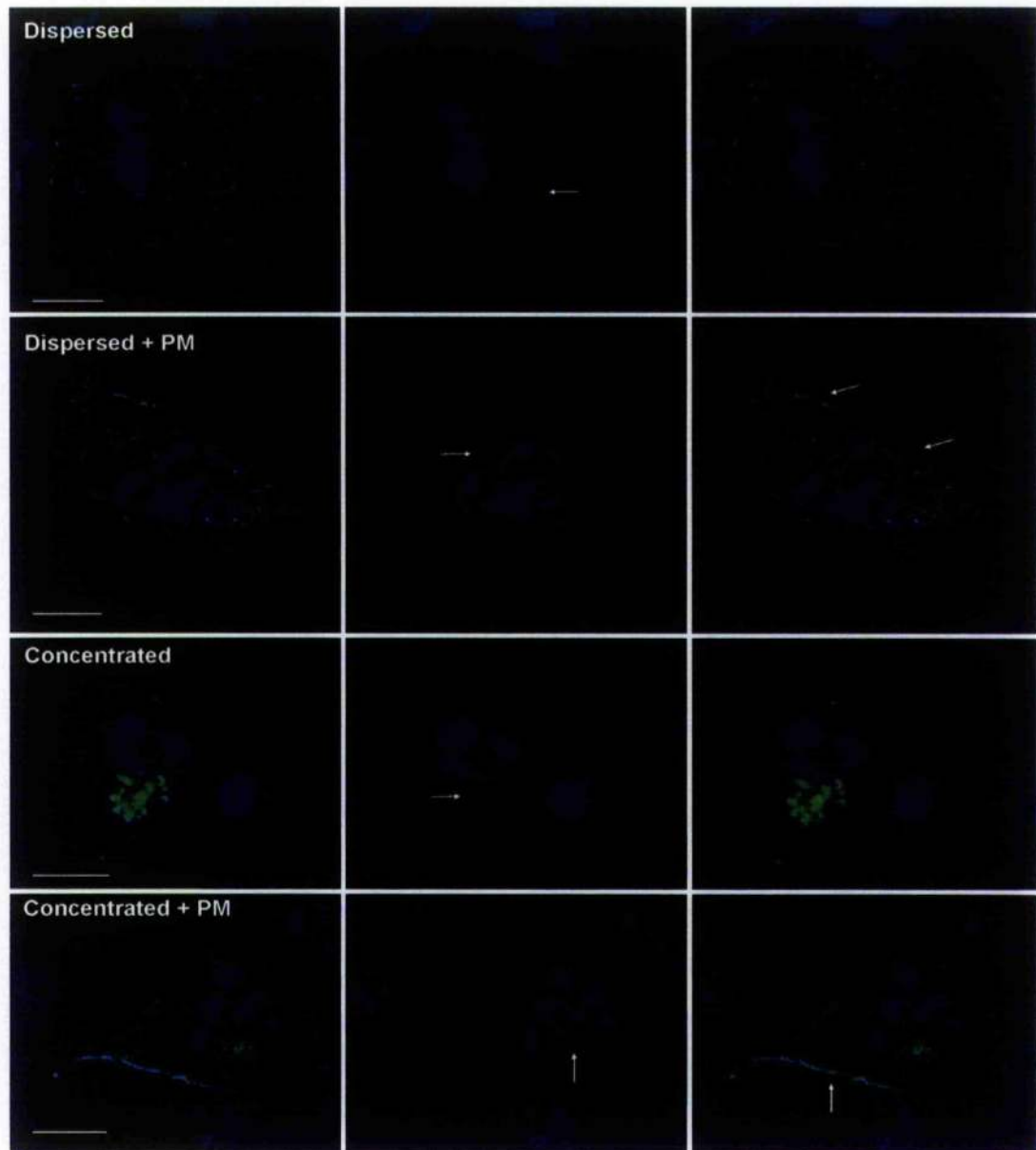
**Figure 36. JLR occurs before JSRV Gag reaches the centrosome.**

Cells expressing the indicated plasmids were immunostained with a JSRV MA antiserum. Positive cells were classified as previously described (see text). The number of JSG2A-expressing cells with a concentrated phenotype is markedly reduced in the presence of enJS56A1 (vertical arrows) indicating that JLR takes place before JSRV Gag is targeted to the pericentriolar region.

IIERV-K and M-PMV also belong to the genus *Betaretrovirus*. Nevertheless, they follow distinct morphogenic pathways: the former assembles at the plasma membrane (Dewannieux, Harper et al. 2006; Lee and Bieniasz 2007) and the latter within the cytoplasm (Vogt 1997). Despite the apparent discrepancy between these two assembly strategies, they must share common requirements since a single amino acid mutation within the CTRS of M-PMV transforms this virus into type-C (Rhee and Hunter 1990). Although both JSRV and M-PMV are targeted to the centrosome, assemble in the cytoplasm, and require Gag myristoylation to reach the plasma membrane (Rhee and Hunter 1987; this work), it should not be assumed that they follow the exact same intracellular assembly pathway. While in M-PMV, the CTRS is apparently responsible for directing Gag nascent molecules to the centrosome (Sfakianos, LaCasse et al. 2003), the presence of a CTRS within JSRV has not been established. Hence the main determinants for JSRV intracytoplasmic assembly remain unknown. Moreover, our previous observations of intracellular virus-like particles in cells coexpressing enJS56A1 and JSRV (Mura 2004), together with our results that indicate that JLR is a pre-centrosomal event, suggest that enJS56A1 does not assemble in the pericentriolar region. Furthermore, results obtained by Manuela Mura in our laboratory show that substitution of arginine 22 with a tryptophan in M-PMV Gag results in partial restriction of wild type virus, although not as manifest as observed with JSR21W (Mura and Palmarini, unpublished).

We wanted to determine whether JSRV and M-PMV shared a common intracellular distribution pattern by confocal microscopy. We assessed wild type M-PMV and two other mutants: a myristoylation-defective M-PMV, where the amino-terminal glycine was replaced by an alanine (M-PMVG2A) (Rhee and Hunter 1987), and a "type-C" M-PMV, where arginine 55 in the CTRS was replaced by a tryptophan as previously reported (M-PMVR55W) (Rhee and Hunter 1990). Cells were transfected with the aforementioned plasmids, fixed at 48 hours post-transfection and immunostained for further analysis by confocal microscopy. Besides the previously described diffuse, dispersed and concentrated phenotypes, we observed several cells with Gag staining at the plasma membrane that were hence classified as such (PM) (Figure 37). It should be noted that we could rarely observe cells with a diffuse phenotype at 24 and 48 hours post-transfection. The PM phenotype was sometimes mixed with a dispersed staining in

the cytoplasm, in which case cells were classified as dispersed + PM. Although in the present study we included a broader range of staining phenotypes, the intracellular distribution of wild type M-PMV was consistent with a previous study where positive cells were classified either as dispersed or concentrated, with 81% and 19% of cells exhibiting the former and latter phenotypes, respectively (Sfakianos, LaCasse et al. 2003). We expected M-PMVG2A to display a similar phenotype to the one observed with JSG2A, with the vast majority of cells exhibiting concentrated Gag staining in the perinuclear region, because wild type M-PMV is directed to the pericentriolar region (Sfakianos, LaCasse et al. 2003) and disruption of the myristoylation signal abrogates membrane targeting but not assembly (Rhee and Hunter 1987). However, that was not the case in four independent experiments performed at late and early timepoints (Figure 38 and see below). M-PMVG2A displayed big positive fluorescent specks and lacked Gag staining at the plasma membrane, which was in agreement with previous electron microscopy observations (Rhee and Hunter 1987). As predicted, the M-PMV mutant bearing an altered CTRS (M-PMVR55W) showed the highest number of positive cells with a plasma membrane phenotype (Figure 38). While these results confirm previous



**Figure 37. Confocal microscopy of COS cells expressing Mason-Pfizer monkey virus.**

Immunofluorescence was performed using a polyclonal antiserum towards M-PMV CA and a monoclonal antibody against gamma-tubulin, a centrosomal marker. Positive cells were classified according to their staining pattern (see text). Phenotypes are indicated on the top left of each row. Gag staining is in green, centrosomes in red, and nuclei in blue. White arrows indicate centrosomes (middle column) or Gag staining at the plasma membrane (right column). Scale bar is approximately 10  $\mu\text{m}$ .

studies (Rhee and Hunter 1987; Rhee and Hunter 1990; Sfakianos, LaCasse et al. 2003), the differences observed between JSRV and M-PMV Gag intracellular distribution may reflect distinct trafficking pathways during assembly.

We decided to perform confocal experiments at different timepoints using the same constructs described above (Figure 39). At 2 hpt, few cells transfected with wild type M-PMV exhibited Gag positive staining and the majority of them displayed a diffuse phenotype (Figure 39, top panel). After 4 hours, cells with dispersed intracellular fluorescence prevailed. Concentrated Gag staining was visible from early timepoints but did not reach a marked peak as observed with JSRV. The number of cells with a PM phenotype increased with time and peaked to 20% at 24 hpt (Figure 39, top panel).

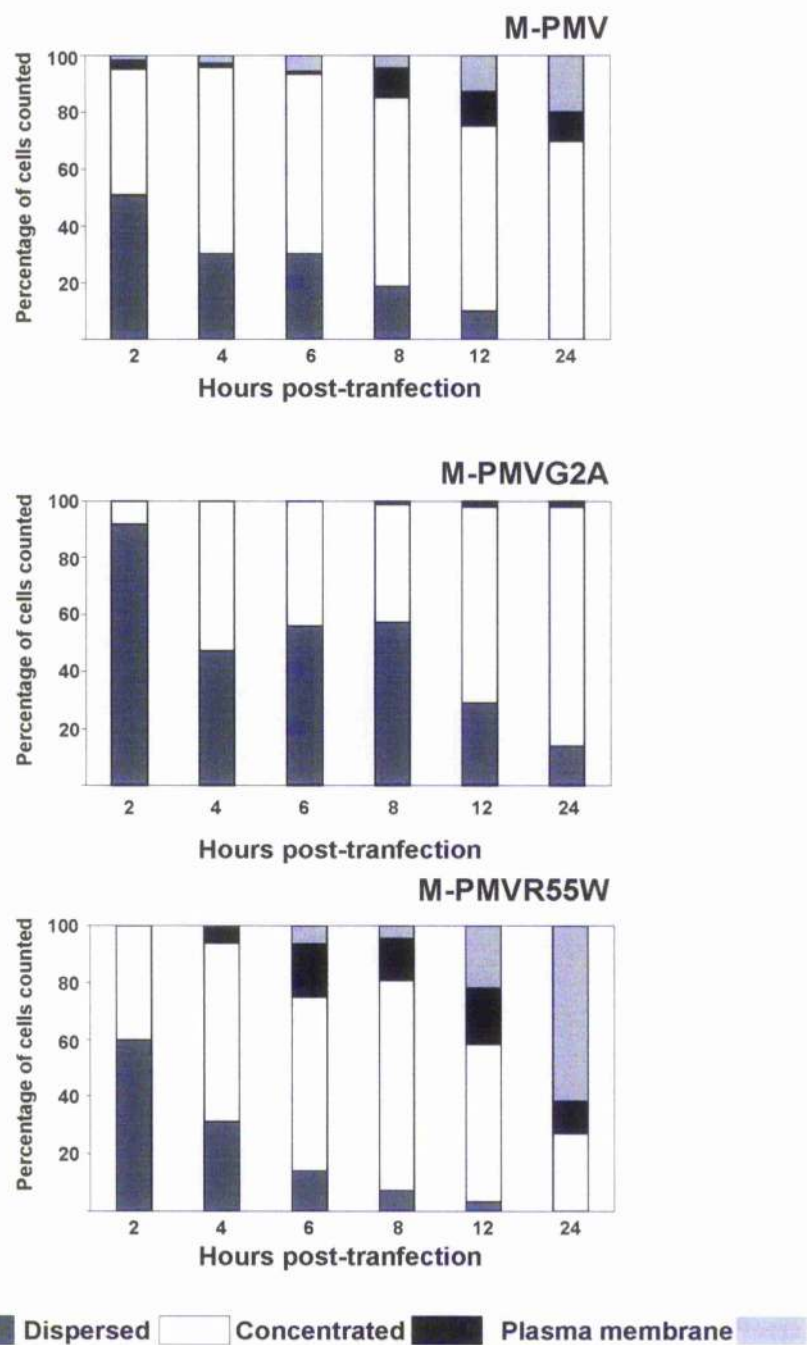


**Figure 38. Confocal microscopy of COS cells expressing M-PMV, M-PMVG2A, and M-PMVR55W.**

Transfected plasmids are indicated on the top left of each photograph. Most representative phenotypes are shown. Note the bigger size of the fluorescent dots in M-PMVG2A when compared with wild type M-PMV. Gag staining is in green and nuclei in blue. Scale bar is approximately 10  $\mu\text{m}$ .

