Development of pseudotyped HIV vectors

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Abstract

Vectors based on human immunodeficiency virus type 1 (HIV-1) offer a means for the delivery of therapeutic transgenes into a wide variety of cell types, both dividing and non-dividing. Virus entry is mediated through interactions between the viral envelope and its cellular receptor(s). For the benefit of gene transfer efficiency and safety it is practical and necessary to complement the vector particle with an envelope protein from another virus (vector pseudotyping) rather than using an HIV-1 envelope that has a limited host-range and cell tropism.

The ability of HIV-1 based vectors to be pseudotyped with gammaretroviral envelope glycoproteins was examined. Modifications to the cytoplasmic tails of the envelope glycoproteins from gibbon ape leukaemia virus (GALV) and the feline endogenous retrovirus RD114 resulted in an increase in HIV-1 vector titre compared to pseudotyping with the wild-type envelope glycoproteins. Attempts were made to understand how these modifications affect vector titre. Pseudotyping of HIV-1 based vectors with alphaviral envelopes glycoproteins was also investigated, and it was found that the envelope glycoproteins of Ross River virus (RRV) and Semliki Forest virus (SFV) could pseudotype HIV-1 vectors.

Stable HIV-1 vector producer cells were established by introducing envelopes suitable for HIV-1 pseudotyping into a novel HIV-1 stable pre-packaging cell line, STAR, which continuously produces HIV-1 Gag-Pol proteins. Pseudotyped vectors harvested from these producer cells were characterised with regard to future ex vivo and in vivo use as the envelope borne by the vector can dictate certain properties of the virion. These data include the stability of each pseudotype in human sera and at 37°C.

Finally, attempts were made to transduce primary human cell cultures, particularly dendritic cells, with different HIV-1 pseudotypes. Although pseudotyped HIV-1 vectors could transduce human dendritic cells to some degree, transduction of dendritic cells with virus from STAR cells appeared to be limited by some facet of the codon-optimised packaging construct used in this cell line.
Acknowledgements

First and foremost, I would like to thank my supervisors Yasuhiro Takeuchi, Mary Collins and Robin Weiss. Without their guidance and perseverance it would simply not have been possible to complete this thesis.

Furthermore, I would like to thank my friends and colleagues, past and present, in the Wohl Virion Centre and Collins Laboratory for all their assistance and suggestions along the way. I would particularly like to convey my gratitude to Keith Aubin, Simon Chowdhury, David Griffiths, Paco Martin, Stuart Neil and, of course, Ike Ikeda for their unflagging encouragement and support.

Outside of the lab, I must mention a number of people who have made life bearable. My sincerest thanks and appreciation to Euan Turner, Mark Davidson and Daniel Hollyman for everything, and to Av and Lorna Mitchison for welcoming yet another stray student into their home.

I would like to take this opportunity to recognise François-Loïc Cosset and David Avram Sanders who kindly provided several plasmids used in this thesis, and the BBSRC and Oxford BioMedica Ltd who funded my studies.

Finally, thanks to all my family. To them I dedicate this work.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ALV</td>
<td>avian leukosis virus</td>
</tr>
<tr>
<td>BaEV</td>
<td>baboon endogenous virus</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CA</td>
<td>capsid</td>
</tr>
<tr>
<td>CCR3</td>
<td>CC chemokine receptor 3</td>
</tr>
<tr>
<td>CCR5</td>
<td>CC chemokine receptor 5</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CDK9</td>
<td>cyclin-dependent kinase 9</td>
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<tr>
<td>cPPT</td>
<td>central polypurine tract</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC chemokine receptor 4</td>
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<tr>
<td>CyA</td>
<td>cyclophilin A</td>
</tr>
<tr>
<td>DARC</td>
<td>Duffy antigen for chemokines</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>DC specific ICAM-3 grabbing non-integrin</td>
</tr>
<tr>
<td>DC-SIGNR</td>
<td>DC-SIGN related</td>
</tr>
<tr>
<td>DIG</td>
<td>detergent-insoluble glycosphingolipid-enriched domains</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EIAV</td>
<td>equine infectious anaemia virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Env</td>
<td>envelope</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
</tr>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>feline leukaemia virus subgroup B</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>g</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>Gag</td>
<td>group antigen</td>
</tr>
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<td>GAG</td>
<td>glycosaminoglycan</td>
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<td>gibbon ape leukaemia virus</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<td>human cytomegalovirus</td>
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<td>human immunodeficiency virus type 1</td>
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<td>IgG</td>
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</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IN</td>
<td>integrase</td>
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<tr>
<td>iu</td>
<td>infectious units</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Vpr</td>
<td>viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>viral protein U</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus G protein</td>
</tr>
<tr>
<td>WPRE</td>
<td>woodchuck hepatitis virus post-transcriptional regulatory element</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
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Chapter 1
Introduction

1.1 The use of viral vectors in gene therapy

Advances in molecular biology and genetics in the last few decades have revealed the molecular basis of many acquired and inherited diseases. In many instances it was found that disease states were the result of one defective gene. These conditions, termed monogenic disorders, were potentially curable with the introduction of genetic material to replace the function of the defective gene. From this notion of gene replacement therapy, gene therapy – the use of DNA or RNA for the treatment or prevention of pathologies – was born. With advances in cell biology and recombinant DNA technology, it is now potentially possible to use a gene therapy approach to modify cells in the treatment of a wide range of conditions. Within this idea is the concept of genetic modification of both somatic and germline cells, so-called somatic and germline gene therapy respectively. The idea of germline gene therapy is controversial and has never been attempted in humans, as modification to the genome would be passed vertically from parent to child. Somatic gene therapy, however, has been widely embraced and has the potential to be used in a wide range of applications. For example, the modification of antigen presenting cells (dendritic cells and macrophage) to modulate the immune response against tumours or invading pathogens, cells of the haematopoietic system to cure inherited immunodeficiencies, airway epithelial cells to fight cystic fibrosis, pancreatic cells to relieve diabetes and neuronal tissue to battle neurodegenerative disease (see Table 1.1).

What is, of course, required is a vehicle, or vector, to introduce the therapeutic genetic material into the cell. Today, vectors fall into two categories: viral and non-viral. The use of non-viral vectors such as naked DNA and liposomes may be attractive but it is generally agreed that most gene therapy applications require sustained, high-level gene expression that can only be provided by viral vectors.
Table 1.1 Statistics of gene therapy protocols and vector usage from completed, ongoing or pending clinical trials up to 2003

Panels A and B shown the range of diseases addressed while panels C and D indicate the vectors used. This data was obtained from the Journal of Gene Medicine, www.wiley.co.uk/genmed
A. Protocols by disease

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<tr>
<th>Condition</th>
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<td>Vascular disease</td>
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B. Patients by disease

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C. Protocols by vector

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<td>Lipofection</td>
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<tr>
<td>Naked/plasmid DNA</td>
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<td>Vaccinia virus</td>
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<td>Others</td>
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D. Patients by vector

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<th>Vector</th>
<th>Number of patients</th>
<th>Percentage of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrovirus</td>
<td>1755</td>
<td>50.2</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>644</td>
<td>18.4</td>
</tr>
<tr>
<td>Lipofection</td>
<td>619</td>
<td>17.7</td>
</tr>
<tr>
<td>Naked/plasmid DNA</td>
<td>123</td>
<td>3.5</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>88</td>
<td>2.5</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>36</td>
<td>1.0</td>
</tr>
<tr>
<td>RNA transfer</td>
<td>30</td>
<td>0.9</td>
</tr>
<tr>
<td>Herpes simplex type 1</td>
<td>21</td>
<td>0.6</td>
</tr>
<tr>
<td>Others</td>
<td>178</td>
<td>5.2</td>
</tr>
</tbody>
</table>
Viruses have evolved to be efficient systems for the delivery of DNA or RNA into cells and many have unique characteristics that would be advantageous in gene therapy. The first step in viral vector design is to identify the viral sequences that are required for the assembly of viral particles, the packaging of the viral genome into the particles, and the delivery of the transgene to the target cells. Next, dispensable genes are deleted from the viral genome to reduce patho- and immunogenicity. The residual viral genome and the gene of interest (also termed transgene) are integrated into the vector construct. Viral vectors available today can be sub-divided into two categories: those which integrate into the hosts chromosomal DNA (such as vectors based on members of the retrovirus family) and those which are non-integrating, for example adenovirus, human parvovirus also known as adeno-associated virus (AAV), herpes simplex virus type 1 (HSV-1), and vaccinia virus. The various characteristics these viral vectors display, compared to their non-viral counterparts, are shown in Table 1.2.

Vectors based on members of the Retrovirus family are the most widely used today (Table 1.1). Retrovirus vectors are favoured for the following reasons. Firstly, their genome has been extensively characterised, which has allowed the creation of a vector system that is replication-incompetent and does not express any viral proteins. Secondly, they can infect a wide range of cell types. Thirdly, these vectors have the ability to integrate into host cell chromatin. Long-term expression of the transgene should, therefore, be possible (Somia and Verma, 2000; Verma and Somia, 1997). The inspiration for the development of retrovirus based vectors comes from members of the retrovirus family that could be described as natural vectors. Some retroviruses found in avians and non-human mammals are the cause of cell transformation and tumour formation in their hosts. Within the genomes of such viruses cellular oncogenes can be found. Most genomes of these viruses lack part or all of the viral structural genes. Their genomes must, therefore, be packaged by a non-defective “helper virus”. By mimicking and modifying this strategy of transferring non-viral genes during retrovirus replication, retrovirus vectors have been created.
<table>
<thead>
<tr>
<th>Vector</th>
<th>retrovirus</th>
<th>lentivirus</th>
<th>AAV</th>
<th>adenovirus</th>
<th>HSV-1</th>
<th>vaccinia</th>
<th>Liposomes</th>
<th>naked DNA</th>
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<tbody>
<tr>
<td>Insert size</td>
<td>~8kb</td>
<td>~8kb</td>
<td>&lt;4kb</td>
<td>~35kb</td>
<td>&gt;20kb</td>
<td>&gt;25kb</td>
<td>&gt;20kb</td>
<td>&gt;20kb</td>
</tr>
<tr>
<td>Functional tire/ml</td>
<td>10⁵</td>
<td>10⁷</td>
<td>10⁷</td>
<td>10¹⁰</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Integration</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes/No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Sustained transgene expression</td>
<td>Variable</td>
<td>Variable</td>
<td>Variable (tissue specific)</td>
<td>Transient</td>
<td>Transient</td>
<td>Transient</td>
<td>Transient</td>
<td>?</td>
</tr>
<tr>
<td>In vivo delivery</td>
<td>Poor</td>
<td>Poor</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Variable</td>
<td>Tissue specific</td>
</tr>
<tr>
<td>Transduction of non-dividing cells</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Immunological problems</td>
<td>Few</td>
<td>Few</td>
<td>Few</td>
<td>Extensive</td>
<td>Few</td>
<td>Few</td>
<td>Few</td>
<td>Few</td>
</tr>
<tr>
<td>Pre-existing host immunity</td>
<td>Unlikely</td>
<td>Unlikely, possible in AIDS patients</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>?</td>
<td>Yes</td>
</tr>
<tr>
<td>Safety problems</td>
<td>Insertional mutagenesis</td>
<td>None</td>
<td>Inflammatory response, toxicity</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 Main features of common gene therapy vectors

Table adapted from (Verma and Somia, 1997) (N/A - not applicable, ? - not known)
The first retrovirus vectors were created by deleting from the viral genome sequences coding for viral structural and enzymatic proteins and replacing them with a transgene. The viral proteins required for assembly of the virion were supplied in a helper virus to package the vector genome. To negate the need for introduction of a helper virus, packaging cells were then created, cells containing virus genomes expressing the viral proteins needed for virion production but unable to package their own genomic transcript (Linial, Medeiros, and Hayward, 1978; Mann, Mulligan, and Baltimore, 1983; Watanabe and Temin, 1983). A vector genome could, therefore, be packaged upon introduction into these cells. In the most recent generations of packaging cells, cells stably express the core, vector genome and viral envelope glycoprotein from different expression cassettes (Cosset et al., 1995; Danos and Mulligan, 1988; Markowitz, Goff, and Bank, 1988). This lowers the risk of recombination that could lead to the production of replication competent virus.