The myristoylation-defective M-PMVG2A exhibited an unexpected temporal distribution pattern as few cells displayed a concentrated phenotype throughout the whole timeframe of the experiment (the maximum observed value was 2% at 12 and 24 hours), in marked contrast with JSRV (compare the middle panels of Figures 32 and 39). Another particular (although predictable) feature of this mutant was the total lack of Gag staining at the plasma membrane, even at late timepoints. The opposite was observed with the CTRS mutant M-PMVR55W, as the number of cells with a plasma





**Figure 39. Kinetics of the intracellular distribution of M-PMV, M-PMVG2A and M-PMVR55W.**

COS cells were transfected with the indicated plasmids and fixed at different times post-transfection. After immunostaining, cells were quantified according to their Gag staining phenotype. Each bar represents a specific time-point at which cells were fixed, immunostained and classified according a predefined staining criterion (see text).

membrane phenotype accumulated with time and reached a peak value of 62% at 24 hours post-transfection (Figure 39, bottom panel).

Overall, these data indicate that although JSRV and M-PMV assemble within the cytoplasm, they display different kinetics of intracellular trafficking (or the same determinants influence Gag trafficking in a different manner). First, despite the fact that both viruses exhibit a concentrated phenotype, JSRV shows Gag accumulation in a perinuclear region at earlier timepoints than M-PMV. Moreover, only JSRV displayed a bottleneck pattern at this stage, as the number of cells with concentrated phenotype increased and decreased in a reproducible temporal fashion. Second, cells expressing JSRV do not show accumulation of Gag staining at the plasma membrane, in contrast with cells transfected with M-PMV at late time points. These differences may reflect either distinct intracellular trafficking pathways or diverse kinetics along the same pathway. However, when the myristoylation signal of these two viruses is disrupted they exhibit different phenotypes, as JSRV accumulates in the pericentriolar region and M-PMV remains mainly dispersed within the cytoplasm.

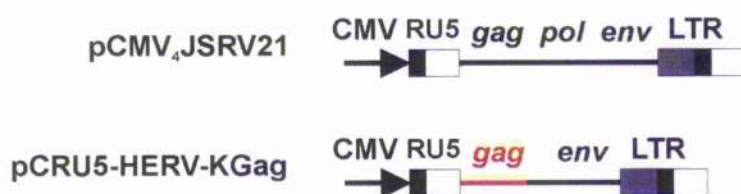
### **A JSRV/HERV-K chimera exhibits different intracellular distribution than JSRV and escapes JLR**

JLR appears to be the result of altered intracellular trafficking of JSRV Gag molecules. In the presence of enJ56A1, JSRV can no longer reach the pericentriolar region, which seems to be a crucial event for the assembly of type-B/D Betaretroviruses. It is therefore plausible to speculate that changing the site of assembly of JSRV by bypassing its need to reach the centrosome could abrogate the sensitivity to JLR. HERV-K is a Betaretrovirus but assembles at the plasma membrane (Dewannieux, Harper et al. 2006; Lee and Bieniasz 2007). We hence constructed a chimeric virus in which HERV-K Gag replaced JSRV Gag (pCRU5-HERV-KGag, Figure 40A). Transfection of pCRU5-HERV-KGag in 293T cells resulted in the release of viral particles into the supernatants (Figure 40B). A tagged version of this plasmid bearing the HA epitope at the end of NC was also constructed. In confocal microscopy experiments, cells expressing HERV-K Gag displayed diffuse, dispersed, and plasma membrane phenotypes (Figure 40D), and

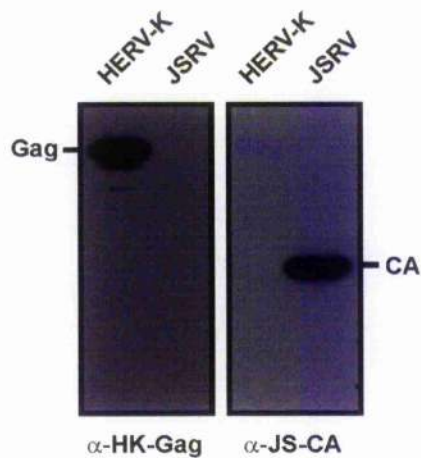


only four cells out of the two hundreds counted exhibited concentrated Gag staining, consistent with a type-C assembly (Figure 40D). Disruption of the myristoylation signal of pCRU5-HERV-KGag resulted in a reduction on the number of cells with fluorescent signal at the plasma membrane and a concomitant increase in the dispersed phenotype (Figure 40C and D). When HERV-K Gag was coexpressed with either JSRV or enJS56A1, we did not observe colocalization (Figure 41), which was consistent with distinct morphogenic pathways. Our antiserum directed toward JSRV MA did not immunodetect HERV-K Gag in cells transfected neither in confocal microscopy (not shown) nor in western blot (Figure 40B). Because the main trafficking signals that direct Gag to the site of assembly lie within the MA domain, we made an HA-tagged HERV-K/JSRV chimeric construct bearing HERV-K MA and the CA, NC and p4 from JSRV (Figure 42A). In theory, this chimera could still multimerize with Gag molecules from wild type JSRV as they share the same CA (Ako-Adjei, Johnson et al. 2005), but because the domains that lie at the N-terminus of CA are derived from HERV-K, should assemble at the plasma membrane, constituting a chimeric “type-C” HERV-K/JSRV. This chimeric virus, referred to as pHVK<sub>MA</sub>JS<sub>CANC</sub>, was assessed by confocal microscopy and western blotting. The confocal phenotypes observed by pHVK<sub>MA</sub>JS<sub>CANC</sub> were reminiscent of cells expressing HERV-K Gag (Figure 42 B). We observed some degree of colocalization in cells expressing pHVK<sub>MA</sub>JS<sub>CANC</sub> and JSRV or enJS56A1, but nevertheless much lower than the one observed when JSRV and enJS56A1 are expressed in the same cell. We could detect viral particles in supernatants of 293T cells transfected with pHVK<sub>MA</sub>JS<sub>CANC</sub> by western blot, indicating that this chimeric virus was competent for viral exit (Figure 42A). Moreover, this chimera was able to release viral particles even in the presence of enJS56A1 (Figure 42A). Because pHVK<sub>MA</sub>JS<sub>CANC</sub> lacks the viral protease, it only releases immature Gag (Figure 42A). When an HA-tagged version of this construct was cotransfected with JSRV or enJS56A1, we could detect cleaved HIA-tagged Gag in the supernatants, indicative of some level of co-assembly (Figure 42A, black arrow). Overall, these results show that this chimera displayed an intracellular distribution resembling a type-C retrovirus, was competent for viral release, coassembled with JSRV (at least partially), and most importantly, could escape JLR.

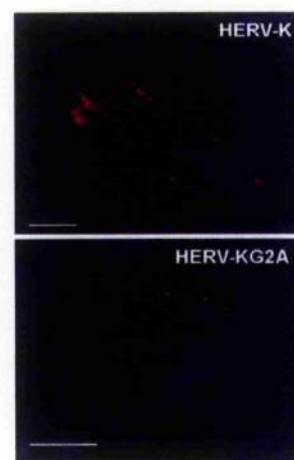
**A**



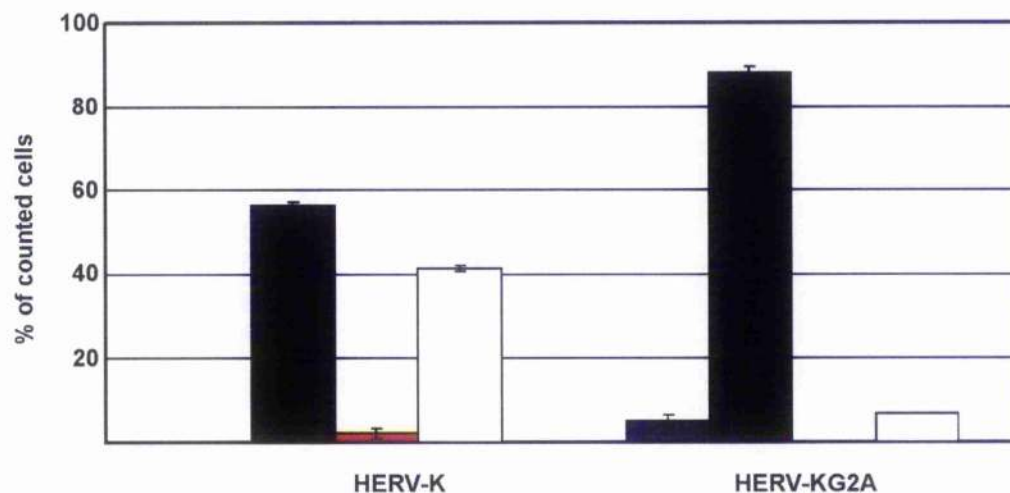
**B**



**C**



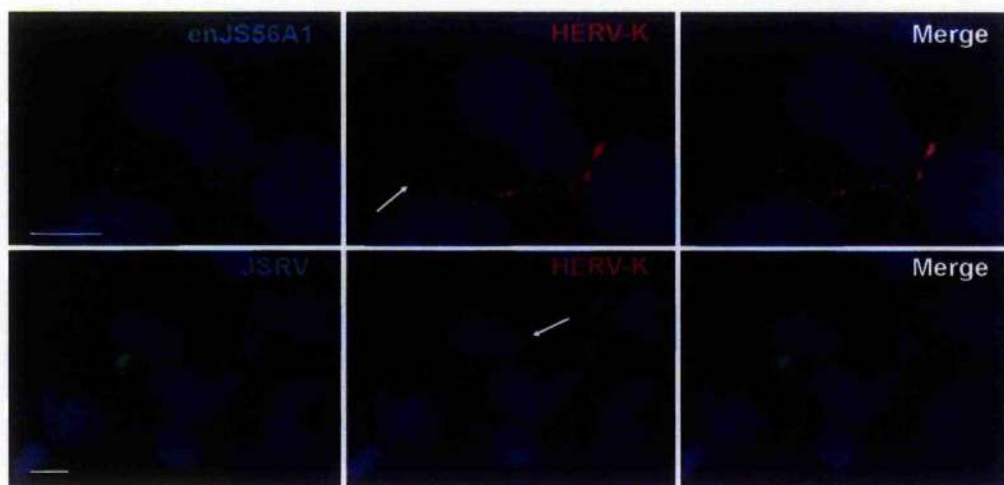
**D**



■ Diffuse ■ Dispersed ■ Concentrated □ Plasma membrane

Figure 40. Characterization of a chimeric HERV-K/JSRV construct (see legend on next page).

(from previous page) pCRU5HERV-KGag is budding competent and exhibits Gag staining at the plasma membrane. (A) pCMV4JSRV and pCRU5HERV-KGag plasmid constructs. In pCRU5HERV-KGag the gag gene of HERV-K replaces JSRV gag and the frameshift required to express pol is disrupted, inhibiting Pol expression. JSRV genes are represented in black and HERV-K gag is represented in red. (B) Viral pellets of 293T cells transfected with HERV-K Gag or JSRV expression plasmids were resolved by SDS-PAGE and immunoblotted with anti-HERV-K Gag or anti- JSRV CA, as indicated below each panel. Note that the construct possessing HERV-K Gag lacks the viral protease and thus releases immature particles. There is no cross-reaction between the two antisera. (C) Confocal microscopy of COS cells expressing HA-tagged versions of HERV-K Gag and a myristoylation defective mutant (HERV-KG2A). Anti-HA staining is displayed in red and nuclei in blue. The scale bar is approximately 10  $\mu$ m. Characteristic phenotypes of each construct are shown. (D) Disruption of the myristoylation signal of HERV-K decreases Gag accumulation at the plasma membrane. COS cells expressing the indicated plasmids were immunostained, assessed by confocal microscopy and counted. HERV-K Gag accumulation at the plasma membrane is dramatically reduced when myristoylation is abrogated, with a consequent increase in cells with a dispersed phenotype.