Vectors based on murine leukaemia virus (MLV), a member of the Retroviridae sub-family gammaretrovirus, are used almost exclusively. There is, however, one major drawback to the use of MLV based vectors in so far as MLV can only cross the nuclear membrane during mitosis (Roe et al., 1993). MLV based vectors, therefore, cannot transduce non-dividing cells and cannot be used for gene transfer into clinically relevant cell types such as antigen presenting cells, brain and muscle.

Lentiviruses, such as human immunodeficiency virus type 1 (HIV-1), are another subfamily of Retroviridae and are unique among the retrovirus family as they are able to transduce both dividing and non-dividing cells (Table 1.2). This property has lead to much interest in the use of vectors based on HIV-1.
1.2 The Retroviridae

HIV-1, as its name suggests, is a human pathogen and is, in a vast number of cases, the etiologic agent for acquired immunodeficiency syndrome (AIDS). HIV-1 is transmitted through sexual contact, direct inoculation of virus, contaminated blood or transplanted organs and from mother to child. The virus replicates in cells of the immune system, principally in macrophage and CD4⁺ T lymphocytes and causes severe immune suppression. The progression from HIV-1 infection to AIDS can be briefly summarised as follows: primary infection; dissemination of the virus to the lymphoid organs; clinical latency; induction of viral expression and replication; cytopathicity; clinical disease characterised by immune disfunction; opportunistic infection by pathogens other than HIV-1; wasting syndrome and neurodegenerative disease. The mean time between primary infection and clinical disease is ten years (Levy, 1998). It must be noted that disease caused by opportunistic infections in the immunosuppressed individual are usually the ultimate cause of death.

Although it is now twenty years since the virus we know as HIV-1 was first identified (Barre-Sinoussi *et al.*, 1983), there are still many questions to be answered surrounding the mechanisms the virus employs to bring about cell death. The lifecycles of HIV-1 and other members of the *Retroviridae* have been extensively studied in order to address these questions. Information from these studies is essential for rational vector design.

The *Retroviridae* are enveloped viruses that possess diploid positive-stranded RNA genomes that are converted to DNA during replication. The DNA viral genome integrates into the host cell chromatin and is transcribed by host cell transcription factors. The progeny virus buds from the cell through the plasma membrane, encompassing the virion in a lipid bilayer.

Traditionally, members of the retrovirus family have been grouped in three sub-families - *Lentiviridae*, *Spumaviridae* and *Oncoretroviridae* - according to their biological activity and disease association. Recent classification, based on the
The genetic relatedness of genomic sequences between the different oncoretroviral groups, has further differentiated seven distinct retroviral genera; the lentiviruses, the spumaviruses, the alpharetroviruses, the betaretroviruses, the gammaretroviruses, the deltaretroviruses, and the epsilonretroviruses (Hunter et al., 2000). As mentioned above, HIV-1 is a member of the subfamily lentivirus, whilst MLV is a gammaretrovirus.

The genomes of each member of the retrovirus family contain some essential genes with structural and functional homology. For example, the retrovirus genome contains two genes that encode polyproteins: *gag* encodes structural proteins such as matrix (MA), capsid (CA) and nucleocapsid (NC) while *pol* encodes the viral enzymes protease (PR) reverse transcriptase/RNase H (RT) and integrase (IN). Furthermore, the retrovirus genome contains a gene, *env*, which encodes the viral envelope glycoprotein. Env itself is composed of two subunits, surface (SU) and transmembrane (TM). The genetic organisation of HIV and MLV is shown in Figure 1.1. In contrast to MLV, HIV-1 has a complex genome that includes a number of accessory genes (*vpr, vpu, vif and nef*), plus *tat* and *rev*, which encode essential regulatory proteins.

Given the structural and functional relatedness of retroviral proteins, retrovirus virions are morphologically similar, but not identical. The basic morphology of a mature HIV-1 virion is shown in Figure 1.2. Briefly, the mature HIV-1 virion consists of two copies of genomic RNA surrounded by the viral structural proteins MA, CA and NC. This core is encompassed by a lipid bilayer in which the viral envelope glycoprotein (a trimer of heterodimers) and a number of host cell membrane proteins are found (see section 1.3.10). The viral enzymes RT, IN and PR can be found within the virion, as are viral accessory proteins and cellular factors needed for replication.

In order to understand how HIV-1 has been adapted for use as a gene transfer vector, vector infection and assembly, the life cycle of HIV-1 (henceforth referred to as HIV) is described in detail below.
Figure 1.1 Organisation of the HIV and MLV genomes in DNA form

Shown above in A and C respectively are the viral exons in the HIV and MLV genomes. The DNA genome of HIV is approximately 10 kb in length, MLV approximately 9 kb. Both RNA forms of the genomes are flanked by long terminal repeats (LTRs) which contain the Unique 3' (U3), Repeat (R) and Unique 5' (U5) regions. During reverse transcription LTRs acquire both U3 and U5 sequences (see section 1.3.2) Transcription starts from R of the 5' LTR (see section 1.3.6). Also shown is the position of the packaging signal, Ψ, in an untranslated region upstream of gag. Ψ mediates incorporation of genomic RNA into nascent virions. Shown in B and D are the positions of the viral proteins in the polyproteins encoded by gag and pol.
Figure 1.2 Basic morphology of the mature HIV virion

HIV is an enveloped virus that buds through the plasma membrane, picking up the viral envelope protein. In the virion the viral structural proteins nucleocapsid, capsid and matrix enclose two copies of viral genomic RNA. A defining morphological feature of lentiviruses is the conical capsid structure. In contrast, the structure of gammaretrovirus capsid is barrel-like. The structural proteins p6, p2 and p1 and the accessory proteins Vif, Vpr and Nef are also found in the virion (not shown).

As shown, the viral enzymes reverse transcriptase/RNase H, protease and integrase are also found in the virion. Also, a number of cellular proteins can be found in the lipid bilayer (Not shown).

Some components of the virion are commonly referred to by their molecular weight, therefore, the molecular weight of some viral proteins is indicated.
1.3 The HIV life cycle

1.3.1 Cell surface attachment

An overview of the HIV life cycle can be seen in Figure 1.3. The first step in the HIV life cycle is cell attachment. This step aids infection and/or transmission between cells and individuals. Many attachment molecules are displayed on a range of cell types. For example, the HIV virion can be adsorbed to the cell surface via interactions between the viral envelope glycoprotein and cell surface glycosaminoglycan (GAG) molecules such as heparan sulphate (Mondor, Ugolini, and Sattentau, 1998; Patel et al., 1993). It must be noted, however, that this interaction is not a general mechanism for attachment as envelope-GAG interactions are envelope- and, therefore, strain-dependent (Moulard et al., 2000; Zhang et al., 2002).

Other cell attachment molecules are cell type specific. DC-SIGN (DC specific ICAM-3 grabbing non-integrin) displayed on submucosal DC and DC-SIGNR (for DC-SIGN related) or L-SIGN (for liver/lymph node-specific ICAM-3 grabbing non-integrin), expressed on endothelial cells in the liver, lymph node, gastrointestinal tract, and placenta both bind HIV in an envelope-dependent fashion (Bashirova et al., 2001; Geijtenbeek et al., 2000b; Pohlmann et al., 2001). Expression of DC-SIGN on the cell surface of T cells promotes infection, presumably by capturing the virus on the cell surface and facilitating interactions between the virus envelope and its cellular receptors (Lee et al., 2001). Furthermore, it has been proposed that DCs then carry the virus to the lymph nodes where it is displayed on the DC cell surface and subsequently infects T cells (Geijtenbeek et al., 2000a). Endothelial cells do not express the appropriate receptor molecules for HIV infection, therefore it is thought that the virus binds to DC-SIGNR, and indeed DC-SIGN, on endothelial cells to aid either vertical or horizontal transmission between individuals (Geijtenbeek et al., 2000b; Jameson et al., 2002; Pohlmann et al., 2001). It is interesting to note that N-linked glycosylation of envelope modulates DC-SIGN and DC-SIGN(R) binding of HIV (Lin et al., 2003). The cell that HIV is produced from can, therefore, have a significant affect on what cells the virus can then attach to.
Also, the erythrocytes from some individuals can bind HIV via the Duffy antigen for chemokines (DARC) (Lachgar et al., 1998). Binding to DARC is thought, like DC-SIGN binding, to present the virus to T cells, increasing infection efficiency.

None of the interactions mentioned above are sufficient to lead to productive virus infection. Recognition and interaction of the envelope glycoprotein with its specific cell surface receptors mediates virus entry in such a way as to lead to productive infection.

### 1.3.2 Cellular receptor usage and entry

The cell surface receptors used by HIV are the main determinants of cell tropism. Two receptors are usually essential for HIV entry: CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) plus a seven-transmembrane spanning co-receptor. Interaction of the envelope SU with CD4 induces conformational changes in the envelope that promote binding to the co-receptors. The major co-receptors for HIV are CXCR4 and CCR5, both of which are members of a seven-transmembrane spanning chemokine receptor family (Clapham and McKnight, 2002; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). Other chemokine receptors or closely related orphan receptors have been shown to act as HIV co-receptors on CD4 cells in vitro, however, there is little evidence to suggest that co-receptors other than CCR5 and CXCR4 are used significantly in vivo (Clapham and McKnight, 2002). It is interesting to note, however, that the presence of DC-SIGN on the cell surface can enable some viruses to use these alternative co-receptors (Lee et al., 2001). Entry of HIV using co-receptors other than CXCR4 and CCR5 may, therefore, by highly strain- and cell type-specific.

CCR5 is found on macrophage and lymphocytes, whereas CXCR4 is mostly expressed on lymphocytes. During horizontal HIV infection, current data supports a model where viruses that predominately use CCR5 as a co-receptor, or “R5 viruses”, are largely transmitted. As infection progresses, strains able to use CXCR4 (X4 viruses) and often several co-receptors (R5X4 viruses), appear (Scarlati et al., 1997). Microglia also become infected and neurotropic HIV strains principally use CCR5 and CCR3 as co-receptors (He et al., 1997). Why R5 viruses should be more “transmissible” and why there should be a switch in co-receptor usage is as yet
unclear. CD4-independent entry of HIV into a number of cell types has been reported
(Hoffman et al., 1999), however, there is little evidence that CD4+ cells are infected in vivo.

Although interactions between SU and CXCR4 and CCR5 can stimulate intracellular signaling which can activate apoptosis (Algeciras-Schimnich et al., 2002; Badley et al., 2000; Kaul, Garden, and Lipton, 2001; Ullrich, Groopman, and Ganju, 2000), intracellular signaling per se via the co-receptors is not thought to be absolutely required for infection (Amara et al., 2003; Aramori et al., 1997). The virus enters the cell via direct fusion of the viral and cellular membranes, a mechanism independent of pH (McClure, Marsh, and Weiss, 1988; Pelchen-Matthews, Clapham, and Marsh, 1995). Fusion may take place on lipid rafts on the cell surface as this is where CD4 molecules are commonly found (Kozak, Heard, and Kabat, 2002). These areas may provide the optimum environments for fusion as these cholesterol and sphingolipid-enriched microdomains mirror the content of the HIV virion lipid bilayer (Campbell, Crowe, and Mak, 2002).