**Figure 41. JSRV and enJS56A1 exhibit distinct intracellular distribution than HERV-K Gag.**

Confocal microscopy of COS cells coexpressing an HA-tagged HERV-K Gag with either JSRV or enJS56A1. Anti-HA staining is displayed in red, anti-JSRV MA in green and nuclei in blue. White arrows indicate HERV-K Gag at the plasma membrane, which contrasts with the intracellular staining of JSRV and enJS56A1 Gag. Scale bar is approximately 10  $\mu$ m.

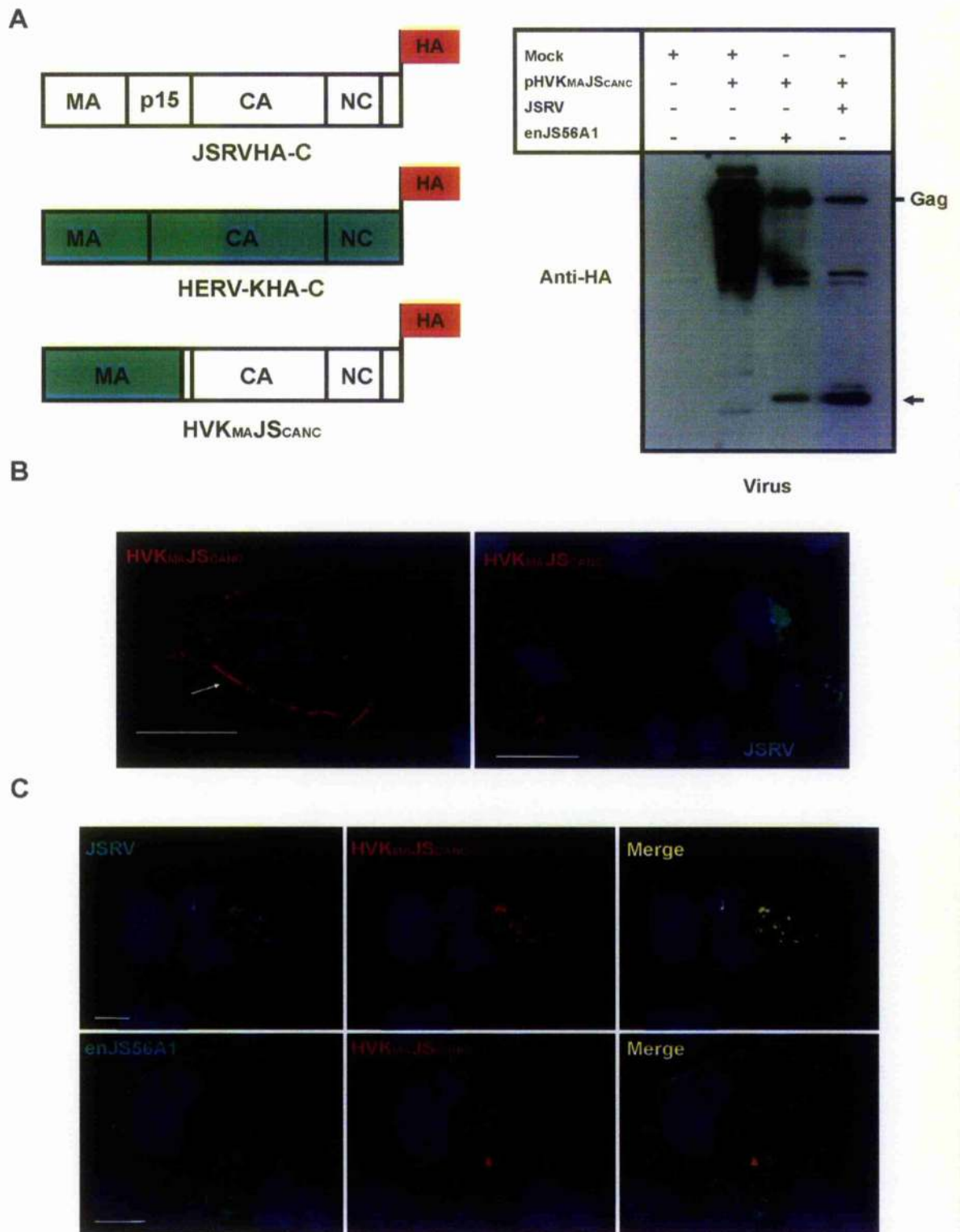


Figure 42. A budding-competent HERV-K/JSRV chimera is directed to the plasma membrane and can escape JLR (see legend on next page)

(From previous page) (A) Left: schematic representation of JSRV and HVKMAJSCANC tagged constructs. The HA epitope (indicated as a red flag) was inserted at the C-terminal end of Gag. JSRV and HVKMAJSCANC are drawn as white and green boxes, respectively. Right: HVKMAJSCANC is budding-competent and escapes JLR. Viral pellets of 293T cells transfected or cotransfected with HVKMAJSCANC, enJS56A1, and JSRV expression plasmids were resolved by SDS-PAGE and immunoblotted with anti-HA. HVKMAJSCANC releases immature viral particles because it lacks the viral protease. Cotransfection of this chimera with JSRV or enJS56A1 results in partial Gag cleavage (horizontal arrow), indicating some level of coassembly. (B) Left: cells expressing HVKMAJSCANC display Gag accumulation at the plasma membrane resembling HERV-K. Right: cells expressing either HVKMAJSCANC or JSRV exhibit distinct intracellular distribution. Note that HVKMAJSCANC is observed near the plasma membrane and JSRV in the perinuclear region. JSRV is in green, HVKMAJSCANC in red, and nuclei in blue. (C) HVKMAJSCANC partially colocalizes with JSRV and enJS56A1. JSRV and enJS56A1 are in green, HVKMAJSCANC in red, and nuclei in blue. Scale bar is approximately 10  $\mu\text{m}$ .

## ***Discussion***

JLR is the result of Gag-Gag interactions between JSRV and enJS56A1. Normal targeting of JSRV Gag is altered in the presence of the enJS56A1 Gag and the viral cycle cannot be completed. Previous studies on Mason-Pfizer monkey virus, a Betaretrovirus closely related to JSRV, have shown that an 18-amino acid sequence within the MA domain, termed the cytoplasmic targeting/retention signal, directs Gag molecules to a pericentriolar region, where assembly is thought to occur (Rhee and Hunter 1990; Sfakianos, LaCasse et al. 2003). Moreover, disruption of the myristoylation signal affects trafficking but not assembly of M-PMV (Rhee and Hunter 1987).

In this chapter we could determine the timeframe in which JLR occurs. We characterized the intracellular distribution of JSRV, enJS56A1 and JSG2A in a temporal fashion and showed that centrosomal targeting of Gag proteins is an obligatory step of the JSRV cell cycle. These experiments allowed us to divide the post-integration events that occur in the JSRV replication cycle as pre- and post-centrosomal. We also determined that JSG2A, a myristoylation-defective mutant, reaches the pericentriolar region, where it accumulates and cannot proceed any further. Because JSG2A cannot reach the centrosome in the presence of enJS56A1, we concluded that JLR takes place at a pre-centrosomal stage. In our experimental setting, we determined that JSRV Gag is targeted to the pericentriolar region as early as 4 hours post-transfection. Frederick Arnaud's observation of cells coexpressing JSRV and enJS56A1 Gag displaying strong colocalization at 4 hours post-transfection supports this view (see paper by Arnaud, Murcia and Palmarini attached to this thesis).

JSG2A proved to be a valuable tool in providing further insight on the molecular biology of JSRV. We could determine that JSRV Gag uses the dynein/dynactin molecular motor complex to reach the centrosome, as JSG2A can no longer accumulate in this region and JSRV viral release is drastically reduced if the dynein-mediated transport is disrupted by overexpression of dynamitin.



Gag myristoylation constitutes a crucial signal for membrane targeting (Rein, McClure et al. 1986; Rhee and Hunter 1987; Swanstrom and Wills 1997). According to studies performed on M-PMV, viral particles assemble in the pericentriolar region, where they colocalize with Env-containing vesicles and are further transported to the plasma membrane by yet uncharacterized trafficking pathways that include recycling endosomes (Sfakianos and Hunter 2003; Sfakianos, LaCasse et al. 2003). JSRV Gag colocalizes with recycling endosomes, and siRNA knockout of Rab11 affects viral release (see paper by Arnaud, Murcia and Palmarini attached to this thesis). We speculate that JSRV binding to its cognate vesicles may require proper interactions with lipid membranes, and hence the lack of amino-terminal myristate in JSG2A could hamper its ability to engage in vesicular transport to the plasma membrane.

Based on previous studies on M-PMV, it has been proposed that type-B/D retroviruses assemble at the pericentriolar region, where nascent Gag molecules are directed by the CTRS (Sfakianos, LaCasse et al. 2003). Different lines of investigation from our laboratory support the notion that assembly of enJS56A1 may not occur in this region. First, viral particles can be observed by electron microscopy in cells expressing enJS56A1 despite the fact that its Gag staining is rarely observed close to the centrosome by confocal microscopy (Mura 2004, and paper attached to this thesis by Mura, Murcia et al.). Second, the data presented here suggest that JLR is a pre-centrosomal event, and virus-like particles have also been observed by EM in cells coexpressing JSRV and enJS56A1 (Mura 2004). This is why we repeated the same temporal confocal experiments using a variety of M-PMV-based constructs. The first noticeable difference was the observation of Gag staining at the plasma membrane with wild-type M-PMV. We have never observed JSRV (or enJS56A1) Gag accumulating at the plasma membrane, the only exception being the double L-domain mutant of JSRV described in the previous chapter. Such difference in Gag staining localization can be due to different budding rates (i.e. JSRV budding out much faster than M-PMV), or to active targeting of M-PMV to the plasma membrane for other reasons than budding.

On the other hand, we expected that disruption of the myristoylation signal in M-PMV would result in Gag centrosomal accumulation as observed for JSRV. To our surprise, M-PMVG2A did not exhibit any accumulation in the perinuclear region. On the



contrary, the majority of M-PMV-expressing cells showed either diffuse or dispersed Gag staining and only a handful of cells displayed a concentrated phenotype. A previous report showed that the CTRS-pericentriolar targeting of M-PMV Gag depends on dynein-mediated transport along microtubules (Sfakianos, LaCasse et al. 2003). Our results suggest that Gag myristoylation may comprise an extra requirement for M-PMV Gag molecules to traffic to the centrosome. An older report from the same laboratory showed that a myristoylation-defective M-PMV assembles intracytoplasmic type-A particles (ICAPS) (Rhee and Hunter 1987). Although in that work Gag pericentriolar targeting was not assessed, the authors described that ICAPs were observed “deep in the cytoplasm”. The aforementioned reports (Rhee and Hunter 1987; Sfakianos, LaCasse et al. 2003) apparently contradict our observations. If the centrosome is the site of assembly for M-PMV (Sfakianos, LaCasse et al. 2003), and myristoylation is required for M-PMV centrosomal targeting (this work), how can the presence of ICAPs be explained for a myristoylation-defective M-PMV (Rhee and Hunter 1987)? A simple explanation could be that M-PMVG2A assembles in a different cell compartment. However, differences in the cell types used or the levels of expression achieved by different transfection protocols could also explain these apparently contradictory results. It is clear that further experimental work should be done to address this issue.

To determine whether the site of assembly had a significant role in JLR, we constructed a HERV-K/JSRV budding-competent chimera (pHVK<sub>MA</sub>JS<sub>CANC</sub>). We chose HERV-K because it belongs to the genus Betaretrovirus (like JSRV) and assembles at the plasma membrane (Dewannieux, Harper et al. 2006; Lee and Bieniasz 2007). Since the trafficking signals that determine the site of assembly are located at the N-terminal region of Gag, we replaced JSRV MA and p15 with homologous sequences derived from HERV-K. To allow this chimeric Gag to interact with wild type JSRV Gag, we kept CA, NC and p4 intact from JSRV (Ako-Adjei, Johnson et al. 2005). Hence, pHVK<sub>MA</sub>JS<sub>CANC</sub> was expected to be targeted to the plasma membrane but still to maintain its ability to bind JSRV-derived Gag molecules. By confocal microscopy this chimera exhibited Gag staining at the plasma membrane, similar to the one observed with HERV-KHA-C, a budding-competent plasmid bearing the full-length HERV-K Gag that displays a type-C phenotype. Notably, this budding-competent chimera was able to “escape” JLR when coexpressed with enJS56A1. The observation of cleaved

Gag in the supernatant of transfected cells suggested some degree of coassembly (and therefore rescue) between HIVK<sub>MA</sub>JS<sub>CANC</sub> and enJS56A1. Although we did not quantify the intensities of the bands detected by western blot, it seemed that our HERV-K/JSRV chimera exhibited a higher degree of cleavage when expressed with JSRV (compare the bands indicated with an arrow in Figure 42A). We experienced a similar impression when colocalization between pHVK<sub>MA</sub>JS<sub>CANC</sub> and JSRV or enJS56A1 was assessed (compare the upper and lower panels of Figure 42C).