The molecular basis of fusion between the virus and cellular membranes can be summarized as follows. Interaction of the envelope SU subunit with CD4 and a co-receptor promotes conformational changes in the SU subunit that expose a highly hydrophilic fusion domain in the TM subunit. Further conformational changes in the envelope result in the insertion of the fusion domain into the cellular membrane that promotes membrane mixing and ultimately delivery of the virion core into the cytoplasm (Clapham and McKnight, 2002).
Figure 1.3 Overview of the HIV replication cycle

Virus binds to the cell and enters via interactions between the envelope (shown in blue) and cellular receptors. Within the viral core (shown in yellow) reverse transcription occurs and viral genomic RNA (shown in green) is converted into DNA (shown in red). The viral genome integrates into host cell chromatin. Transcription from integrated genomes provides viral RNA coding for all viral proteins and progeny genomic RNA. The virus assembles at and buds through the plasma membrane picking up viral envelope protein(s).
1.3.3 Reverse transcription

Uncoating of the virus is poorly understood. Several proteins (the viral accessory proteins Vif and Nef and the cellular protein cyclophilin A (CyA)) are thought to play a role (reviewed in (Greene and Peterlin, 2002)) but further work is required to ascertain what factors, cellular or viral, are involved in the uncoating process. Once liberated from the membrane the virion "docks" with the cytoskeleton. It is thought this occurs for the following reasons. Firstly, association of the virion with actin microfilaments appears to promote efficient viral DNA synthesis (Bukrinskaya et al., 1998). Why this should be remains unclear. Secondly, recent studies suggest that the virus moves to the nuclear membrane along microtubules via interactions with the cellular molecular motor protein dynein (McDonald et al., 2002).

Viral DNA synthesis (or "reverse transcription") is mediated by the viral enzyme reverse transcriptase (RT) which catalyses both the RNA- and DNA-dependent DNA polymerisation reactions necessary for the reverse transcription of the viral genome from RNA to single stranded DNA to double stranded DNA. This enzyme also contains an RNase H subunit for the degradation of the RNA strand of the RNA-DNA hybrid generated by the reverse transcription of the RNA genome. Figure 1.4 shows reverse transcription of the HIV genome. During reverse transcription the viral complex is commonly termed the reverse transcription complex or RTC. Once reverse transcription is complete, the viral complex consists of double stranded DNA genome plus a number of viral proteins that have been shown to have karyophilic properties - MA, IN and the accessory protein Vpr (Bukrinsky et al., 1993b; Fassati, 2001; Miller, Farnet, and Bushman, 1997). It must be noted, however, that there is evidence that most, if not all, MA and RT molecules dissociate from the viral complex before completion of reverse transcription (Fassati, 2001). As the viral complex has completed reverse transcription, but has yet to enter the nucleus, it is now given the name pre-integration complex (PIC). There is much speculation as to what cellular proteins interact with or are a part of the PIC. What is known is that the DNA binding protein HMG I (Y) is present in the PIC at this time (Miller, Farnet, and Bushman, 1997). This protein has a function in integration of the viral genome in the host cell chromatin (see section 1.3.5).
Figure 1.4 Overview of the molecular mechanism of HIV reverse transcription

The conversion of genomic retroviral RNA to a double stranded DNA is mediated by reverse transcriptase and is thought to occur in the following stepwise fashion. Step 1; Reverse transcription is initiated when tRNA^{Lys} (red) binds genomic RNA at the primer binding site (PBS) allowing RT mediated elongation of the DNA negative strand (shown in white). Step 2; “Strong stop” DNA is produced and part of the genomic RNA degraded by the RNase H subunit of RT. The strong stop DNA element then translocates to the other end of the genome. Step 3; Elongation of the DNA negative strand from the strong stop primer. Step 4; RNase H degrades the genomic RNA leaving two RNA sequences at the two polypurine tracts (shown as PPT and cPPT) as primers for positive strand elongation. Step 5; After priming of the positive strand DNA (yellow), RNase H removes the RNA primers and transcription occurs from the downstream polypurine tract to the tRNA primer. Step 6; RNase H removes tRNALys and the DNA primed from the PPT jumps to the opposite end of the genome. Step 7; Transcription of complimentary DNA sequence occurs. Step 8; The positive strand DNA primed from the cPPT is displaced, leaving an overhang or DNA flap in the complete DNA provirus.
1.3.4 Nuclear entry

Mitosis is not necessary for entry of HIV PIC into the nucleus; therefore HIV can infect non-dividing cells (Stevenson et al., 1990; Weinberg et al., 1991). The HIV PIC is, however, approximately two-fold larger than the diameter of a nuclear pore (Pemberton, Blobel, and Rosenblum, 1998), therefore HIV nuclear entry most likely requires the energy-dependent action of host and viral proteins working in concert. What viral proteins and/or elements are involved in nuclear entry is still in dispute.

It has been proposed that there are nuclear localisation signals (NLS) in MA (Bukrinsky et al., 1993a), IN (Gallay et al., 1997) and Vpr (Heinzinger et al., 1994) that could mediate passage of the PIC through the nuclear pore. This, however, is quite controversial. With regard to MA, it was thought that a basic region in the proteins amino terminus was a NLS (Bukrinsky et al., 1993a) but it has not yet been possible to biochemically demonstrate that this is the case (Fouchier et al., 1997). It was also proposed that phosphorylation of a tyrosine residue in the carboxyl terminus region of MA promotes association of MA with IN and this complex promotes nuclear entry. Mutation of this tyrosine residue was reported to block nuclear entry in non-dividing cells (Gallay et al., 1995a; Gallay et al., 1995b). Although others have reposted that serine and tyrosine phosphorylation of MA play a role in HIV nuclear entry (Bukrinskaya et al., 1996) blocking of nuclear entry by mutation of tyrosine could not be confirmed in subsequent studies (Freed et al., 1997). Moreover, the results presented by Fassati and Goff (Fassati, 2001) call into question if MA is even a constituent of the PIC.

It has been reported that IN contains an NLS that can be recognised by the cellular proteins importin-α and importin-β, components of the classical nuclear import pathway that interact with constituents of the nuclear pore (Gallay et al., 1997). Vpr does not contain an NLS that can be recognised by these proteins (Heinzinger et al., 1994; Sherman et al., 2001). Instead, it is thought Vpr functionally resembles importin-β, therefore the PIC could interact with components of the nuclear pore directly via Vpr (Vodicka et al., 1998).
With regard to viral proteins involved in nuclear entry there would appear to be some functional redundancy within the PIC. This is most likely not accidental as the viral proteins discussed above could function cooperatively or have larger individual roles in different cell types. For example, whilst Vpr is dispensable for the infection of non-dividing T cells (Eckstein et al., 2001) its presence in macrophage significantly enhances infection (Eckstein et al., 2001; Vodicka et al., 1998).

As an alternative to passage through the nuclear pore, it is speculated that HIV can gain entry into the nucleus by acting directly on the nuclear membrane. It has been shown that Vpr can influence the integrity of the nuclear membrane, causing herniations to appear (de Noronha et al., 2001). It is thought that this may allow entry of the HIV pre-integration complex into the nuclear compartment. This, however, cannot be the complete explanation regarding HIV nuclear entry, as virions lacking Vpr can efficiently traverse the nuclear membrane of some non-dividing cells (Popov et al., 1998; Zufferey et al., 1997).

Perhaps the most controversial viral element thought to be instrumental in HIV nuclear entry is the DNA “flap”. First described by Zennou and co-workers (Zennou et al., 2000) the DNA flap is produced in the double stranded DNA form of the viral genome during reverse transcription. As shown in Figure 1.4, one DNA strand is primed for elongation at the central polypurine tract (cPPT) while another is primed at a polypurine tract proximal to U3. As synthesis progresses the strand primed at the cPPT is displaced. Mutation of the cPPT by Zennou et al. (Zennou et al., 2000) produced a linear reverse transcription product in the PIC. The ability of this PIC to cross the nuclear membrane in non-dividing cells was poor (Zennou et al., 2000). This data argued that the DNA flap plays a fundamental role in HIV nuclear entry. The data has been challenged by Dvorin and co-workers (Dvorin et al., 2002) and Limon and co-workers (Limon et al., 2002). Here repetition of the work described by Zennou and colleagues indicates that the presence or absence of the DNA flap has no effect on the levels of HIV nuclear entry in non-dividing cells.

Before dismissing the role, if any, of the DNA flap in nuclear entry, data accrued with HIV vectors must be taken into account. The addition of the cPPT to an HIV vector increases the ability of the vector to transduce a range of non-dividing cells.
This work was, however, conducted before the publication of Dvorin et al. (Dvorin et al., 2002) and Limon et al. (Limon et al., 2002) and may, therefore, warrant re-examination. In conclusion, while the existence of the DNA flap is not in question, its function clearly is.

1.3.5 Integration

Once in the nucleus the viral genome can undergo several different fates, only one of which, integration of the viral genome into host cell chromatin, leads to the production of infectious virions, as it would appear that it is only possible to produce all viral proteins and genomic RNA from an integrated genome.

Integration is mediated by the viral enzyme IN which binds to the ends of the viral DNA. Briefly, both ends of the genome are brought together (but not covalently joined) while IN removes two nucleotides from the viral DNA leaving a 5' overhang in each LTR. The enzyme then breaks the phosphodiester bonds in the host cell chromosomal DNA to form an integration site and proceeds to covalently join the 3' end of the viral DNA to the 5' end of the chromosomal DNA. IN is a member of a structurally related superfamily of polynucleotidyl transferases that are proposed to use acidic residues to coordinate divalent metal ions during catalysis (Dyda et al., 1994; Rice, Craigie, and Davies, 1996). It is, therefore, proposed that the breakage of phophodiester bonds during HIV integration is mediated by a conserved motif of acidic amino acids (D,D-35-E) at the IN active site which coordinate with a Zn$^{2+}$ ion bound by a conserved motif (HHCC) at the protein's amino terminus (Zheng, Jenkins, and Craigie, 1996). Finally, repair of the single-strand gaps between the viral and cellular DNA is carried out by cellular enzymes (Brown, 1997). A schematic diagram of retroviral integration is shown in Figure 1.5. (For the purposes of clarity the viral genome is not shown as a circle in this schematic diagram). Cell proteins such as HMG I(Y) and barrier to autointegration factor (BAF) are required for efficient integration, although what their exact roles are remains to be seen (Chen and Engelman, 1998).
Integration occurs at random sites, but it is generally agreed that HIV integrates into euchromatin. This is supported by the fact that retrovirus integration in general is favoured near DNase I hypersensitive sites (Panet and Cedar, 1977; Rohdewohld et al., 1987; Vijaya, Steffen, and Robinson, 1986) and active genes (Mooslehner, Karls, and Harbers, 1990; Scherdin, Rhodes, and Breindl, 1990), plus HIV integration in vitro is disfavoured in heterochromatic regions (Carteau, Hoffmann, and Bushman, 1998). Moreover, recent data utilising global analysis of cellular transcription and integration site mapping using the human genome sequence has indicated that active genes are preferential targets for HIV integration, in particular those that are activated upon infection (Schroder et al., 2002). Regional integration "hotspots" were also found in this study, most noticeably a 2.4 kb region in chromosome 11 which contained 1% of the 524 total integration sites analysed.

There is, of course, the possibility that integration into euchromatin could cause the inactivation of tumour suppressor genes or the activation of protooncogenes and, consequently, cell transformation. This, however, is not a commonly observed phenomenon during HIV infection. Indeed, there are few reports indicating that insertion of the HIV genome into host cell chromatin has lead to cell transformation (Herndier et al., 1992; Ruff, Bagg, and Papadopoulos, 1989). In the best characterised example to date, HIV genome insertion into the fur gene, lead to the upregulation of the cellular protooncogene c-fes/fps, located upstream of fur (Shiramizu, Herndier, and McGrath, 1994).

If not integrated into host cell chromatin, the viral genome can be maintained in the nucleus in several forms. Firstly, the ends of the viral genome can be joined by cellular ligases to produce a circular structure known as a 2-LTR circle or, secondly, the viral genome undergoes homologous recombination to produce a 1-LTR circle. Furthermore, it is possible for the viral genome to integrate itself into one of these circular structures producing a rearranged circular structure. As mentioned above only integration of the genome into the host cell chromatin leads to virion production. It is
Figure 1.5 Overview of the molecular mechanism of retroviral integration

This process is mediated by the viral protein integrase (IN). Steps 1 and 2; The LTRs are cleaved to produce a 5' overhang. Steps 3 and 4; Host cell chromatin is digested to form the integration site. Steps 5 and 6; The viral genome integrates leaving a two base pair mismatch. Step 7; Host cell proteins repair the mismatch and fill in the gaps. Also shown are the approximate positions of the gag, pol and env genes.
possible, however, for these circular DNA forms to be transcriptionally active and produce the regulatory protein Tat and the accessory protein Nef (Wu and Marsh, 2001). The production of Tat and Nef in this fashion in T lymphocytes resulted in T cell activation and increased viral replication.

1.3.6 Transcription of the HIV genome

Transcription of the viral genome is directed by a promoter/enhancer in the U3 sequence of the 5' LTR. Transcription commences from the beginning of R in the 5' LTR and terminates at a polyadenylation signal in the end of the R region in the 3' LTR. Transcription is catalysed by the cellular RNA Pol II complex, thus producing mRNA which is "capped" at the 5' end with the addition of methylated guanine residue and has a polyadenylic acid or poly(A) "tail" at the 3' end. Without the aid of the viral regulatory protein Tat, however, transcription is inefficient. Newly initiated transcripts can terminate prematurely, around 200 nucleotides from the transcription start site (Kao et al., 1987). It is thought that Tat aids transcription in the following manner. Once a viral genomic transcript has been produced, Tat along with the cellular protein Cyclin T1 binds to an RNA stem loop structure, the transactivation response element or TAR, in the repeat region of the LTR, where they recruit another cellular protein, cyclin dependent kinase (CDK9) (Wei et al., 1998; Zhu et al., 1997). This complex on TAR, known as P-TEFb, or the positive transcription-elongation factor b complex, then promotes transcription of genomic viral RNA from the integrated DNA viral genome (Bieniasz et al., 1999). It is thought that this complex, via CDK9, phosphorylates RNA Pol II, which in turn increases processivity of the RNA Pol II transcription complex (Cullen, 1998a; Price, 2000). The integrated viral LTR also contains enhancer sequences to which nuclear factor κB, nuclear factor of activated T cells (NF-AT) and Ets family members can bind, thus upregulating transcription in response to various extracellular stimuli (Jones and Peterlin, 1994). It is thought that this is a mechanism evolved by the virus so that the highest levels of transcription take place when the extracellular environment favours virus replication.
1.3.7 Nuclear export of viral RNA

Splicing of viral genomic RNA is shown in Figure 1.6. Newly synthesised genomic RNA, as well as variously spliced forms of viral RNA, must be brought out of the nucleus so that the viral structural, enzymatic and accessory proteins can be translated and the genomic RNA can be packaged into progeny virions. This creates a problem as unspliced, or incompletely spliced, RNAs are normally retained in the nucleus. To counter this, HIV possesses a virus-specific RNA export system controlled by the regulatory protein Rev.

A member of the importin family, importin-β (Henderson and Percipalle, 1997) brings Rev into the nucleus. In the nucleus, importin-β interacts with Ran-GDP, releasing Rev (Cullen, 1998b). Rev then binds specifically to the HIV genomic transcript via the Rev responsive element, or RRE, an RNA-stem loop structure in the env gene sequence (Malim et al., 1990). This binding leads to the multimerisation of Rev on the viral genomic transcript (Malim and Cullen, 1991). Rev contains a nuclear export signal and interacts with a cellular protein, Crm1, via this signal to bring the RNA-Rev complex out of the nucleus through the nuclear pore (Neville et al., 1997). Crm1 is a member of a group of proteins related to the importins and is sometimes referred to as exportin-1 (Greene and Peterlin, 2002). Crm1 can directly interact with components of the nuclear pore, nucleoporins, and, although the movement of the complex through the nuclear pore is not fully understood, it may be similar to the mechanism of importin-β mediated import into the nucleus (Mattaj and Englmeier, 1998). Also bound to the Rev-Crm1 complex is Ran-GTP. Once in the cytoplasm Ran-GTP is converted to Ran-GDP and the Rev-Crm1 complex dissociates from the viral RNA (Cullen, 1998b). It is interesting to note that this is the inverse relationship of importin-β where Ran-GTP stimulates cargo release.
Figure 1.6 HIV RNA and protein products

RNA spliced or otherwise is shown as solid black line. The protein products of the viral coding sequences are shown in blue. (A) The HIV provirus and its protein products. (B) Genomic RNA for packaging into nascent virions and production of Gag and Gag-Pol. The site of the ribosomal frameshift in gag to produce gag-pol is marked with an arrow. The position of the RRE is also indicated (C) Splicing of genomic RNA that produces all other viral proteins.
1.3.8 Assembly and maturation

Assembly and maturation of the HIV virion occurs at the plasma membrane. Here two copies of genomic RNA are packaged into immature virions formed by Gag and Gag-Pol polyproteins. Gag-Pol is N-terminally colinear with Gag and is derived from ribosomal frameshifting in the p1 coding sequence at a rate of approximately 5% (Jacks et al., 1988; Wilson et al., 1988) (Figure 1.6.B). Due to the myristoylation and phosphorylation of Gag (Gallay et al., 1995a; Gottlinger, Sodroski, and Haseltine, 1989), Gag and Gag-Pol polyproteins are targeted to, and preferentially associate with, cholesterol and sphingolipid rich microdomains, or lipid rafts, in the plasma membrane (Ono and Freed, 2001; Spearman et al., 1994). A highly basic region in MA, the so-called M domain, is responsible for binding of the viral polyproteins to the membrane (Parent et al., 1995). Once at the plasma membrane, Gag interacts with the cellular chaperone HP68 that induces conformational changes in the polyprotein in preparation for processing into mature virions (Zimmerman et al., 2002). The association of Gag and Gag-Pol molecules at the membrane make up the immature virion. It is thought that these molecules interact via the interaction or “I” domain in NC (Swanstrom and Wills, 1997).

The mature virion consists of the proteins that make up Gag and Gag-Pol. Maturation of the virion takes place as the viral protease (PR) cleaves Gag and Gag-Pol, liberating viral structural and enzymatic proteins. PR is an aspartic acid protease and as such contains conserved aspartic acid residues at its active site that coordinate a water molecule for the hydrolysis of the peptide bond. The structural proteins MA, CA, NC and p6 are derived from Gag. MA, CA and NC are also derived from Gag-Pol polyproteins. The enzymatic proteins RT/RNase H, IN and PR are derived from Pol in the Gag-Pol polyprotein (Swanstrom and Wills, 1997). A schematic of the mature HIV virion is shown in Figure 1.1 and the Gag-Pol polyprotein is shown in Figure 1.2.
The C-terminal domain of CA acts to oligomerise Gag (Gamble et al., 1997). MA forms the outer layer of the virion, while CA forms the core of the virus particle, which in the mature virion is conical. The third component of the Gag polyprotein, NC, binds genomic RNA placing it within the virion during assembly. Whilst assembly is underway, NC coats the viral RNA within the virion core, compacting the genome, presumably protecting it from nuclease activity. NC binding to the genomic RNA occurs via an interaction with four independent RNA stem loops, the $\Psi$ packaging sequence (Berkowitz, Luban, and Goff, 1993; Luban and Goff, 1994). Two copies of genomic RNA are found in the mature virion. The two genomic RNAs have been shown to dimerise via interactions between the stem loops of the $\Psi$ sequence (Clever and Parslow, 1997). It is thought that unwinding of genomic RNA allows the tRNA$^{Lys}$ primer needed for reverse transcription to anneal to the PBS (Swanstrom and Wills, 1997). p6, also known as the late domain, interacts with the accessory protein Vpr, incorporating it into the virion (Cohen, Subbramanian, and Gottlinger, 1996) and the cellular protein tumour susceptibility gene 101 (Tsg101) (Garrus et al., 2001; Martin-Serrano, Zang, and Bieniasz, 2001). Tsg101 binds to a P(S/T)AP tetrapeptide motif located within p6 (VerPlank et al., 2001) where it recruits other cellular factors that help to complete the budding process (see below). Two other proteins, p2 and p1, are liberated from Gag by PR cleavage. p2 and p1 are found separating CA and NC, and NC and p6 respectively. They are known as spacer proteins and their function is unclear at present, although it is thought that cleavage of p2 may regulate PR processing of Gag (Pettit et al., 1998). As mentioned above, the accessory proteins Nef and Vif are incorporated into the virion.

Several cellular proteins are also incorporated into the virion during maturation. For example, molecular chaperone proteins such as cyclophilin A, Hsp60, Hsp70 and Hsc70 are incorporated into the HIV virion (Franke, Yuan, and Luban, 1994; Gurer, Cimarelli, and Luban, 2002; Luban et al., 1993). There is much speculation as to where the proteins mentioned above act in the virus life cycle as they could act both in virus assembly to modulate the conformation of viral proteins and during the early stages of infection when the viral proteins are shed from the virion in the cytoplasm. The best studied interaction, between CA and cyclophilin A, indicates that cyclophilin A regulates HIV infectivity in human T lymphocytes (Braaten and Luban, 2001). The
molecular basis of this regulation remains unclear but it has been suggested from the crystal structure of Cyclophilin A bound to CA that Cyclophilin A may act to weaken CA-CA interactions, thus promoting disassembly of the viral core (Gamble et al., 1996).

1.3.9 Budding from the plasma membrane

HIV buds through the cholesterol and sphingolipid rich microdomains mentioned above (Nguyen and Hildreth, 2000). In order to do this it appears that the virus has commandeered a cellular pathway. Within the cytoplasm several proteins of the vacuolar protein sorting pathway associate to form a complex, ESCRT-1. ESCRT-1 is responsible for introducing ubiquitinated molecules into the multi-vascular body (MVB) pathway that is responsible for the formation of endosomal vesicles (Katzmann, Babst, and Emr, 2001). Such vesicles are responsible for the trafficking of molecules between the trans-Golgi network, the plasma membrane and the lysosome. Tsg101, which, as mentioned above, binds to p6 (VerPlank et al., 2001), is part of the ESCRT-1 complex. Therefore, it is thought that the virus harnesses the MVB pathway via Tsg101 so that, at the site of virion assembly, the MVB pathway constituents encapsidate the virion with a lipid bilayer and mediate membrane fusion so that the lipid bilayer completely covers the virion (Garrus et al., 2001; Martin-Serrano, Zang, and Bieniasz, 2001). Furthermore, the observations that MVB targeting requires substrate ubiquitination (Katzmann, Babst, and Emr, 2001), and that Tsg101 binds ubiquitin (Garrus et al., 2001), are consistent with previous observations that ubiquitination of Gag plays an important role in virion budding and release from the cell (Schubert et al., 2000; Strack et al., 2000). It remains unclear, however, if all members of the ESCRT-1 complex are required for the budding process and if sequestering these cellular factors contributes in any way to cell death (Luban, 2001).
1.3.10 Incorporation of molecules into the virion membrane

The viral envelope protein (Env) is incorporated into the virion as it matures and buds through the plasma membrane. Env is translated as a polyprotein that is cleaved by cellular protease(s) in the endoplasmic reticulum into the surface (SU) and transmembrane (TM) subunits (Swanstorn and Wills, 1997). This process occurs in the Golgi and is mediated by member(s) of the subtilisin-like protein convertase family (Miranda et al., 1996). Whilst in the trans-Golgi network, the SU subunit is glycosylated and the envelope forms a trimer of SU and TM heterodimers. Envelope trimers arrive at the plasma membrane and are anchored in lipid rafts by palmitic acid residue found in the cytoplasmic tail of the TM subunit (Rousso et al., 2000). Env incorporation into the virion is dependent on specific interactions between the envelope's cytoplasmic tail and MA (Dorfman et al., 1994; Freed and Martin, 1995; Mammano et al., 1995).