These data may suggest that a “type-C” JSRV escapes JLR because it can reach the site of budding by an alternative pathway that cannot be blocked by enJS56A1. However, in the previous chapter we have shown that an intact enJS56A1 Gag was required for JLR, and also that an enJS56A1-derived construct bearing a major deletion in the NC but with an intact CA was able to coassemble with JSRVΔPro in functional complementation assays. Those experiments suggest that proper assembly between enJS56A1 and JSRV is required for JLR to occur. Hence, it is possible that enJS56A1 cannot block our HERV-K/JSRV simply because their Gag molecules cannot interact/assemble appropriately.

Data generated by Manuela Mura in our laboratory have shown that an M-PMV single mutant bearing an arginine to tryptophan substitution (M-PMVR22W) like the one observed in enJS56A1 partially blocks the exit of wild-type M-PMV. Notably, when a second mutation that disrupts M-PMV CTRS is added, the degree of the restriction observed is comparable to JLR (Mura and Palmarini, unpublished). Based on those results and the ones presented in this thesis, we could speculate that because M-PMV can potentially assemble within the cytoplasm or at the plasma membrane (Rhee and Hunter 1987; Rhee and Hunter 1990), a blockade that takes place before centrosomal targeting could result in partial restriction as the virus possesses the information to assemble at the plasma membrane. It is also feasible that coassembly between M-PMVR22W and wild-type M-PMV abrogates the dominant activity of the CTRS in a similar way as the R55W mutation does, and thus M-PMV is targeted to the plasma membrane. This model should require some degree of interaction between Gag molecules before centrosomal targeting. JSRV may, instead, have only one available

pathway as the presence of a CTRS has not been determined yet. If that is the case, blockade of centrosomal targeting could explain the dramatic effects of JLR.

It is appealing to think that restriction mechanisms similar to JLR can be extended to other retroviruses. Although encouraging results have been obtained in our laboratory, more experiments are required to determine such possibility.

## **Chapter 5**

### **Recombinant expression and purification of JSRV matrix protein.**

#### ***Summary***

This section will cover the development of a protocol for recombinant overexpression and purification of the matrix protein of JSRV. This scheme was aimed as a first step of future high resolution structural studies (i.e. nuclear magnetic resonance and/or crystallography) to characterize JSRV MA at the atomic level. Given the crucial role played by JSRV MA in JLR, such studies could provide insight in the structural basis of this late block. This part of the project was mainly developed at Jeff Urbauer's laboratory, located in the Department of Chemistry of the University of Georgia.

#### ***Introduction***

From a structural point of view, retroviruses could be regarded as an arrangement of proteins surrounding two molecules of viral RNA that constitute the central core of the viral particle, which is enveloped in a cell-derived lipid coat. The inner architectural platform of the viral particle is provided by the polyprotein Gag, where the viral structural proteins constitute at least three linear domains that always follow the same order (from amino to carboxy terminus): matrix (MA), capsid (CA), and nucleocapsid (NC). During maturation, Gag is cleaved at specific sites by the viral protease into the individual domains. Maturation is required for viral infectivity, takes place upon viral release, and does not change the spatial distribution of the cleaved products. Within the viral particle, the amino terminal end of Gag faces the viral envelope while the carboxy terminal end faces the centre of the particle.

Because Gag is the major structural retroviral protein, its sole expression is sufficient to assemble into virus-like particles. As described in previous sections, retroviruses can follow two different morphogenic pathways. The so-called type-C viruses are targeted

to the plasma membrane, where assembly and budding take place concurrently. In contrast, for type-B and type-D retroviruses, those two events are separated in time and space, as they first assemble within the cytoplasm of the infected cell and then traffic to the plasma membrane to be released. Gag possesses different motifs that are essential for viral particle trafficking and morphogenesis, such as targeting of Gag molecules towards specific subcellular sites (i.e. the pericentriolar region or the plasma membrane); interaction among Gag proteins, or viral budding once viral particles have been assembled. The spatio-temporal timing of these events is finely regulated.

Regardless of the morphogenic pathway followed, Gag molecules must travel to the plasma membrane. The trafficking signals responsible for membrane targeting lie within the matrix protein, whose interaction with biomembranes is highly dynamic. During viral egress, MA associates with the inner leaflet of the plasma membrane (which will be then part of the viral envelope) during or after assembly. This association is transient, since MA dissociates from the viral envelope when entering a target cell. Most retroviruses possess two membrane targeting signals within MA: a positively charged surface area that interacts with the acidic heads of the membrane phospholipids in an electrostatic fashion, and an amino-terminal myristate which partitions into the membrane (Conte and Matthews 1998). Although not all retroviral matrix proteins are myristoylated, they all bear a basic surface patch (Murray, Li et al. 2005). Myristoylation is a cotranslational modification that consists on the addition of a myristate moiety to the amino terminal glycine of the Gag polyprotein. The hydrophobic nature of the fatty acid chain of the myristate allows myristoylated proteins to partition into the membrane hydrocarbon. Structural studies performed on HIV MA suggest that exposure of the myristate is regulated by an "entropic switch" that depends on the oligomeric state of the protein (Tang, Loeliger et al. 2004).

Another important trafficking motif that lies within MA is the cytoplasmic/targeting retention signal (CTRS), first described in Mason-Pfizer monkey virus. This 18-residue long sequence is responsible for targeting M-PMV Gag molecules to the pericentriolar region, the proposed site of assembly for this virus (Rhee and Hunter 1990; Choi, Park et al. 1999; Sfakianos, LaCasse et al. 2003). It has been shown that a single mutation within the CTRS is sufficient to direct assembly of M-PMV to the plasma membrane

(Rhee and Hunter 1990). Conversely, introduction of the M-PMV CTRS sequence into MoMLV causes the latter to assemble immature capsids in the cytoplasm (Choi, Park et al. 1999).

Structural biochemical studies on retroviral proteins are of utmost importance to understand molecular events at to the atomic level and to design specific tailored-made drugs that could block viral infections with minimum (or null) side-effects to the host. The structure of the entire Gag in any retrovirus has not been solved yet. However, several individual retroviral proteins (structural and non-structural) have been solved at the atomic level by means of nuclear magnetic resonance (NMR) or crystallography. To date, the three-dimensional structures of the MA proteins of eight retroviruses have been solved: HIV, SIV, MLV, BLV, M-PMV, HTLV-II, RSV, and EIAV (Matthews, Barlow et al. 1995; Rao, Belyacv et al. 1995; Christensen, Massiah et al. 1996; Matthews, Mikhailov et al. 1996; Conte, Klikova et al. 1997; McDonnell, Fushman et al. 1998; Hatanaka, Iourin et al. 2002; Riffel, Harlos et al. 2002).

*In vitro* expression of the endogenous locus enJS56A1 blocks the release of Jaagsiekte sheep retrovirus. The main determinant for JSRV late restriction is a conserved arginine residue in position 21 (R21) of the matrix protein, which is naturally substituted by a tryptophan in enJS56A1 (Mura, 2004; this work). Gag residue R21 is absolutely required for viral release, as replacement of this residue even with a conservative lysine abrogates viral exit (this work). During the retroviral cycle, JSRV Gag is targeted to the centrosome in a dynein-dependent fashion (see Chapter 4 and paper attached by Arnaud, Murcia and Palmarini). We have shown in previous sections of this thesis that centrosomal targeting is a required step for efficient completion of the JSRV retroviral cycle. Expression of dynamitin (which disrupts the dynein-mediated transport to the pericentriolar region) dramatically reduces the number of JSRV-positive cells with Gag accumulation near the centrosome as well as the amount of virus in the supernatant of transfected cells. Data generated by Frederick Arnaud in our laboratory suggest that centrosomal targeting of JSRV Gag is altered in the presence of enJS56A1, and the experiments described in this thesis indicate that such mislocalization is due to the formation of chimeric multimers/viral particles. Such Gag multimers are formed at a pre-centrosomal stage, hampering their ability to reach the pericentrosomal region.

Because in previous sections we studied JSRV and enJS56A1 Gag proteins from a functional perspective, we wanted to address the structural basis of their distinct biological behaviour. First, we tried to overexpress and purify recombinant Gag proteins of JSRV and enJS56A1 to study their conformation in solution. Unfortunately we did not manage to express the entire Gag in bacteria. The most probable reason for the lack of protein expression is that the codons of numerous amino acid residues of enJS56A1 and JSRV Gag are rare in several strains of bacteria. Therefore, we tried to overexpress the MA proteins of JSRV and enJS56A1 but we only managed to obtain good levels of expression of JSRV MA. Consequently, the work described in this section is focused solely on the MA protein of JSRV.

Since residue R21 is located within the MA and our data suggest that it is the main determinant for JLR and centrosomal targeting, we modelled the tertiary structure of JSRV and enJS56A1 MA based on the solved structure of the highly homologous matrix protein of M-PMV (Conte, Klinkova et al. 1997). To pursue biophysical and biochemical studies on JSRV MA, we developed a method for high level overexpression and high-yield/high purity production of this protein. We cloned JSRV MA into a commercially available vector bearing an inducible T7 promoter for overexpression in *Escherichia coli*. The recombinant protein produced was then purified using a classical purification scheme. We obtained a high yield of protein with an excellent level of purity. Moreover, heteronuclear NMR spectra of isotopically labelled JSRV MA indicate that the purified protein is properly folded. On the other hand, native JSRV MA formed crystals that diffracted at 1.4 Å (William Lanzilotta, personal communication). We therefore developed a modified purification method for the production of JSRV MA labelled with selenomethionine for crystallographic studies. Finally, we determined the secondary structure of JSRV MA by circular dichroism and the quaternary structure using analytical ultracentrifugation. The high yield/high purity method that we developed for JSRV MA production will allow future high resolution studies either by NMR or crystallography.



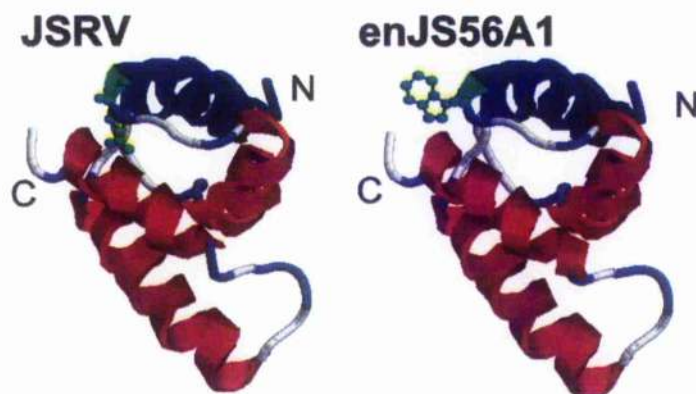
## **Results**

### **Modelling of the matrix proteins of JSRV and enJS56A1**

As aforementioned, eight retroviral matrix proteins have been hitherto solved. Despite their low sequence similarity, their three dimensional structures display a remarkable resemblance (Conte and Matthews 1998; Murray, Li et al. 2005) since they all exhibit a compressed fold with a core of four  $\alpha$  helices (Murray, Li et al. 2005). Because the matrix proteins of JSRV and M-PMV display 39% identity and 64% similarity, we modelled JSRV MA using M-PMV-MA as a template to estimate the spatial position of arginine 21 and the possible structural consequences of an R to W substitution. There is only one available model of M-PMV MA at the protein databank of the Research Collaborative for Structural Bioinformatics (RCSB), which is based on NMR data (Conte, Klikova et al. 1997) and can be found at the RCSB PDB website under the PDB ID **1bax** (<http://www.rcsb.org/pdb/home/home.do>). Unfortunately, this structure lacks the side chains of the proteins because the pdb file deposited is only an alpha-carbon trace. Therefore, we first added the side chains by using MaxSprout, a web-based algorithm for generating side chains coordinates from a Carbon (alpha) trace (<http://www.ebi.ac.uk/maxsprout/>). To model JSRV MA, we used Swiss-Model, a web-based fully automated protein structure homology-modelling server (<http://swissmodel.expasy.org/SWISS-MODEL.html>). Based on the model we obtained, R21 is positioned at the end of the first  $\alpha$ -helix of MA, in an exposed area of the protein (Figure 43). As described in Chapter 3, two other basic residues lie by R21: K19 and H20. This basic patch may provide a favourable electrostatic charge to promote interactions between the matrix protein (or Gag) and phospholipid membranes at different stages of the retroviral replication cycle. The R to W mutation in Gag position 21 present in enJS56A1 alters the net charge of the MA protein and furthermore introduces a highly hydrophobic amino acid that could potentially change the three dimensional conformation of the protein.

Based on this model, we can hypothesize that the mutation observed in enJS56A1 can result in structural changes that could affect the association between MA and

phospholipid membranes, and/or the normal folding of the protein, thus altering the biological functions of MA/Gag and resulting in JLR.



**Figure 43. Modelling of the putative JSRV and enJS56A1 MA proteins.**

The tertiary structures of the matrix proteins of enJS56A1 and JSRV were modelled by using the Swiss Model Server (<http://swissmodel.expasy.org>). The matrix protein of MPMV (Protein Data Bank ID code 1BAX [PDB] ) was used as a template (see text). Models are displayed in ribbons, and the W21 and R21 are displayed in green in a ball-and-stick format. The N and C terminals are indicated. The first  $\alpha$ -helix is shown in blue. Both models were derived from the amino-terminal 92-aa residues of JSRV and enJS56A1 Gag.

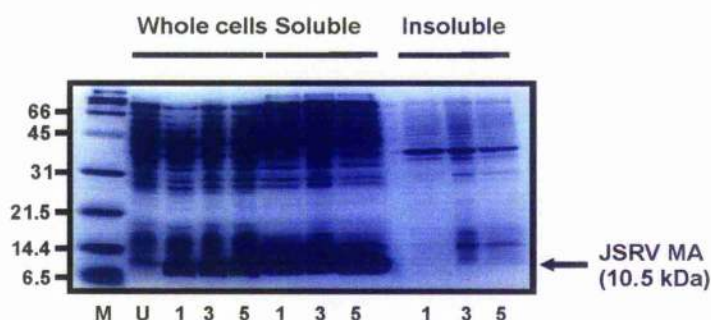
### Cloning of the JSRV MA

We amplified by PCR the sequence corresponding to the amino-terminal 90 and 120 amino acid residues of the JSRV MA using the proviral clone JSRV<sub>21</sub> (Palmarini, Sharp et al. 1999) as a template. PCR products were cloned into the *NheI* and *EcoRI* sites of pET-24b, a bacterial overexpression vector bearing the T7 promoter. We introduced a stop codon at the 3' end of the cloned sequence to avoid fusion of the JSRV MA with the histidine tag included in pET24b. We decided not to include the histidine tag as a fusion protein with JSRV MA to avoid possible technical complications such as decreased expression, alteration in structure and/or function of the fused protein, and proteolytic tag removal with subsequent proteolysis of the target protein. Because

overexpression of JSRV MA was excellent and we could further purify it using classical methods rapidly and with high yields, it was not at this stage beneficial to include an affinity tag.

### Growth and overexpression of JSRV MA in *E. coli*

We transformed the BL21(DE3) *Escherichia coli* cell strain with the expression plasmid construct for overexpression of JSRV MA (pET24bJSMA). To allow easy isotopic labelling, we used minimal media in all our overexpression studies and growths. In our hands, the use of minimal media was suitable to grow BL21(DE3) because it does not reduce the overall yield of the target protein. We assessed overexpression levels of JSRV MA by analyzing aliquots of induced cultures at specific time intervals by SDS-PAGE. We observed high yield expression of JSRV MA at all time points (Figure 44), with the majority of the recombinant protein localized in the soluble fraction, even at 5 hours post-induction. Based on this expression profile, we limited our induction time for JSRV MA to 3 hours. The predicted molecular weight of JSRV MA (1-90) is 10563.3, and is in agreement with the mobility displayed in SDS gels (Figure 44).



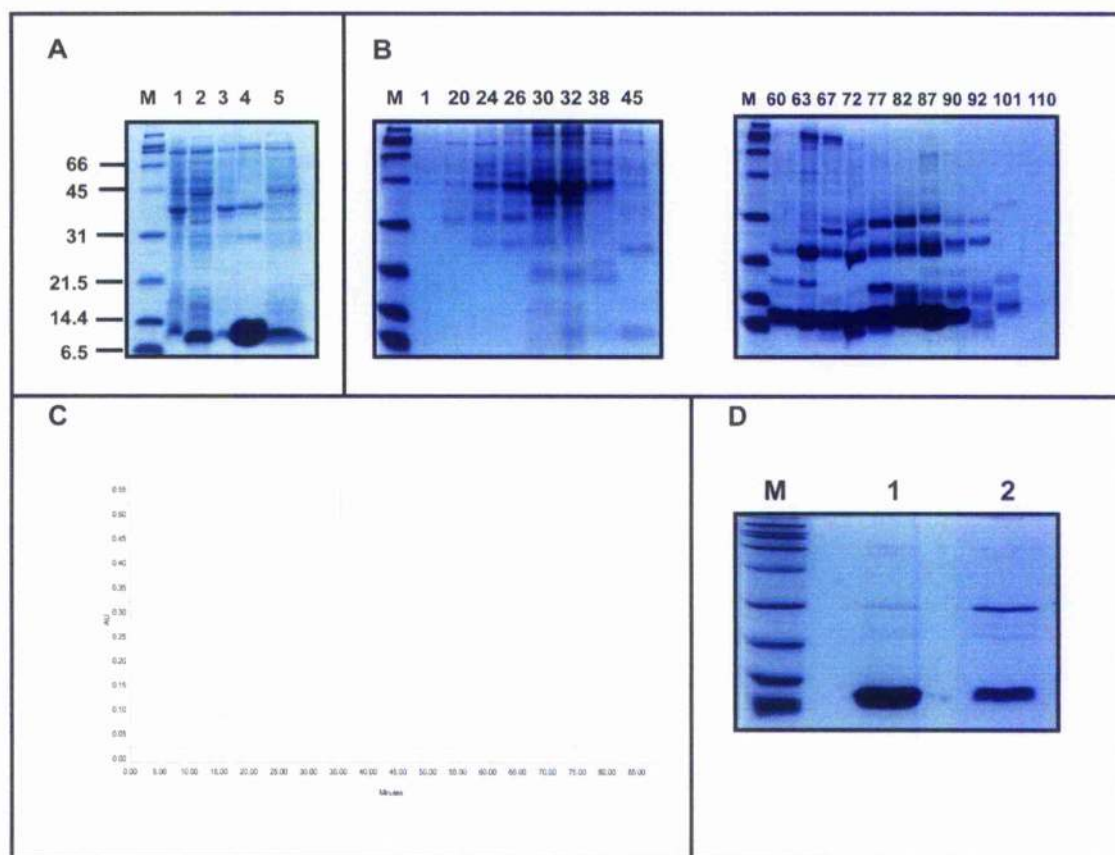
**Figure 44. Recombinant overexpression of JSRV MA.**

Lysates of BL21(DE3) cells expressing JSRV MA were analyzed by SDS-PAGE (15%) at different post-induction times. Samples of whole cells, soluble and insoluble fractions were taken at 1, 3, and 5 hours after the addition of IPTG. M: molecular marker, U: uninduced. The band corresponding to JSRV MA is indicated with an arrow. A high yield expression of soluble JSRV MA was obtained.

## Purification of the JSRV MA

The purification protocol for JSRV MA was based on classical methods, starting with precipitation and removal of contaminant proteins with ammonium sulphate, followed by ion exchange chromatography and further high pressure liquid chromatography (HPLC) (see Figure 45). Panels A, B, and D of Figure 45 depict the SDS-PAGE analysis of samples taken at each purification step. The whole procedure was performed at 4°C. Because the theoretical pI of JSRV MA was 8.71 we used an ion exchange step with a cation exchanger (SP Sepharose Fast Flow) equilibrated at pH 6.8. JSRV MA-containing fractions were subsequently subjected to HPLC, and high yield/high purity protein was recovered after this latter stage. Good levels of purification were achieved with the cation exchange chromatography step (compare the soluble fraction lanes in Figure 44 with the lanes corresponding to fractions 60 to 90 shown in Figure 45B), although some contaminant proteins still remained. The subsequent HPLC step proved successful to “clean” JSRV MA of contaminants (Figure 45D). HPLC fractions containing JSRV MA were pooled, dialyzed against buffer (50 mM KCl, 50 mM  $\text{KH}_2\text{PO}_4$ , 1 mM DTT, pH 6.2), and concentrated. JSRV MA kept its stability in solution during the whole purification scheme. The level of purification achieved with this protocol is shown in Figure 46. In three consecutive rounds of purification, we consistently obtained over 7 mg/ml of pure protein. The yield and purity of the protein was not affected when we isotopically labelled JSRV MA with  $^{15}\text{N}$  (not shown). We also labelled JSRV MA with selenomethionine (SeMet) for crystallographic studies. SeMet-labelled JSRV MA purification scheme was essentially similar to the one described above, with the only difference that the second step of purification was performed by using size exclusion chromatography instead of HPLC. The purity and yield of the protein were not affected by this modification, although the latter was modestly reduced (to 5.5 mg/ml). The identity of the overexpressed purified protein was confirmed by electrospray mass spectrometry.





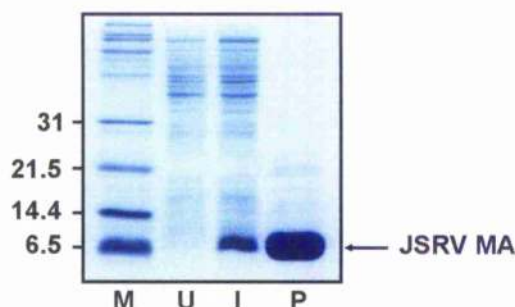
**Figure 45. Purification scheme of JSRV MA.**

(A) 15% SDS-PAGE analysis of samples containing JSRV MA at various stages during the purification procedure. Lane 1, sonication pellet; lane 2, sonication supernatant; lane 3, salt precipitation pellet; lane 4, dialysis pellet; lane 5, dialysis supernatant. (B) SDS-PAGE analysis of selected samples taken after cation exchange chromatography. Fraction numbers are indicated above. (C) HPLC spectrum of pooled fractions obtained by cation exchange chromatography. Peak 1 corresponds to JSRV MA. (D) SDS-PAGE of samples corresponding to peaks 1 and 2 in panel C.

### Structural studies on the JSRV MA

We performed preliminary structural studies on JSRV MA. We used circular dichroism spectroscopy, nuclear magnetic resonance, and analytical ultracentrifugation to assess secondary, tertiary, and quaternary structure, respectively. The CD spectrum indicates that JSRV MA possesses an abundant percentage of  $\alpha$ -helices. We calculated the approximate percentages of secondary structural elements using the CDSSTR algorithm of the DICHROWEB online server for protein secondary structure analysis (Lobley, Whitmore et al. 2002; Whitmore and Wallace 2004) (Figure 47A). Obtained results

suggest that JSRV MA possesses 63% of helical structure, 4% of strands, 16% of turns, and 18% is unordered.



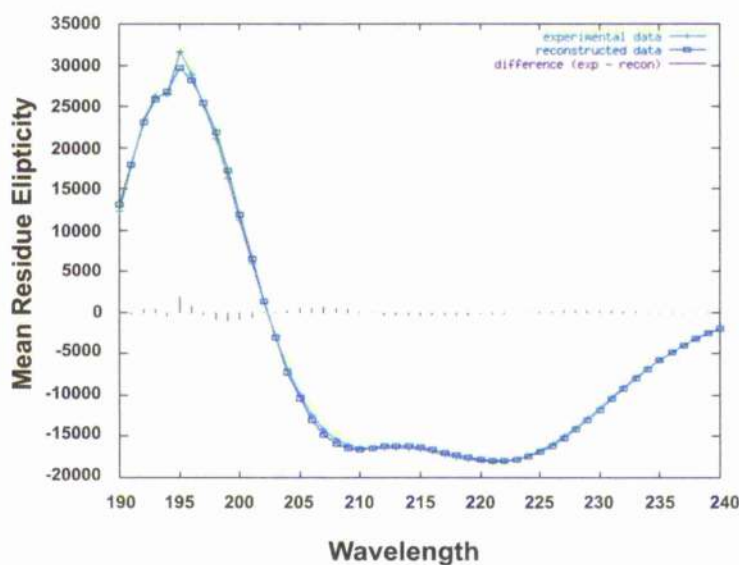
**Figure 46. Analysis of JSRV MA purification.**

Samples containing JSRV MA at different stages of the purification scheme were analyzed by SDS-PAGE. M: molecular marker; U: uninduced whole cell lysate; I: induced whole cell lysate; P: purified protein. The band corresponding to JSRV MA is indicated with a horizontal arrow.