The list of cellular membrane proteins found in the lipid bilayer that the virion acquires as it buds is extensive (for review see (Ott, 1997)). It is not known on what basis some molecules but not others are incorporated, but many incorporated cellular proteins have been proposed as having roles in viral pathogenesis. Often these molecules have specific functions in the modulation of the anti-viral immune response. For example, human lymphocyte antigen Class II (HLA-II) molecules are the most frequently detected and abundantly incorporated cellular molecules in the HIV virion (Tremblay, Fortin, and Cantin, 1998). It is thought that the presence of these molecules on the virion surface aid functional interactions between the virus and T cell receptor molecules on T lymphocytes (Arthur et al., 1992). Virus binding to the T cell receptor via HLA molecules incorporated in the lipid bilayer can lead to a state where the T cell is unresponsive to stimuli (anergy) or undergoes apoptosis (Greenfield, Nguyen, and Kuchroo, 1998). Furthermore, displaying complement-regulatory molecules such as CD46, CD55 and CD59 on the virion is sufficient to protect the virion from complement-mediated cell lysis (Saifuddin et al., 1997). Also, the incorporation of class I and class II major histocompatibility molecules (MHC I and MHC II, respectively) is proposed to have a major impact on recognition of the virus by the immune system (Esser et al., 2001). The presence of these molecules on the virus cell surface should not allow the immune system to recognise the virus as
“non-self”. Interestingly, the HIV co-receptors CCR5, CXCR4 and CCR3 are not incorporated in the virions lipid bilayer (Lallos et al., 1999), presumably to prevent virion-virion fusion.

HIV is not the only virus to bud through lipid rafts. Data has been presented showing that members of the gammaretrovirus, paramyxovirus, orthomyxovirus, and gammaherpesvirus families assemble in, and by extension bud from lipid rafts (Pickl, Pimentel-Muinos, and Seed, 2001). This could, in part, explain the longstanding observation made of many RNA and DNA viruses that should mixed infection in vitro occur virus particles consisting of combinations of different core and envelope proteins are produced (Huang et al., 1974; Rubin, 1965; Zavada, 1982). Such virions are termed pseudotypes (Rubin, 1965). In the case of HIV it has been shown that several different HIV pseudotypes can be produced during mixed infections in vitro. Viruses that can undergo phenotypic mixing with HIV include human immunodeficiency virus type 2 (HIV-2) (Le Guern and Levy, 1992), the deltaretroviruses human T-cell leukaemia virus types 1 and 2 (HTLV-I, HTLV-II respectively) (Landau, Page, and Littman, 1991; Le Guern and Levy, 1992; Lusso, Lori, and Gallo, 1990), xenotropic (Canivet et al., 1990; Lusso et al., 1990), polytropic (Chesebro, Wehrly, and Maury, 1990) and amphotropic (Chesebro et al., 1990; Lusso et al., 1990; Spector et al., 1990) strains of the gammaretrovirus murine leukaemia virus (MLV), the alphaherpesvirus herpes simplex type 1 (HSV-1) (Calistri et al., 1999; Zhu, Chen, and Huang, 1990) and the rhabdovirus vesicular stomatitis virus (VSV) (Weiss et al., 1986; Zhu, Chen, and Huang, 1990). In each case, phenotypic mixing produced HIV pseudotypes with an extended cellular tropism, capable of infecting CD4+ cells. To what extend, if at all, phenotypic mixing of HIV and other viruses occurs in vivo is unknown.
1.4. HIV accessory proteins

The HIV accessory proteins, Vpr, Vif, Vpu and Nef have a number of functions in the virus life cycle, for the most part interacting with host cell factors and adding an interesting layer of complexity to HIV replication and pathogenesis.

1.4.1 Vpr

Vpr appears to have several functions in HIV pathogenesis. The presence of Vpr in an infected cell arrests the cell cycle in the G2 phase by interfering in some fashion with cellular proteins that regulate the G2-M transition in the cell cycle (Re et al., 1995). This may enhance virus production, as expression of the viral genome is optimal in the G2 phase of the cell cycle (Goh et al., 1998).

Furthermore, as discussed earlier, Vpr is also proposed to have a function in nuclear entry (de Noronha et al., 2001; Heinzinger et al., 1994; Sherman et al., 2001; Vodicka et al., 1998).

1.4.2 Vif

Much of the research performed on Vif centres around the observation that, in the absence of Vif, virions produced from T lymphocytes, monocytes or macrophage are non-infectious (von Schwedler et al., 1993). Subsequent investigations revealed that the presence of Vif is essential only when HIV is produced from "restricted" cells such as those named above (Madani and Kabat, 1998; Simon et al., 1998). Recently it has been shown that Vif acts to suppress an innate antiviral mechanism present in T cells, mediated by a cellular gene, CEM15. The function of this gene had not previously been described and the molecular basis of its anti-viral action is as yet unclear (Sheehy et al., 2002). It is interesting to note that, firstly, Vif binds viral genomic RNA (Dornadula et al., 2000; Zhang et al., 2000) and, secondly, CEM15 has a high degree of homology to the cellular RNA editing protein APOBEC-1 (Sheehy et al., 2002). This has led to the intriguing suggestion that CEM15 could edit viral genomic RNA, making progeny virus non-infectious (Pomerantz, 2002).
1.4.3 Vpu

Vpu is an integral membrane protein associated with the Golgi and endoplasmic reticulum (Maldarelli et al., 1993). Unlike the other HIV accessory proteins, Vpu does not appear to be incorporated into the virion at detectable levels (Klimkait et al., 1990; Strebel et al., 1989).

Vpu appears to have three distinct functions. Firstly, Margottin and co-workers have shown that Vpu binds CD4 (Margottin et al., 1998), and enhances its degradation via ubiquitin-mediated proteolysis. This depletes the pool of CD4 molecules available for interaction with Env, promoting Env expression at the cell surface and inhibiting superinfection. Secondly, Vpu is required for efficient virus maturation and release. In the absence of Vpu viral proteins accumulate in the cell under the plasma membrane and, of those virions that do bud, a large proportion are malformed and remain attached to the cell (Klimkait et al., 1990). Finally, it has been shown that Vpu acts as a monovalent cation channel (Ewart et al., 1996). The need for such a function is as yet unclear, but there is some data suggesting that there is a requirement for the movement of ions during budding as drug-induced blockage of ion channels in infected cell negates the budding-enhancement function of Vpu (Ewart et al., 2002).

1.4.4 Nef

Like Vpu, Nef also down-regulates CD4 in infected cells (Garcia and Miller, 1994). Nef helps create CD4-specific clathrin-coated pits that rapidly endocytose the viral receptor (Piguet et al., 1999). Nef is also involved in the down-regulation of MHC class I molecules from the cell surface which inhibits CTL mediated lysis of HIV infected cells (Collins et al., 1998). Here it appears Nef acts on a protein sorting pathway which directs MHC class I molecules to the cell surface (Piguet et al., 2000).

Nef can also enhance virion infectivity in some cell types, particularly in the human cell line HeLa or primary T cells during activation (Spina et al., 1994). The mechanism surrounding this enhancement has yet to be elucidated, but it is speculated that it involves a Nef-associated protein kinase (p21-activated kinase or PAK) that
phosphorylates serine in MA (Swingler et al., 1997). The role of serine phosphorylation in MA is as yet unclear, but may aid the uncoating process in some fashion (Bukrinskaya et al., 1996). Nef also enhances infection at a cellular level as the expression of Nef in macrophage induces the production of chemotactic factors to attract and stimulate lymphocytes, attracting a pool of infectable cells to sites of virus replication (Swingler et al., 1999). In order to prevent macrophage cell death during replication and attraction, Nef induces constitutive activation of the cellular transcription factor Stat-3 (Briggs et al., 2001).

1.5 Development of vectors based on HIV

The basic concept behind the creation of a viral vector is the replacement of viral genes with one or more exogenous genes or transgenes so that once a target cell has been transduced by the virus the transgene(s) in question can be expressed in that cell. The first attempt at HIV mediated gene transfer involved the introduction of the bacterial marker chloramphenicol transferase into a replication-competent genome (Terwilliger et al., 1989). During virus replication this marker was introduced into the host cell genome as a part of the HIV provirus. This work was furthered by a replication-defective construct where the viral envelope sequence had been deleted and replaced by the Escherichia coli guanine phosphoribosyl transferase (gpt) gene under the control of the simian virus 40 (SV40) promoter, conferring mycophenolic acid resistance to infected cells. As the envelope sequence is absent in the vector genome, virus produced by infected cells is devoid of an Env glycoprotein and is, therefore, non-infectious. The HIV envelope was supplied to the virus in the producer cell making it competent to infect human CD4+ cells. The envelope of the amphotropic MLV virus could also be supplied in trans pseudotyping the virus, conferring tropism to a wide range of human and murine cells (Page, Landau, and Littman, 1990).

As HIV is a human pathogen, the occurrence of replication competent virus (RCV) from HIV vectors by recombination is of much concern. In the development of HIV vectors, attempts have been made to delete as much viral gene sequence as possible.
The most commonly used HIV vectors, constructed by Naldini and co-workers (Naldini et al., 1996), have further reduced the risk of replication competent virus occurring by separating gag-pol and vector genome expression cassettes onto different plasmids. Virus is produced by transiently transfecting these plasmids into human 293T cells with a heterologous viral envelope expression vector, thus pseudotyping the vector (the need for vector pseudotyping is discussed below). To construct the vector genome, a molecular clone of HIV, R7, was used. R7 is a chimera of the HIV molecular clones NL4-3 and HXB2. R7 was constructed by substituting the sequence encoding CA, p2, NC, p1, p6 and Pol in HXB2 for that in NL4-3. The env gene was deleted along with the majority of the gag-pol coding sequence. The RRE sequence remains intact, however. The gag-pol sequence is replaced with a transgene expression cassette, a transgene under the control of a heterologous promoter. To construct the packaging component, env gene and packaging sequence are deleted along with the 5'LTR. As expression of the packaging component (gag-pol) is under the control of the human cytomegalovirus (hCMV) early promoter there is no requirement for Tat in the expression of the packaging construct. Both tat and rev are retained in the packaging plasmid, however. Tat is required for the expression of the vector transcript, whose transcription is controlled by the viral LTR. The mRNA encoding gag-pol and the vector transcript both contain the RRE and, therefore, require Rev to bring them into the cytoplasm. As a further safety measure, all the HIV accessory genes were subsequently removed from the packaging construct (Zufferey et al., 1997). It must be noted that, in the context of the vector system, removal of the viral accessory genes had no significant effect on vector production from 293T cells (Zufferey et al., 1997) and thus far has not been shown to impact on the ability of the vector to transduce primary cell cultures ex vivo.

As gag-pol expression occurs only in the producer cell, the vector is replication incompetent. Furthermore, as no viral proteins are produced in the transduced cell there is a reduced chance that an immune response against the vector will be raised in the host (Kafri et al., 1999).

As the LTRs in the integrated genome are intact, the vector genome can be mobilised from the infected cell by replication competent HIV (Bukovsky, Song, and Naldini, 1999). Although the vector genome can compete with the virus genome for
encapsulation, thus limiting HIV transmission (Bukovsky, Song, and Naldini, 1999), mobilisation of the genome in a therapeutic setting is not desirable as this can lead to inappropriate transgene expression which, in turn, could lead to a disease pathology. Moreover, the vector genome could recombine with the virus genome (Bukovsky, Song, and Naldini, 1999). To limit these concerns, the promoter and enhancer binding sequences in U3 of the 3’LTR of the vector construct have been deleted in most vector genome constructs. The 3’LTR copies to the 5’ end of the genome during reverse transcription. Thus, there is little or no expression of the vector genome from the LTR in the infected cell (Zufferey et al., 1998). These vector genomes are termed self-inactivating.