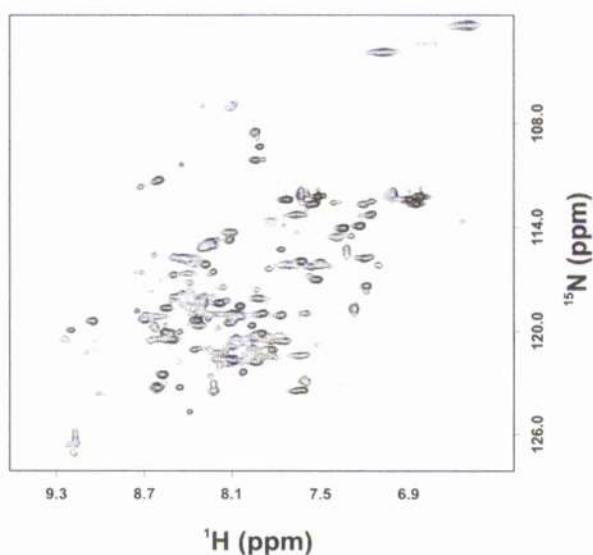
To further study JSRV MA using heteronuclear NMR methods, we assessed the presence of tertiary structure of the purified protein. We produced JSRV MA uniformly labelled with  $^{15}\text{N}$  and acquired a variety of NMR spectra on samples under different conditions of temperature, pH, DTT and EDTA concentration. Figure 47B shows a two-dimensional  $^{15}\text{N}$ -HSQC spectrum of  $^{15}\text{N}$ -labeled JSRV MA. The spectrum is consistent with the presence of tertiary structure.

We also determined the quaternary structure of JSRV MA by performing sedimentation equilibrium experiments. Recombinant JSRV MA was purified as described above and analytical ultracentrifugation data were collected in triplicate at different concentrations and speeds. Velocity profiles were obtained and analyzed using the Ultrascan 6.2 software package. The values recorded in three experiments were fitted to a single ideal species model and resulted in random residuals and a monomer molecular weight of 10,488 kDa (Figure 47C), which is in good agreement with the molecular weight derived from the protein sequence. Overall, JSRV MA seems to be folded and stable at fairly high concentrations, which makes it an attractive protein for high resolution structural studies using NMR.

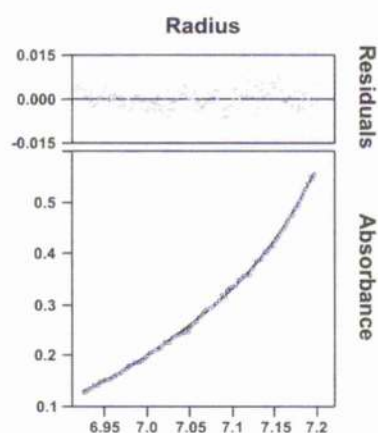
A



B



C



**Figure 47. Structural studies on purified recombinant JSRV MA.**

(A) Purified JSRV MA exhibits tertiary structure. Two-dimensional  $^{15}\text{N}$ -HSQC spectrum of isotopically labelled ( $^{15}\text{N}$ ) JSRV MA. (B) Purified recombinant JSRV MA behaves like single ideal species in solution. Equilibrium analytical ultracentrifugation data acquired at 280 nm for JSRV MA was fitted to a single ideal species model. (C) Circular dichroism spectrum of JSRV MA. This spectrum was acquired on a sample of  $^{15}\text{N}$ -labelled JSRV MA. The percentage of secondary structural elements of JSRV MA was calculated based on these data (see text).



## ***Discussion***

The matrix protein of JSRV possesses the main determinants for Gag centrosomal and membrane targeting. We have previously shown that enJS56A1 blocks JSRV by hampering the ability of the former to reach the pericentriolar region. Notably, arginine 21 is the main determinant for both centrosomal targeting and JLR. A thorough understanding of the structure of these two polyproteins will provide insight on JLR from a different angle than the biochemical approach described in previous sections. Moreover, comparison of the three dimensional structure of JSRV and enJS56A1 MA with solved retroviral matrix proteins will also contribute to our knowledge of basic aspects on retroviral biology. Unfortunately our attempts to overexpress the whole Gag polyproteins or the enJS56A1 MA were fruitless despite the use of a variety of strains of bacteria as well as different culturing and induction conditions (not shown). Analysis of JSRV and enJS56A1 Gag coding sequences indicates that several amino acid residues are encoded by codons that are rare (or present in very low amounts) in such bacterial strains. A possible way to overcome this issue is by reconstructing the Gag coding sequences with codons that are preferentially used in bacteria (codon optimization). Here, we have shown that JSRV MA can be considerably overexpressed using minimal media, labelled isotopically and purified using classical schemes with exceptional yield and high purity, without the need of incorporating affinity tags. Structurally, JSRV MA exhibits a high level of  $\alpha$ -helices that are in agreement with other retroviral matrix proteins (Conte and Matthews 1998; Murray, Li et al. 2005). Moreover, JSRV MA is stable with seemingly well-defined tertiary structure and does not aggregate at the high concentrations required for NMR studies. These results encourage future functional and high resolution structural studies that could pave the way for the design of molecules with antiretroviral activity.

## Chapter 6

### Conclusions and general discussion

The aim of this final chapter is to discuss the data presented in this thesis and their implications. Retroviruses have been studied during the years for different reasons and under different perspectives. In the early days of retrovirology, retroviruses were studied because of their ability to cause cancer and to unravel the mechanisms of carcinogenesis. Even today we look for retroviruses as aetiological agents of tumours in several species (Murcia 2002; Paul, Quackenbush et al. 2006; Bindra, Muradrasoli et al. 2007; De las Heras, Murcia et al. 2007). On the other hand, the AIDS epidemic and the discovery of HIV over 20 years ago resulted in the investment of a vast amount of resources with the aim of developing either an efficient therapy or a vaccine for this devastating disease. As a consequence, HIV is probably the most thoroughly studied virus in science history and the whole field of retrovirology has benefited from this. However, the scientific and social challenge posed by HIV/AIDS is far from being over.

Retroviruses have not been studied solely as causative agents of diseases. Because of their unique replication strategy that includes integration into the host's genome, endogenous retroviruses (ERVs) have heavily colonized every animal species (Gifford and Tristem 2003; Weiss 2006). Thus, ERVs can be used as DNA fossils to study their own evolution, the coevolution between virus and host, and the evolution of the host *per se* (Coffin 2004).

Retroviruses have been also successfully used as molecular tools. The ability of reverse transcriptase to synthesize cDNA revolutionised molecular biology. Despite some recent problematic experiences (Hacein-Bey-Abina, Von Kalle et al. 2003), retroviral vectors are still a promising tool for gene therapy.

This thesis focused specifically on the interaction between endogenous and exogenous retroviruses. From a traditional perspective, the host responds to a viral infection by mounting an immune response that, depending on the cells and cytokines involved, can be classified as innate or acquired. In recent years however, a new kind of immunity

against viral infections has been proposed. This “intrinsic immunity” comprises a collection of genes that encodes for restriction factors that have the ability to block viral replication. In contrast with immune responses, which generally take place after viral infection, genes involved in intrinsic immunity are constitutively expressed (Bieniasz 2004). Host and tissue-specific expression of such factors are fundamental in determining viral tropism. Several antiretroviral molecules have been discovered, such as APOBEC3G, TRIM5 $\alpha$ , and Fv1 and it is likely that many more will be discovered in the future. Some of these restriction factors are cellular in origin while others are derived from ERVs. Thus, in some cases ERVs have been co-opted by the host against incoming retroviral infections.

Sheep are an exceedingly interesting model system to study the evolutionary interplay between exogenous and endogenous retroviruses in a natural setting. The exogenous and pathogenic JSRV/ENTV coexist with at least 27 copies of enJSRVs that are highly expressed in the sheep genital tract (Palmarini, Hallwirth et al. 2000; Carlson, Lyon et al. 2003; DeMartini, Carlson et al. 2003; Palmarini, Mura et al. 2004). enJSRVs can be taken as an example of the evolutionary symbiosis between ERVs and host. Work done in our laboratory has provided evidence on the necessity of the expression of the *env* genes from enJSRVs during placental morphogenesis in sheep (Dunlap, Palmarini et al. 2006). In this particular case, enJSRVs have become assimilated to the extent of playing specific roles in the reproductive physiology of the host species. On the other hand, because sheep is a candidate species for xenotransplantation strategies based on a “sheep-human” chimera, the existence of enJSRVs should also be taken into consideration (Almeida-Porada, Porada et al. 2004; Narayan, Chase et al. 2006). In this proposed system, human stem cells are inoculated into foetal sheep, thus the potential risk of transmitting xenotropic viruses that could cause iatrogenic zoonoses should be evaluated.

The fact that the great majority of ERVs do not possess exogenous counterparts supports the view of endogenous retroviruses playing a protective role against related exogenous infections. ERVs can block infection of exogenous viruses by different mechanisms, the most common being receptor interference. Fv-1, another ERV-derived restriction factor that acts at a post-entry stage has also been described. Although it has

not been formally proved, such genetic traits could contribute to the elimination of the virus from the species.

The work presented in this thesis has aimed to better understand the molecular basis of JLR. In our experimental setting we used a variety of *in vitro* approaches that included the construction of several JSRV and enJS56A1 mutants bearing either deletions or amino acid substitutions. Chimeras between JSRV and Human endogenous retrovirus-K (HERV-K) were also constructed. Because JLR blocks viral exit, the experiments performed in this thesis mainly consisted on transfection (and cotransfection) of cell lines with virus-expressing plasmids followed by western blot analysis of cell lysates and viral pellets obtained from supernatants. Within this experimental approach, we performed assays to address interference, interaction, and complementation between Gag molecules. We also performed confocal microscopy to study intracellular distribution (and its kinetics) and colocalization of the tested constructs. Finally, for future studies aimed to resolve the tridimensional structure of the JSRV matrix protein either by crystallography or nuclear magnetic resonance (NMR), we developed a protocol for overexpression and purification of recombinant JSRV MA. It should be also mentioned that we did not succeed in expressing JSRV and enJS56A1 Gag proteins in different strains of bacteria for *in vitro* assembly experiments (not shown).

The studies presented here cover relevant biological aspects of the JSRV/enJS56A1 biology, and how their simultaneous expression within the same cell results in JLR. First, we functionally characterized JSRV and enJS56A1 Gag proteins, and in doing so we provided basic information about JSRV/enJS56A1 biology that had not been previously available. For example, we determined the identity and boundaries of the individual proteins that constitute Gag. We also identified and assessed the function of well known retroviral domains such as late (L) and membrane (M) domains. Moreover, we determined that JLR is not due to a transdominant M or L domain phenotype and provided evidence that supports the necessity of an intact enJS56A1 Gag to block JSRV. In addition, we showed that arginine 21 within the matrix protein (which is highly conserved among different members of the genus Betaretrovirus) is required for viral exit, and that non-conservative substitutions of this residue within JSRV result in transdominant mutants. We also proposed a model for JSRV intracellular kinetics that

includes dynein-dependent centrosomal targeting as an obligatory step in the viral cycle. Within this model, myristoylation is required for the transport of viral particles to the plasma membrane but only after JSRV has reached the centrosome. With regard to JLR, the experimental data generated in this thesis, together with complementary results obtained in our laboratory by Frederick Arnaud suggest that enJS56A1 blocks JSRV before the latter reaches the centrosome. enJS56A1 and JSRV Gag molecules seemingly assemble into chimeric multimers or viral particles that cannot reach the pericentriolar region and are therefore unable to complete the viral cycle. These chimeric particles appear to form microaggregates that are further degraded by the proteasome. Finally, a chimeric construct bearing the matrix protein of HERV-K (a type-C Betaretrovirus), but the capsid, nucleocapsid and p4 of JSRV, was targeted to the plasma membrane and displayed reduced susceptibility to JLR.

A key point to address in this section is the relevance of the work presented here. In our opinion, the importance of JLR can be considered from different perspectives. From an evolutionary point of view, JLR constitutes a further example of the interplay or “arms race” that takes place between virus and host. As mentioned above, endogenous retroviruses arise from exogenous infections of the germ line, or, in rare cases they can also originate by retrotransposition. When enJS56A1 was originally isolated and characterized, it was estimated that it integrated into the sheep genome between 0.9 to 1.8 million of years ago (Palmarini, Hallwirth et al. 2000). Based on the results presented in this thesis, we hypothesized that the exogenous virus from which enJS56A1 derives (that integrated into the germline) must have possessed an arginine in position 21 of the matrix protein in order to be able to exit from infected cells. This assumption was firstly based on the fact that even a conservative mutation (R21K) abolished the capacity of JSRV to be released (see Results section in chapter 3), and secondly because R21 is highly conserved among different members of the genus *Betaretrovirus* (see Figure 18A). Therefore, the R to W substitution displayed by enJS56A1 in position 21 must have taken place after entering the germ line. A recent study performed in our laboratory further supports this view: while enJS56A1 displays W21 in all domestic sheep tested so far, samples obtained from the Argali sheep (*Ovis ammon*, a wild sheep) exhibited enJS56A1 as a provirus bearing either R21 or W21. Moreover, all the samples examined from other wild sheep species, such as *Ovis*

*nivicola*, *Ovis Canadensis*, and *Ovis dalli*, present the non-interfering R21 within enJS56A1 MA (Arnaud, Caporale et al. 2007), indicating that the dominant-negative version of enJS56A1 must have been positively selected before or after domestication, approximately 9000 years ago (Ryder 1983). We reason that domestication produced ecological changes to the host's environment that favored the spread of infectious diseases and hence the selection of resistant animals. Because animals were forced to live in dense populations that had not existed before, it is likely that the incidence of a disease associated to a JSRV-related virus of the time rose. Morbidity and mortality (if any) must have exerted selective pressure for animals bearing an interfering enJS56A1, as they would have been protected against such disease. A further piece of evidence supports this hypothesis: another enJSRV, termed enJSRV-20, has been recently isolated. This endogenous JSRV, like enJS56A1, harbors W21 within the matrix protein in all samples so far examined from domestic sheep. enJSRV-20 effectively blocks JSRV exit in cotransfection experiments similar to the ones described in this thesis (Arnaud, Caporale et al. 2007). Moreover, this locus displays a parallel polymorphism to the one observed in enJS56A1 as enJSRV-20 also possesses either R21 or W21 in Argali sheep. However, *Ovis nivicola*, *Ovis canadensis* and *Ovis dalli* lack enJSRV-20 (Arnaud, Caporale et al. 2007). The fact that arginine 21 was found substituted by the dominant-negative tryptophan in two proviruses highlights the evolutionary importance of this specific codon.

Within the context of the aforementioned "arms race" between virus and host, the emergence of animals bearing transdominant-interfering enJS56A1 and enJSRV-20 must have exerted selective pressure for the appearance of exogenous counterparts that could escape JLR. Exogenous viruses could have used different ways to escape transdominant enJSRVs. A possible strategy for exogenous viruses could be the development of a different tissue tropism than the one exhibited by the interfering endogenous viruses. If the LTR of the transdominant virus can only be active in specific tissues, a given JSRV-like virus could avoid JLR by replicating in tissues where interfering ERVs are absent. This view is supported by the high level of sequence identity observed between JSRV and enJS56A1 within the coding regions, in contrast with the much lower levels of similarity at the LTR level (Palmarini, Hallwirth et al. 2000). The fact that enJSRVs are highly expressed in the genital tract of the ewe,

whereas JSRV expression reaches high levels in tumor cells in the lungs let us speculate that JSRV may have adopted this strategy. (Spencer, Stagg et al. 1999; Palmarini, Datta et al. 2000; Palmarini, Gray et al. 2001; Spencer, Mura et al. 2003). Alternatively, since JLR is saturable (JSRV can be released in the presence of enJS56A1 when its level of expression is 15 times higher than enJS56A1 [Mura 2004]), an exogenous JSRV-related virus with a much higher level of expression than enJS56A1 can overcome JLR simply by outnumbering the quantity of interfering Gag molecules (this could be achieved by developing a more efficient promoter). Another strategy to escape this restriction could be by establishing highly specific Gag-Gag interactions with such elevated affinity that only identical molecules will assemble into viral particles, hence excluding the transdominant Gag molecules from forming chimeric particles of multimers. Such strategy would possibly require some amino acid substitutions at the level of the capsid protein. A third possibility would include an exogenous, JSRV-related virus that was targeted to a different intracellular compartment than enJS56A1, for example, by assembling at the plasma membrane (see below). Finally, another possible way to overcome JLR could be by selective shutting off of the expression of interfering endogenous viruses. Obviously, we do not rule out other possible strategies to escape JLR.

Curiously, a further example of selective fitness between endogenous and exogenous JSRVs has recently been reported: enJSRV-26 has been isolated from a single Texel sheep among more than 330 sheep examined (including direct relatives) (Arnaud, Caporale et al. 2007). enJSRV-26 can escape JLR when cotransfected either with enJS56A1 or enJSRV-20 (Arnaud, Caporale et al. 2007). Results obtained in that study argue that enJSRV-26 derives from an exogenous retrovirus that integrated into the host within the last 200 years. Possibly, enJSRV-26 is the result of a unique event infecting the single sheep where it has been detected. This finding raises the possibility that an exogenous counterpart of enJSRV-26 may be circulating with unknown (if any) pathogenic effects. A similar situation has been recently documented with the koala retrovirus (KoRV) that is currently in the transition between an exogenous and an endogenous element (Tarlinton, Meers et al. 2006). How does enJSRV-26 escape JLR? Further studies are needed to address this question and are currently under way.



Overall, JSRV, enJS56A1, enJSRV-20 and enJSRV-26 are examples of the evolutionary interplay that takes place between retroviruses and host, and how the environment affects this interaction. It is noteworthy that two endogenous proviruses (enJS56A1 and enJSRV-20) have acquired an analogous mutation (R21W) in two temporally distinct events. Although this fact may suggest selective pressure over this specific codon (Bush 2001), the high level of similarity between enJS56A1 and enJSRV-20 and the identity of their 3' flanking region makes the presence of a transdominant Gag in enJSRV-20 more likely due to a gene conversion event rather than independent mutations (Arnaud, Caporale et al. 2007). Further supporting this view is the fact that in both proviruses the observed substitution was arginine to tryptophan, and we showed that W21 is not strictly required for JLR. In general, few codons per gene are positively selected and the residues they encode are usually located in distinct positions within the protein that are generally exposed to external selective forces. Interestingly, the modeling of JSRV matrix that we showed in Figure 43 shows that R21 is likely located in an exposed loop, where it may interact with other proteins.

What is the function of R21? What is the importance of an arginine in that position for the overall topology of JSRV Gag? Structural and biochemical studies are needed to address this question, and in this work we developed a protocol for recombinant expression and purification of the JSRV matrix protein that will pave the way to solve its tridimensional structure. However, it should be considered that the folding of the matrix in the context of the Gag polyprotein may be different from the tertiary structure of MA once it has been released by the viral protease. Since JLR takes place before maturation, it would be probably more appropriate to determine the structure of the whole JSRV Gag and then establish how MA is folded within this structural frame. Alternatively, studies to determine the conformation of JSRV and enJS56A1 Gags in solution could also provide clues on the importance of R21 for the overall folding of the protein. Unfortunately, no full retroviral Gag protein has been solved to date due to technical constrains (Gag proteins are too dynamic to crystallize and too big for NMR studies). However, many individual domains have been determined either by crystallography, NMR, or both, and we consider that solving the structure of JSRV MA will still provide valuable data. Therefore, at the present time we can only speculate

that R21 is part of a functional trafficking domain unrelated to the ones so far described (M, L, and I domains).

From a functional perspective, we showed that R21 is necessary for JSRV to reach the pericentrosomal region. This observation is linked to another relevant aspect regarding JSRV biology: while dissecting the molecular mechanisms of JLR, we determined the nature and chronology of distinct events of the JSRV replication cycle. We showed that JSRV must get to the pericentriolar region in order to complete its cycle. Although we do not know why Gag traffics to the centrosome, we established that this targeting is dependent on the presence of R21 and dynein-mediated transport. Based on this finding we arbitrarily divided JSRV late events into “pre-centrosomal” and “post-centrosomal”. Once the centrosomal region has been reached, JSRV must travel to the plasma membrane. Work done in our laboratory indicates that this specific step requires an intact endocytic pathway and slow recycling endosomal trafficking. We also showed here that myristoylation is absolutely necessary to reach the plasma membrane, as a myristoylation-deficient JSRV is defective for viral exit and accumulates at the pericentriolar region. Our results seem to indicate that the myristoyl group of JSRV Gag is important for the interaction with membranes of the recycling endosomes instead of the plasma membrane as for type-C assembly. Moreover, our findings suggest that in a normal cycle, JSRV Gag molecules assemble –or at least interact with each other and form multimers- before they are targeted to the centrosome, because JLR requires interaction between Gag molecules and takes place before centrosomal targeting. M-PMV, another Betaretrovirus, has been proposed to assemble at the microtubule organizing center (MTOC) (Sfakianos, LaCasse et al. 2003). Because the MTOC is located at the pericentriolar region, and M-PMV Gag is targeted to this subcellular compartment via dynein-mediated transport depending on intact microtubules like JSRV, we assayed the intracellular kinetics of JSRV, cnJS56A1, and M-PMV. Based on the results we obtained, and despite their close relatedness and targeting to similar compartments, JSRV and M-PMV, display different intracellular kinetics and moreover, when myristoylation is abrogated they exhibit different intracellular distribution. It is hence possible that Gag molecules from JSRV and M-PMV bind different intracellular proteins during assembly and/or trafficking. On the other hand, it has been previously shown that M-PMV possesses an 18-amino acid sequence within the MA domain,

regarded as cytoplasmic targeting/retention signal (CTRS). The CTRS acts as a dominant signal for the assembly of intracytoplasmic immature capsids (Rhee and Hunter 1990; Choi, Park et al. 1999). Substitution of R55 with a W causes disruption of the CTRS and an M-PMV virus bearing this mutation assembles at the plasma membrane (Rhee and Hunter 1990). Although we do not know whether JSRV bears a CTRS, we believe that the R21W mutation that causes JLR is different from a CTRS disruption simply because enJS56A1 and JSR21W (a JSRV single mutant bearing an R21W substitution) do not assemble at the plasma membrane nor do they exit (Mura 2004; this work). Manuela Mura in our laboratory observed that mutation of R22 to W in M-PMV partially abrogates viral exit of wild type virus, although not as significantly as enJS56A1-mediated block (Mura and Palmarini, unpublished). However, an M-PMV double mutant bearing a double mutation (R22W and R55W) effectively blocks the release of M-PMV to similar levels as observed by JLR (Mura and Palmarini, unpublished). A possible explanation for this extra requirement is that because M-PMV can follow two different morphogenic strategies, a dominant-negative M-PMV Gag must block both pathways to be effective, and therefore a double mutation is necessary. An alternative explanation from a structural point of view could be based on the fact that both R22 and R55 are located in exposed loops of M-PMV matrix (Conte, Klikova et al. 1997) and hence it is feasible that they cooperate for the specific overall topology (or electrostatic charge) required for binding either cellular partners or membrane phospholipids.

How does JLR occur? Based on the data presented here and other studies performed in our laboratory, we postulate that JSRV and enJS56A1 Gag molecules interact *in trans* at a pre-centrosomal stage, giving rise to chimeric particles or multimers. These JSRV/enJS56A1 chimerae cannot reach the pericentriolar region and form microaggregates that are degraded by the proteasome (see paper by Arnaud, Murcia and Palmarini attached to this thesis), failing to complete the retroviral cycle. Because addition of proteasome inhibitors does not abrogate JLR but results in the formation of *bona fide* aggresomes bearing JSRV and enJS56A1 Gag, we think that JLR affects trafficking of Gag rather than targeting proteasomal degradation in an active fashion. Although cells transfected with enJS56A1 alone or with enJS56A1 and JSRV exhibit intracytoplasmic viral particles without major morphologic alterations (Mura 2004), it is

feasible to think that enJS56A1/JSRV chimeric particles (or multimers) are sensed by the cell as aggregates of misfolded protein. When the intracellular amount of protein aggregates/ misfolded proteins exceeds the capacity of the proteasome, the cell responds by forming aggresomes (Johnston, Ward et al. 1998).