Other vectors based on HIV have been constructed (Kim et al., 1998; Kotsopoulou et al., 2000) but do not shown any significant advantage over that described by Zufferey et al. (Zufferey et al., 1997). It has been argued that vectors based on non-human lentiviruses should be safer in a therapeutic setting as these viruses do not replicate in human cells. Vectors based on simian immunodeficiency virus (SIV) (Negre et al., 2000), feline immunodeficiency virus (FIV) (Poeschla, Wong-Staal, and Looney, 1998), equine infectious anaemia virus (EIAV) (Mitrophanous et al., 1999; Olsen, 1998) and the bovine lentivirus Jembrana disease virus (Metharom et al., 2000) have been constructed. These vectors can transduce growth arrested human cell lines but it is still questionable whether or not they will be useful for the transduction of a wide range of primary human cell cultures. Firstly, it has been shown that EIAV vectors are significantly less efficient in the transduction of human cells compared to HIV based vectors (Ikeda et al., 2002). This is due to restriction of EIAV infection by the human cellular factor Ref1 (Hatziioannou et al., 2003). Secondly, efficient transduction of primary human DC cell cultures with SIV is possible only when a certain SIV accessory gene, Vpx, is added to the vector (Mangeot et al., 2002). It has yet to be seen if this accessory protein is dispensable for the transduction of other primary human cell cultures or if others are required. Chimeric vectors composed of SIV and HIV elements have also been described (White et al., 1999). Construction of vectors of this type may reduce risk of recombination between packaging and vector sequences, but in this case the titre of the vector is less than that achieved using the prototype vector constructed by Zufferey et al. (Zufferey et al., 1998).
1.6 Development of stable HIV packaging cell lines

Production of HIV vectors by three plasmid transient transfection is not a realistic method of producing high titre vector stocks suitable for future experimental or clinical gene transfer protocols. Firstly, virus preparations could be contaminated with replication-competent virus produced by recombination during the transfection procedure. Using stable packaging lines extensive safety controls can be applied to large batches of virus more easily than to relatively small batches produced by transfection. Secondly, from a practical perspective, it would be simpler, more efficient and economical to produce virus particles from stable packaging cell lines in certain settings.

Stable MLV packaging cells have been successfully created. To produce these cell lines separate packaging, vector and envelope expression constructs have been stably transfected into the cell line of choice, whereupon these cells constitutively produce retrovirus vectors without adverse effects to the producer cell.

The parallel situation using HIV vectors is, however, more complex, as the introduction of the packaging components of the vector by transfection appears to have adverse effects to the producer cell, ultimately leading to cell death, or gene silencing (Buchschacher and Wong-Staal, 2000). It is at present unclear why this might be the case, although it has been speculated that the long term expression of HIV Gag and Pol proteins, in particular PR, are cytotoxic (Krausslich, 1992; Krausslich et al., 1993; Snasel et al., 2000).

Thus far, no stable cell lines constitutively producing high titre HIV vectors have been produced by transfection, although a number of inducible systems where expression of the vector components is regulated have been reported (Kafri et al., 1999; Kaul et al., 1998; Klages, Zufferey, and Trono, 2000; Sparacio et al., 2001; Yu et al., 1996). The duration of virus production from these cells after induction is short. A further drawback to the use of such cell lines is the presence of molecules that regulate virus production in the harvest supernatant. It also may not be economical to use such molecules in large-scale virus production.
Stable, high-level expression of HIV proteins can occur after infection (Hoxie et al., 1985). To produce stable HIV packaging cells Ikeda and co-workers (Ikeda et al., 2003) used MLV vector infection to introduce the codon-optimised HIV \textit{gag-pol} (Kotsopoulou et al., 2000) into the human cell lines 293T and HT1080. Stable, long-term, expression of HIV \textit{gag-pol} in the absence of Rev was then observed in these cells. The regulatory genes \textit{tat} and \textit{rev} were subsequently introduced by MLV vector infection, as these proteins are required for the transcription and movement of a vector genome into the cytoplasm. These pre-packaging cells were named STAR (293T based) and HT-STAR (HT1080 based). STAR serves as an acronym for - synthetic \textit{gag-pol} plus \textit{tat} and \textit{rev}. Infectious vectors could be produced at high titre with the introduction by infection of a vector genome and by transfection of an envelope coding sequence. A self-inactivating vector cannot be used in this context. A self-inactivating vector can be transfected into cells, but in general this produces lower titre virus and many weeks of screening is required to find clones stably expressing the vector genome (Ikeda et al., 2003). The use of STAR cells appears to be a realistic approach to the production of high titre HIV vector stocks for gene transfer protocols. Why stable expression of HIV Gag-Pol after infection, but not transfection, is possible remains unclear. As mentioned above, it has been shown that HIV integration preferably occurs at transcriptionally active sites (Schroder et al., 2002). It has, therefore, been speculated that the site of integration dictates the level at which HIV Gag-Pol is expressed (Ikeda et al., 2003).

\textit{1.7 Thesis aims}

With the ability to transduce both dividing and non-dividing cells, minimal immunogenicity, low incidence of insertional mutagenesis and many engineered safeguards against the generation of replication competent virus, vectors based on HIV have great potential for use in a wide variety of gene therapy applications.

The ultimate aim of this thesis is the development of HIV-based vector technology for the transduction of clinically relevant cell types, specifically focusing on the production and characterisation of HIV vector pseudotypes. Pseudotyping of HIV-
based vectors is necessary as it may not be advantageous to use an HIV envelope with an HIV vector in the field of gene therapy.

As mentioned, HIV gains entry into the cell via interactions between the viral envelope glycoprotein and its receptors, CD4 plus one of a number of chemokine receptors such as CXCR4 or CCR5 (Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). An HIV vector bearing the HIV envelope on its surface would, therefore, be able only to gain entry into cells displaying CD4 plus a co-receptor. This would confer a relatively narrow tropism to the vector, limited to certain cell lineages, including macrophage and lymphocytes.

There are also safety issues to consider concerning the use of an HIV envelope. Firstly, there are concerns that replication competent virus could arise during vector production via non-homologous recombination (Buchschacher and Wong-Staal, 2000). Secondly, a compelling reason not to use the HIV envelope in a therapeutic setting is that the interaction between the envelope surface subunit, CD4 and the co-receptor CXCR4 can trigger apoptosis in certain cell types, such as lymphocytes (Badley et al., 2000), endothelium (Ullrich, Groopman, and Ganju, 2000) and neurons (Kaul, Garden, and Lipton, 2001). Recently it has also been shown that HIV envelope/CD4/CCR5 interactions can also trigger apoptosis in lymphocytes (Algeciras-Schimmich et al., 2002).

The use of an HIV envelope may not be the most suitable or efficient means to mediate HIV vector entry. Alternatives to the HIV envelope for mediating HIV vector infection must, therefore, be used, hence the need for pseudotyping.

HIV-based vectors have, to date, most commonly been pseudotyped with the G protein from the rhabdovirus vesicular stomatitis virus (VSV-G), as this pseudotype can be transiently produced at high titre. The use of HIV(VSV-G) has been favoured as this pseudotype can transduce many clinically relevant human cell types ex vivo (Follenzi et al., 2000; Naldini et al., 1996). Whilst HIV(VSV-G) has the potential to transduce a wide range of cell types, it is unknown if the use of VSV-G is the most efficient method of mediating vector entry into a number of cells that are gene therapy targets. The ultimate aim of this thesis is to test the transduction of a range of different
HIV pseudotypes versus HIV(VSV-G) on several clinically relevant cell cultures to determine which is optimal in this regard.

The first goal of this thesis, therefore, is to assess the ability of a number of viral envelope glycoproteins to pseudotype HIV based vectors using a transient production method.

The viral envelope glycoproteins in question are those from (i) members of the gammaretrovirus family: amphotropic murine leukaemia virus (MLV-A), gibbon ape leukaemia virus (GALV) and the feline endogenous virus RD114, and (ii) from the alphaviruses Ross River virus (RRV), Semliki Forest virus (SFV) and Sindbis virus (SIN).

The cellular receptors for members of the gammaretrovirus family are found on a wide range of human cell types and these envelopes have been used successfully with MLV vectors to transduce several clinically relevant cell types, for example CD34\(^+\) cells (Demaison et al., 2002; Kelly et al., 2000), T lymphocytes (Blaese et al., 1995; Bordingnon et al., 1995; Gladow et al., 2000; Uckert et al., 2000) and cells from primary human melanomas (Palmer et al., 1999; Porter et al., 1996). Alphaviruses are human pathogens and exhibit a wide cell tropism. Pseudotyping HIV-1 with the envelopes from human pathogens that infect clinically relevant cell types \textit{in vivo} may be a more efficient method of mediating vector entry than using either VSV-G or gammaretrovirus envelopes.

Data from transient production experiments will be used to determine which envelopes should be introduced into stable packaging cells (STAR) to determine if long-term production of high titre pseudotypes is possible. Using virus from these cells it will be possible to compare properties that each envelope confers to the virion, such as resistance to inactivation in human sera, compared to HIV(VSV-G). This data can then be used for consideration of what HIV pseudotypes should be used in future \textit{ex vivo} and \textit{in vivo} applications.
Finally, infection of primary human cell cultures with a range of different pseudotypes will be carried out to determine which is optimal in this regard and also with a view to optimisation of the vector packaging and genome constructs.
Chapter 2
Materials and Methods

2.1 Plasmid preparation and manipulation

2.1.2 Preparation of heat shock competent *E. coli*

1ml of an overnight culture of *E. coli* HB101 in Luria Broth (LB) (GibCoBRL) was subcultured into 100ml of fresh LB and cultured for 2 hours on a shaker at 37°C. The culture was then cooled on ice for 10 min and pelleted at 4°C and the supernatant then discarded. The pellet was resuspended in 30ml ice-cold transformation buffer (250mM PIPES, 2.5mM CaCl$_2$, 2 H$_2$O, 60mM KCl, pH is adjusted to pH 6.7 using KOH then 55mM MnCl$_2$ is added to this solution). The bacteria were pelleted and resuspended in 10ml cold transformation buffer containing 10% DMSO and frozen at -80°C in 200μl aliquots.

2.1.2 Introduction of plasmid DNA into *E. coli*

10 - 50ng of plasmid were mixed with 50μl of heat shock competent *E. coli* HB101 and incubated on ice for 10 min. The cells were then heat shocked for 90s at 42°C, cooled on ice and pelleted. Pellets were resuspended in 20μl of LB and plated on LB-agar + 50μg/ml ampicillin (Sigma) and incubated overnight at 37°C.

2.1.3 Plasmid DNA mini-preps

Single colonies of *E. coli* HB101 were picked into 5ml of LB supplemented with 50μg/ml ampicillin and agitated at 37°C overnight. Mini preps of plasmid DNA were produced from 4ml of 5ml overnight cultures of transformed bacteria using a Concert™ Rapid plasmid DNA Mini-prep kit (GibCoBRL) as per the manufacturer’s instruction.
2.1.4 Restriction enzyme digests

All restriction enzymes were purchased from Promega. 0.5-2 μg of plasmid DNA was mixed with 2μl of the appropriate enzyme buffer (x10 stock) and diluted to 20μl in water with 1 μl of enzyme. Digests were performed at 37°C for 1 – 2 hr and 10 μl was mixed 6:1 with Orange G loading buffer then loaded on a 1% TAE:agarose (SeaKem) gel containing 0.4 μg/ml ethidium bromide. Gels were run at 100V and observed under UV on a Syngene gel documentation system.

2.1.5 Isolation of DNA restriction fragments

DNA fragments were isolated from bands cut from TAE/agarose gels using a Concert™ Rapid DNA fragment gel purification kit (GibCoBRL) as per the manufacturer’s instructions.

2.1.6 Sub-cloning of DNA fragments

Isolated fragments were mixed at a ratio of 3:1 with vectors linearised by restriction enzyme digest. Vector and insert were mixed 1:1 with Takara DNA ligase solution and incubated at 15°C for 30 min. Ligation solutions were transformed by heat shock into E. coli HB101 and subsequently plated onto LB-agar + 50μg/ml ampicillin. Resultant colonies were mini-prepped and screened for insert and orientation by restriction digest.

2.1.7 Site directed mutagenesis

Site directed mutagenesis was performed using a QuikChange site directed mutagenesis kit (Stratagene) as per the manufacturers instructions. 10ng of plasmid DNA was mixed with 1x Pfu Turbo buffer plus 1μl of each primer (from 10μM stock) and 2.5mM dNTPs in a final volume of 50 μl. PCR was performed for 30 cycles using 1.5 U of Pfu Turbo DNA polymerase (30s 92°C, 1min 55°C, and 1min/kb extension at 72°C). PCR products were then digested using DpnI, added directly to the PCR mix. Undigested plasmid was then transformed into XL1-Blue E.coli by electroporation.
and subsequently plated onto LB-agar + 50μg/ml ampicillin. Resultant colonies were mini-prepped as above and mutagenesis was confirmed by DNA sequencing using an ABI Prism – 3100 Genetic Analyzer.