Since deletion of the major homology region (MHR) of enJS56A1 abrogates its ability to restrict JSRV, we speculate that proper assembly between JSRV and enJS56A1 is required for JLR to occur. Moreover, enJS56A1 $\Delta$ NC2, a construct bearing an in-frame deletion in NC and a functional protease, was able to co-assemble into exit-competent chimeric viral particles and complement JSRV $\Delta$ Pro, a budding-competent JSRV that lacks the viral protease. We also evaluated the possibility that differential intracellular targeting could provide means for JLR-escape variants and hence constructed a HERV-K/JSRV chimera that is targeted to the plasma membrane. This chimeric virus was able to release viral particles in the presence of enJS56A1. However, this result should be considered cautiously. Although it is tempting to conclude that such “type-C” chimera escapes JLR by bypassing the obligatory centrosomal step, it could simply reflect the lack of proper interaction/assembly between HERV-K/JSRV and enJS56A1 Gag molecules required for JLR that was shown for enJS56A1 $\Delta$ NC2.

Is JLR occurring *in vivo*? Although the existence of OPA and the circulation of JSRV in flocks all around the world would indicate that JLR is not taking place *in vivo*, certain aspects about the natural progression of the disease should be initially considered. First, OPA is a very slow disease: it has been shown that in a natural setting, only few infected sheep develop OPA during their commercial lifespan (Caporale, Centorame et al. 2005). Second, JSRV is more easily detected in peripheral leucocytes and lymphoid organs than in the lungs of infected animals. Based on this observations, a model on disease progression has been proposed in which the former tissues constitute the principal reservoir of virus, and infection of lung cells is opportunistic (Caporale, Centorame et al. 2005). What triggers OPA, as well as the cause of the long incubation period of the disease remains to be elucidated, but it is appealing to think that JLR is one possible factor that keeps the spread of the virus “on hold” within the infected animal. Further *in vivo* studies are required to address this issue. However and despite these speculations, the natural horizontal transmission of JSRV clearly shows that JLR

(even if it occurs *in vivo*) can be overcome. A possible reason for this, as described above, could be that enJS56A1 (and enJSRV-20) are either expressed at very low levels or not expressed at all in the lungs. Alternatively, it is feasible that transdominant enJSRVs are expressed in the same tissues as JSRV but they could be “diverted” by other Gag molecules derived from non-interfering, budding-competent enJSRVs. Finally, evidence on the evolution of enJSRV-like exogenous variants has been found with the isolation of the aforementioned enJSRV-26, which escapes JLR in cotransfections (Arnaud, Caporale et al. 2007).

From a therapeutic perspective, JLR can provide insight for the development of novel antiretroviral strategies. Such strategies will include the design of OPA-resistant transgenic sheep. These animals would harbour a transdominant gag as a transgene that would be selectively expressed in specific cell types, for example Clara cells and type II pneumocytes. This ambitious project is actually being carried out by our laboratory together with other groups that include the Roslin Institute. With regard to therapeutics, new antiretroviral drugs based on JLR could target a distinct stage of the viral cycle, although it is possible that such drugs could only apply to viruses closely related to JSRV, like M-PMV (see below). At the present time, highly active antiretroviral therapy (HAART) is the paradigm treatment for HIV infection. HAART consists on a cocktail of individual antiretroviral drugs that block reverse transcription, maturation, and viral fusion (Temesgen, Warnke et al. 2006). Since its introduction in 1996, this combined therapy has provided considerable progress in the treatment of HIV-patients, the simultaneous targeting of multiple stages being crucial for the success of this treatment. Because JLR acts on assembly, this restriction factor provides another “susceptible” stage for retroviral blockade that effectively works *in vitro*. Although at first sight it could be argued that sheep Betaretroviruses are not closely related to HIV and hence JLR does not constitute a valid model for developing novel anti-HIV drugs, distantly related viruses share conserved biological requirements to complete their cycle, assembly being one of them. The principle of a JLR-based antiretroviral could be the synthesis of Gag-like molecules that can form hetero-multimers with wild type retroviral Gag that could be further degraded by the proteasome. As aforementioned, studies performed in our laboratory by Manuela Mura have shown that a defective M-PMV double mutant is transdominant over wild type M-PMV. Such dominant-negative

mutant (that has been discussed above) has been designed based on the natural substitutions observed in enJS56A1 and shows that this strategy could potentially be extended to another retrovirus closely related to JSRV.

From a clinical and epidemiological perspective, therapeutic drugs based on JLR could help preventing disease transmission and progression. In a given retroviral infection, once the virus integrated into the host cell, the late phase of the cycle starts and expression of the provirus leads to production of viral progeny. Hence, cells bearing proviruses constitute reservoirs of infection within the infected individual. If such cells can escape recognition by the immune system, persistent infections can be developed. The more cells persistently infected, the more progeny virus is produced, and thus disease progression and transmission to other individuals is likely to develop faster. Because the restriction mechanism described in this thesis blocks viral exit at a late stage of the replication cycle, a JLR-based therapeutic approach could be extremely valuable to avoid the spread of the infection within the infected individual, probably delaying or attenuating the course of the disease, and avoiding the spread of virus to in-contact individuals.

Unfortunately, there has not been developed a tissue culture system to grow JSRV yet. Therefore, our assays have to be performed in transfected rather than in infected cells. We are aware that transient transfections could provide in some cases misleading results if proteins are overexpressed. However, in our case, JSRV exit is blocked by enJS56A1 even when the former is overexpressed. Indeed, it has previously been shown that enJS56A1 can still block JSRV when expression plasmids driven by the same promoter are transfected at a ratio of 1 (for enJS56A1) to 15 (for JSRV) (Mura 2004).

The study of sheep Betaretroviruses has developed beyond the frontiers of veterinary medicine as proved intellectually challenging and scientifically enriching. Different lines of investigation that have been opened in the last few years shed light on mechanisms of cell transformation, lung cancer, reproductive physiology, evolution of retroviruses, and specifically in this thesis, retroviral restriction. Paradoxically, and despite all these achievements, our advice as veterinarians to a sheep farmer when JSRV strikes a flock is still the same as was 20 years ago: eliminate the diseased

animals. This double-faced reality clearly reflects the necessity of *in vivo* studies to address unknown aspects of OPA pathogenesis. Hopefully, in the near future we will be able to devise novel strategies based on JLR to select for JSRV-resistant animals either by genetic engineering or by breeding flocks with the “right” proviruses. An alternative strategy would include the treatment of infected sheep with drugs that mimic enJS56A1 Gag. Despite the feasibility of these strategies, and even if they succeeded, we should bear in mind that the dynamics of the interaction between virus and host will likely select for new variants of exogenous, JSRV-related viruses.



## Appendices

### Appendix I. Oligonucleotides used in this project.

Primer	Plasmid*	Sequence
PP201AG Fw	pJSΔLD201	5'CCTCATAATAATACCTTTGCCGGTGCTCC GCCTTTTCCTCCC3'
PP201AG Rv	pJSΔLD201	5'GGGAGGAAAAGGCGGAGCACCGGCAAA GGTATTATTATGAGG3'
PP207AA Fw	pJSΔLD207	5'CTCTGCTCCGCCTTTTGCAGCCGCCTATA CTCCTTC3'
PP207AA Rv	pJSΔLD207	5'GAAGGAGTATAGGCGGCTGCAAAAGGC GGAGCAGAG3'
Double LD Fw	pJSΔLD201-7	5'CCTCATAATAATACCTTTGCCGGTGCTCC GCCTTTTGCAGCC3'
Double LD Rv	pJSΔLD201-7	5'GGCTGCAAAAGGCGGAGCACCGGCAAA GGTATTATTATGAGG3'
xK19E Fw	JSK19E	5'ACATGCTATCTGTAATGTTGGAACATAG AGGGATTACTGTTTC3'
xK19E Rv	JSK19E	5'GAAACAGTAATCCCTCTATGTTCCAACA TTACAGATAGCATGT3'

Primer	Plasmid*	Sequence
xK19/H20E Fw	JSK19E/H20E	5'GTACACCTGCTATCTGTAATGTTGGAAG AAAGAGGGATTACTGTTTCTAAACCTAA3'
xK19/H20E Rv	JSK19E/H20E	5'TTAGGTTTAGAAACAGTAATCCCTCTTTC TTCCAACATTACAGATAGCATGTGTAC3'
xG22A Rv	JSG22A	5'TAATTTAGGTTAGAAACAGTAATCGTCT ATGTTTCAACATTACAGATAG3'
xH20E Fw	JSH20E	5'ACACATGCTATCTGTAATGTTGAAAGAA AGAGGGATTACTGTTTCTAAACC3'
xH20E Rv	JSH20E	5'GGTTTAGAAACAGTAATCCCTCTTCTTT CAACATTACAGATAGCATGTGT3'
xR21A Fw	JSR21A	5'CATGCTATCTGTAATGTTGAAACATGCA GGGATTACTGTTTCTAAACCTAA3'
xR21A Rv	JSR21A	5'TTAGGTTTAGAAACAGTAATCCCTGTCAT GTTTCAACATTACAGATAGCATG3'
xR21E Fw	JSR21E	5'CATGCTATCTGTAATGTTGAAACATGAA GGGATTACTGTTTCTAAACCTAA3'
xR21E Rv	JSR21E	5'TTAGGTTTAGAAACAGTAATCCCTTCATG TTTCAACATTACAGATAGCATG3'
xR21K Fw	JSR21K	5'ATCTGTAATGTTGAAACATAAAGGGATT ACTGTTTCTAAACC3'

Primer	Plasmid*	Sequence
xR21K Rv	JSR21K	5'GGTTTAGAAACAGTAATCCCTTTATGTTT CAACATTACAGAT3'
enG2A Fw	en56G2A	5'GAGAGTATAAATATGGCACAGACGCATA GTCG3'
enG2A Rv	en56G2A	5'CGACTATGCGTCTGTGCCATATTTATACT CTC3'
en56A1MHR Fw	penJS56A1ΔMHR	5'GGTAAGATAATGTCAGATGAACAGGCTG GG3'
en56A1MHR Rv	penJS56A1ΔMHR	5'TTTTGAAAGATCCTCTGTCTTAGTACTGG A3'
enp12stop Fw	penJS56A1ΔCA-NC	5'TGCTTTTCCTGTCTTTTGAAAATACAACC AGCG3'
enp12stop Rv	penJS56A1ΔCA-NC	5'CGCTGGTTGTTATTTTCAAAGACAGGAA AAGCA3'
enCAstop Fw	penJS56A1ΔNC	5'GGCGGCATTACAGGGATAAAAAGCATAAA AGAAG3
enCAstop Rv	penJS56A1ΔNC	5'CTTCTTTTATGCTTTTATCCCTGTAATGCC GCC3'
NotICMVI Fw	pCMV <sub>4</sub> en56A1 <sub>xs</sub>	5'ATATGCGGCCCGCCGATGTACGGGCCAGA TA3'

Primer	Plasmid*	Sequence
en56A13381 Rv	pCMV <sub>4</sub> en56A1 <sub>xs</sub>	5'CAAAGTCTAGACAGTCACGAATTAGTGT CC3'
en56A11030 Fw	penJS56A1HA-MA penJS56A1FLAG- MA	5'AACCGACAGCAAGGTATACAG3'
en1827-1848 Rv	penJS56A1HA-MA penJS56A1FLAG- MA	5'AGAATTCATACTGTGTAGGAGGTGGCAC A3'
JS21MA1-18 Fw	pET24bJSMA	5'CGCGCATATGATGGGACAAACGCATAGT CGTCAG3'
JS21MA270 Rv	pET24bJSMA	5'CGCGGAATTCTCATCAATCCAAGCAATC ACGAATTA3'
HERV-K Gag Fw	pCRU5-HERV-KGag	5'CGCGAATTCGTGATAATGGGGCAAAC TA AAAG3'
HERV-K Gag Rv	pCRU5-HERV-KGag	5'AGGGAAGCTTCTACTGCTGCACTGCTGC TTGTGG3'
IIKG2A Fw	pCRU5-HERV- KG2A	5'AAATGAATTCGTGATAATGGCGCAAAC T AAAAGTAAAATTA3'
HKG2A Rv	pCRU5-HERV- KG2A	5'TAATTTTACTTTTAGTTTGCGCCATTATC ACGAATTCATT3'

Primer	Plasmid*	Sequence
HK1061 ClaI Rv	pHVK <sub>MAJ</sub> SCANC	5'GTCGATCGATAGGTGGTGCCGTAGGATT AAGTCT3'
MPMVG2A Fw	pM-PMVG2A	5'CGCTCGGATATGGCGCAAGAATTAAGCC 3'
MPMVG2A Rv	pM-PMVG2A	5'GGCTTAATTCTTGCGCCATATCCGAGCG 3'

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