2.1.8 Plasmid DNA midi-preps

1ml of 5ml of bacteria cultured in LB + 50μg/ml ampicillin (37°C, overnight) was subcultured into 250 ml LB + ampicillin and shaken at 37°C for 16 hrs. Midi preps of plasmid DNA were produced from this culture using Plasmid Midi Kit (Qiagen) as per the manufacturer’s instruction.

2.1.9 Plasmid DNA maxi-preps

Plasmid DNA for transfection was prepared by polyethylene glycol extraction as described. Single colonies of *E coli* HB101 carrying the desired plasmid were picked into 5 ml of LB supplemented with 50μg/ml ampicillin (Sigma) and agitated at 37°C for 8 to 10 hours. 500μl of bacteria was subcultured into 500 ml of LB + 50μg/ml ampicillin and shaken at 37°C for 16 hrs. Bacteria were then harvested by centrifugation (15 mins at 4000g), washed once in STE and resuspended in 10 ml of ice cold Solution I (50 mM glucose, 1mM EDTA, 10 mM Tris-HCL (pH 8.0)).

20ml of Solution II (0.2M NaOH, 1% SDS) was added, and the cells inverted and left lyse for 5 min at room temperature. The mixture was neutralised by adding 15 ml of Solution III (3M Sodium Acetate buffer (pH 6.0)) and left on ice for 15 min. Protein/SDS precipitates were removed by centrifugation at 10,000g for 15 min at 4°C and supernatants were filtered through tissue paper.

Crude nucleic acid preparations were isolated by adding 0.6 x volumes of isopropanol and centrifugation at 10,000g for 15 min at 4°C. Pellets were washed once in 70% ethanol and re-suspended in 0.5 ml of TE (pH 8.0).
An equal volume of cold 5M LiCl was added to precipitate RNA, centrifuged as above and the supernatant transferred to an equal volume of isopropanol and the resulting precipitate harvested and washed as previously. The pellet was resuspended in 0.5 ml of TE pH 8.0 + 20μg/ml RNase (Sigma) and incubated at room temperature for 30 mins. Supercoiled plasmid DNA was precipitated by the addition of an equal volume of 1.6M NaCl, 20% PEG 8000 (Sigma) solution and harvested by microfuge centrifugation. PEG pellets were resuspended in 500 μl TE (pH 8.0), phenol:chloroform extracted twice, ethanol precipitated and resuspended in TE. DNA purity was assessed by the ratio of optical densities at 260 and 280 nm and was typically >1.75. Plasmids were checked by restriction digest, and aliquots diluted to 1 mg/ml and stored at −20°C.

2.2 Plasmids

2.2.1 Packaging and vector genome expression plasmids

The HIV packaging plasmid pHCMVΔR8.91 (Figure 2.1.A), a kind gift from D. Trono (Geneva, Switzerland), expresses gag, pol, tat, and rev and does not express the accessory genes vif, vpr, vpu, and nef (Zufferey et al., 1997). Expression of gag, pol, tat, and rev is controlled by the human cytomegalovirus (hCMV) immediate early promoter. pSYNGP, expressing the codon-optimised HIV gag-pol (Kotsopoulou et al., 2000) can also be seen in Figure 2.1.A. The HIV vector plasmid pHR'SINcPPT-SE (Figure 2.1.B) contains the reporter gene enhanced green fluorescent protein (eGFP) under the control the promoter/enhancer sequences from the U3 region of the spleen focus forming virus (SFFV) strain P long terminal repeat (LTR), with the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) in the 3’ untranslated region of the eGFP sequence. The WPRE significantly up-regulates the expression of reporter proteins in a non-promoter, non-transgene and non-vector dependent fashion when placed in the sense orientation in the 3’ untranslated region of the transgene (Zufferey et al., 1999). pHR'SINcPPT-SE also contains the HIV central polypurine tract (cPPT) sequence (Zennou et al., 2000). This plasmid is described in Demaison et al. (Demaison et al., 2002) and was a gift from A. Thrasher (London, United Kingdom). pH7G, a gift from K.Mitrophanous (Oxford BioMedica, UK) has not been previously described. pHV was a gift from Y.Ikeda (UCL,UK) and its
construction is described in Ikeda et al. (Ikeda et al., 2003). Both pH7G and pHV are shown in Figure 2.1.B.

MLV vectors were assembled from pHIT60 carrying MLV gag-pol (Soneoka et al., 1995), and pCNCG. pCNCG encodes an MLV vector genome incorporating a hCMV promoter-driven eGFP reporter gene. This plasmid is based on pHIT111 described in Soneoka et al. (Soneoka et al., 1995). Both pHIT60 and pCNCG were kindly provided by Oxford BioMedica plc. (Oxford, United Kingdom).

2.2.2 Envelope glycoprotein expression plasmids

pMD-G (Naldini et al., 1996), also provided by D. Trono, encodes the vesicular stomatitis virus G protein (VSV-G) under the control of the hCMV promoter. The envelope expression cassette found in pMD-G is shown in Figure 2.2.
Figure 2.1 HIV packaging and vector constructs

(A) The HIV packaging constructs pHCMVΔR8.91 and pSYNGP. Transcription of \textit{gag-pol} in both constructs is controlled by the hCMV promoter and terminates in the LTR in pHCMVΔR8.91 or at the SV40 poly(A) site in pSYNGP. pHCMVΔR8.91 also encodes Tat and Rev, while pSYNGP encodes the neomycin resistance gene \((\text{neo}^R)\). The protein products from the protein coding regions contained in the mRNA are shown in blue and \textit{cis}-acting elements in white. The packaging signal that does not overlap \textit{gag} is also deleted. (B) The HIV vector constructs pHR'SINcPPT-SE, pH7G and pHV. Transcription of the vector transcript is controlled at the 5' end of the genome by the LTR or hCMV and terminates in the 3' LTR. The positions of the packaging signal (\(\psi\)), RRE and cPPT are shown. The position of the eGFP expression cassette contained within the vector construct is also shown. The protein products from the protein coding regions contained in the mRNA are shown in blue and \textit{cis}-acting elements in white.
A

pHCMVΔR8.91

\[ \text{hCMV}^{\gamma A} \rightarrow \text{gag-pol} \rightarrow \text{RRE} \rightarrow \text{LTR} \]
\[ \text{tat + rev}_1 \rightarrow \text{tat + rev}_2 \]

pSYNGP

\[ \text{hCMV} \rightarrow \text{codon optimised gag-pol} \rightarrow \text{SV40} \rightarrow \text{neo}^{\theta} \]
\[ \beta\text{-globin/IgG chimeric intron} \rightarrow \text{SV40 poly(A)} \rightarrow \text{Synthetic poly(A)} \]
B

pHR'SINcPPT-SE

LTR RRE cPPT SFFV eGFP WPRE LTRAU3

pH7G

hCMVLTRAU3 RRE/rev cPPT hCMV eGFP LTR

pHV

hCMVLTRAU3 RRE cPPT SFFV eGFP WPRE LTR
2.2.2.1 Gammaretrovirus envelope glycoprotein expression plasmids

All retroviral envelope expression plasmids are found in the constructs called phCMV (Figure 2.2) and unless stated otherwise were kindly provided by F-L Cosset (Lyon, France). In these constructs the hCMV promoter also controls expression. phCMV-A (MLV strain 4070A envelope expression vector) is based on a Clonetech expression vector pIRES2-eGFP as are the wild-type and chimeric RD114 envelope expression vectors (phCMV-RD114wt, phCMV-RD114-PR and phCMV-RD114/MLV). phCMV-GALVwt, phCMV-GALV-KQ and phCMV-GALV/MLV (GALV/MLV expression vector formerly called phCMV-GALV (Negre *et al.*, 2000)) are based on pcDNA3.1 (Invitrogen). These plasmids differ from the other retroviral envelope expression constructs as transcription from the promoter reads through the envelope gene and a phleomycin resistance gene until the SV40 polyadenylation signal is reached. The envelope and phleomycin resistance genes are separated by a 74 bp sequence described in Cosset *et al.* (Cosset *et al.*, 1995). Translation of the phleomycin resistance gene occurs by reinitiation of translation.

phCMV-GALVwt and phCMV-GALV-KQ were constructed in this study. The wild-type GALV expression plasmid pMD-pCEII (provided by F-L Cosset) contains the GALV SEATO envelope sequence under the control of hCMV. This plasmid was used as the template to introduce the mutation K618Q into GALVwt. This was achieved by PCR site-directed mutagenesis using *Pfu* Turbo polymerase (Stratagene) according to the manufacturer’s protocol. The following primers were utilised; (5’-3’)

**Forward:** ATTCCTGGTGCTTAGACTTGACGTGAATAGG  
**Reverse:** CCTATTCACGTCAAGTCTAAGACCAGGAAT

Mutagenesis was confirmed by DNA sequencing (ABI Prism – 3100 Genetic Analyzer) using the primer; (5’-3’) GGATGGTTCAATAACTCCCCT positioned 50 bp upstream of codon 618. This plasmid was renamed pMD-CEII-KQ. The GALV cytoplasmic tails from pMD-CEII and pMD-CEII-KQ were removed using *NsiI* and *NorI* digestion and replaced the MLV cytoplasmic tail in phCMV-GALV/MLV resulting in the envelope expression vectors phCMV-GALVwt and phCMV-GALV-KQ respectively.
Note that the MLV-A, GALV/MLV and RD114/MLV envelopes contain the cytoplasmic tail of the Friend strain of MLV. The amino acid sequence of the FrMLV cytoplasmic tail is identical to that of MLV-A save for the conservative substitution Ile/Leu at codon 628 in the cytoplasmic tail of the envelope.

2.2.2.2 Alphavirus envelope glycoprotein expression plasmids

All alphavirus envelope glycoprotein expression plasmids were kindly provided by D.Sanders (Indiana, USA). All alphavirus envelope coding sequences were cloned into the expression vector pcDNA3.1/Zeo(+) (Invitrogen). The Ross River virus envelope expression vector pRRV-E2E1A has been previously described (Sharkey et al., 2001). Neither the Semliki Forest virus envelope expression vector pSFV-E2E1, nor the Sindbis envelope expression vector pSIN-E2E1A has been previously described. To construct pSFV-E2E1A and pSIN-E2E1A the E3-E2-6K-E1 coding regions from the cDNA described in (Garoff et al., 1980) and the Sindbis virus heat resistant small plaque (hrsp) strain (Arias et al., 1983) respectively were inserted into pcDNA3.1/Zeo(+).
Figure 2.2 The envelope expression vectors for VSV-G (pMD-G) and the gammaretroviral envelopes (phCMV)

In pMD-G transcription is controlled by the hCMV promoter and terminates at a β-globin p(A) signal. The protein product of the expression constructs is shown in blue. Two versions of phCMV with different backbones have been used. The pcDNA3.1 version of phCMV differs from the pIRES2-eGFP version transcription from hCMV reads through the phleomycin resistance gene $Sh\ ble$. This protein is expressed by reinitiation of translation.
pcDNA3.1/Zeo(+)

Figure 2.3 The envelope expression vectors for the alphavirus envelopes

Transcription of the envelope sequence. Transcription of the zeocin resistance gene *Sh ble* is controlled by the SV40 promoter and terminates at an SV40 p(A) signal. In above figure the protein products are shown in blue and the *cis*-acting elements in white.
2.3 Cell culture

2.3.1 Tissue culture adapted cell lines

The tissue culture adapted cell lines used in this thesis are listed in Table 2.1. All cell lines used were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (GibCoBRL) supplemented with 10% Foetal Calf Serum (FCS), Penicillin (100 units/ml) and Streptomycin (100 µg/ml), with the exception of NIH 3T3 cells that were maintained in DMEM supplemented with 10% Donor Calf Serum (DCS), Penicillin (100 units/ml) and Streptomycin (100 µg/ml).

Cells were grown at 37°C in a humidified incubator at 10% CO₂. Upon confluence, cells were washed in PBS and detached in a minimal volume of Typsin-EDTA (GibCoBRL) and replated at a concentration of 1/4 to 1/10 or at the desired density for transfection or infection. All tissue culture ware was purchased from Nunc.

2.3.2 Maintenance of hybridoma and harvest of hybridoma supernatant for immunoblotting

The rat hybridoma 42/411 (Pinter et al., 1982) was grown in RPMI media (GibCoBRL) supplemented with 10% Foetal Calf Serum (FCS), Penicillin (100 units/ml) and Streptomycin (100 µg/ml) plus 1mM 2-mercaptoethanol (Sigma). Cells were grown at 37°C in a humidified incubator at 5% CO₂.

Cells were grown under these conditions in 30ml of medium at a starting density of 6 x 10⁶ cells/ml for 48 hours. The supernatant was then centrifuged (3000g, 7 mins, 4°C) to remove cells but not filtered. 0.15mM NaN₃ was then added. The supernatant was then aliquoted and frozen at -20°C until needed.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>293T</td>
<td>Human Embryonic Kidney</td>
<td>M. Calos, Stanford University, USA</td>
</tr>
<tr>
<td>TE671</td>
<td>Human Rhabdomyosarcoma</td>
<td>85111502; European collection of animal cell cultures</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human Cervical Carcinoma</td>
<td>93021013; European collection of animal cell cultures</td>
</tr>
<tr>
<td>HT1080</td>
<td>Human Fibrosarcoma</td>
<td>CCL-121; American Tissue Culture Collection</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>Murine Fibroblast</td>
<td>CRL-1658; American Tissue Culture Collection</td>
</tr>
</tbody>
</table>

Table 2.1 Tissue culture adapted cell lines
2.3.3 Human primary cell cultures

The procedure to isolate and infect the primary human cell cultures used in this thesis is outlined in Figure 2.4.

2.3.3.1 Isolation of human dendritic cells

120ml of venous blood was drawn from healthy volunteers into heparinised syringes. Blood was diluted 1:1 with Hanks Balanced Salt Solution (HBSS – Gibco) without Ca\(^{2+}\)/Mg\(^{2+}\). 30ml of blood and Hanks was then layered onto 15ml of Lymphoprep (Nycomed-Amersham) in 50ml centrifuge tubes and then centrifuged at 700g for 30 minutes at room temperature. The mononuclear cell fraction was removed, washed 3 times in HBSS and plated on 6 well dishes (10\(^6\) cells per dish) in RPMI supplemented with 10% FCS, previously heat inactivated at 56°C for 45 mins. After 2-3 hours incubation at 37°C in 5% CO\(_2\), non-adherent cells were washed off and the medium was replaced with RPMI + 10% FCS supplemented by 1000U/ml IL-4 and GM-CSF 100 ng/ml (Peprotech). After 5 days in culture (37°C in 5% CO\(_2\)) live suspension cells were incubated with 4 μg/ml each of mouse-anti-human monoclonal antibodies against CD2, CD8 and CD19 (eBioscience) and contaminating T and B cells depleted by incubating with goat-anti-mouse conjugated Dynabeads (Dynal) (4x10\(^6\) beads/10\(^6\) cells to be removed) and magnetic sorting. Cells were then replated at the required density for infection in 96-well plates.
120 ml heparinised peripheral blood drawn from healthy volunteers and layered onto lymphoprep

Isolation of mononuclear cell faction by centrifugation

blood

lymphoprep

centrifugation

serum

mononuclear cells

red blood cells

Mononuclear cells isolated by adherence to tissue culture plastic

Adherent cells

Culture in RPMI + 10% FCS + IL-4 and GM-CSF

DENDRITIC CELLS
(Depleted of contaminating B and T cells by magnetic removal of cells expressing CD2, CD3 and CD19)

Vector transduction

Further culture with cytokines

FACS analysis

Non-adherent cells

Culture in RPMI + 10% FCS + IL-2 (Stimulated with Concanavalin A)

T LYMPHOCYTES

Vector transduction

Further culture with cytokines

FACS analysis

Figure 2.4 Isolation and infection of primary human cell cultures
2.3.3.2 Isolation of human T lymphocytes

T lymphocytes were isolated on the basis of non-adherence to tissue culture ware after isolation of PBMC by centrifugation. T lymphocytes were then cultured in RPMI + 10% FCS supplemented with 100U/ml IL-2 (Peprotech) and 1μg/ml Concanavalin A (SIGMA) for 48 hours. Cells were then washed, replated and cultured for a further 3 days in RPMI + 10% FCS supplemented with IL-2. Cells were then replated in 96-well dishes at the required density for infection.

2.4 Production of pseudotyped vectors

2.4.1 Virus production utilising liposome mediated transfection of 293T cells

Transfection of 293T cells was conducted using LipoFectamine (Invitrogen) and the method essentially as described in the manufacturer’s instructions. 2x10^5 293T cells per well in a 24-well plate were seeded in 24 hours before transfection. The medium was replaced by 400μl of OptiMEM (GibCoBRL) prior to transfection.

Plasmids were diluted in OptiMEM to the following concentrations: Vector – 150ng/μl, Packaging - 100 ng/μl, and envelope - 50ng/μl. For each well 2.6μl of each plasmid was mixed together conserving the weight ratio 3:2:1 or in the case of VSV-G pseudotypes 3:2:0.5. In each experiment an unenveloped control was also included. In this case OptiMEM replaced the solution that should contain envelope expression plasmid DNA.

5μl of OptiMEM and 3μl of LipoFectamine were added to the plasmid mix and incubated at room temperature for 25 min. The volume was adjusted to 100μl in OptiMEM and added drop wise to the cells. After 4-6 hours at 37°C cells were then washed and grown for 48-72 hours in serum-free OptiMEM at 32°C. Supernatants were harvested, diluted 1/5 with OptiMEM and passed through a 0.45-μm-pore-size filter.
Note that for virus production from 6-well plates or 10cm dishes the same protocol is followed except all volumes are increased by a factor of 2 or 4 respectively. Virus produced from the 24-well transfection protocol is diluted 1/5 with OptiMEM before filtration through a 0.45µm pore filter and infection. All other infections are carried out using neat filtered supernatant.

2.4.2 Isolation of STAR cells producing alphavirus pseudotypes

STAR cells producing HIV(RRV) pseudotypes were generated by transfection of 0.8 µg of the RRV envelope expression vector pRRV-E2E1 into 2 x 10^5 STAR cells per well of a 24 well plate. 48 hours later transfectants were selected with the addition of 200 µg/ml zeocin to the culture media. Resistant colonies were selected 15 days later. After a further 5 days in culture the selected colonies were transduced with HIV(VSV-G) generated by transient transfection and carrying the vector HV at a multiplicity of infection of 40 as determined by infection of 293T cells. Cells were washed extensively and the media replaced. After 48 hours the supernatant from each well was titrated onto 293T cells to test for pseudotype production.

To confirm the RRV envelope sequence was indeed present in STAR-RRV cells, 2 x 10^5 cells were added to 200 µl of lysis buffer (5mM MgCl₂, 0.5% Tween-20, 0.5% NP40, 0.1% gelatin and 100µg/ml proteinase K) and incubated at 56°C for at least 2 hours, then heated to 95°C for 10 minutes. 5 µl of lysate was added to a PCR reaction in a total volume of 50 µl. PCR was carried out using Taq Polymerase and 10x Taq Polymerase Buffer (Promega) and the primers;

RRV-F (5'-3') – AGGATGTCTGCGCGCTGATG
RRV-R (5'-3') – TTGAGGTAGTCGCCTGGCGGA
The PCR product from this reaction was gel purified and ligated into the vector pGEM-T Easy (Promega) using 2x ligation buffer supplied with the vector as per the manufacturers instructions. The insert was then PCR amplified as above using the following primers and sequenced using an ABI Prism – 3100 Genetic Analyzer.

T7 (5’-3’) – TAATACGACTCACTATAGGG  
SP6 (5’-3’) – ATTTAGGTGACACTATAGAAT

2.4.3 Viral harvest from STAR cells

For viral harvest from 10cm plates, 6 well plates or 24-well plates 4 x 10⁶, 1 x 10⁶ and 2 x 10⁵ cells respectively were plated 24 hours before the start of virus harvest. Virus was harvested in 8ml, 2ml and 1ml of media from 10cm plates, 6 well plates and 24-well plates respectively after 48 hours incubation at 37°C.

2.4.4 Concentration of viral supernatant from STAR cells

Where specified in the text viral supernatant was concentrated by one of three methods. (i) Ultracentrifugation; 10ml of supernatant was spun down at 15,000 rpm using a Beckman SW41 rotor for 3 hours at 4°C. The resulting pellet was resuspended in 100µl of OptiMEM. (ii) Low-speed centrifugation; 6ml of supernatant was spun down at 3,200g for 6 hours at 4°C. The resulting pellet was resuspended in 100µl of OptiMEM. (iii) Filtration; 6 ml of supernatant (except MLV-A pseudotype - 3ml and RD114/MLV pseudotype - 4ml) were concentrated using a Centricon-20 (100 kDa cut-off) filter (Amicon). This gives a supernatant with a final volume of 150µl.

2.5 Infection of tissue culture cell lines

293T cells were plated at 1 x 10⁵ per well in 24-well plates 24 hours before infection. On the day of infection filtered supernatant was serially diluted in OptiMEM and added to the wells in the presence of 8 μg/ml polybrene (hexadimethrine bromide (Sigma)) unless stated otherwise. The cells incubated overnight at 37°C. The virus was then washed off the cells and replaced with DMEM and 10% FCS.
Spinoculation of virus onto cells was performed as described by O'Doherty and co-workers (O'Doherty, Swiggard, and Malim, 2000). Filtered viral supernatant was diluted onto cells as above in 24-well plates and spun at 1,200g for 2 h at 25°C. The plates were then incubated overnight at 37°C. Virus containing supernatant was removed the next day and replaced with DMEM + 10% FCS.

After infection cells were cultured for a further 48-72 hours then removed in Versin (GibCoBRL) for analysis. The number of infected cells (those expressing eGFP) was determined by fluorescence-activated cell sorting (FACS) using a FACScan and CELL QUEST software (Becton Dickinson).

2.6 Infection of human primary cell cultures

See Figure 2.4. 5 days post-isolation, monocyte-derived dendritic cells or T lymphocytes 5 x 10⁴ were plated in 100μl of culture media and exposed to 100μl of virus supernatant overnight. eGFP in viral supernatants can lead to pseudotransduction of phagocytic cells, with cells displaying punctate staining due to phagocytosed eGFP for up to 3 days (Neil et al., 2001). Cells were therefore cultured for 7 days post infection, until infected cells displayed uniform cytoplasmic eGFP. The percentage cells infected was then determined by FACScan and analysed using CELL QUEST software.

2.7 Determination of infectious titre on tissue culture cell lines by FACS

Uninfected cells were passed through the FACS to analyse their side vs. forward scatter characteristics. This profile gives an indication of the internal complexity and size of the cells. Typically, apoptotic cells are smaller than their healthy counterparts and, therefore, can be differentiated by their side vs. forward scatter profile. The region in which cells that have the appropriate profile can be found was then gated. For each infection 10,000 cells in this region were recorded and analysed for eGFP expression.
The side scatter vs. green fluorescence (or FL-1 value) plot of uninfected cells determines the region in which cells not expressing eGFP fall. A region of FL-1 higher than an uninfected control (see below) was gated and the percentage of recorded cells that fall within this gate could be determined. Titre, expressed as infectious units/ml, was determined using this percentage.

To set the minimum level of detection by FACS five uninfected controls were analysed for the percentage of cells to be found in the “eGFP positive” gate. The mean of these values was 0.02% (or 2 cells). In these experiments an arbitrary minimum level of detection of five times the mean of uninfected cells (0.1% infected or 10 positive cells) was set. Virus titre (infectious units/ml) was calculated from the percentage of cells infected (using values of less than 10% and greater than 0.1% infected cells) and the number of cells per well on the day of infection using the equation: (%-infected cells x number of cells at infection / 100) x dilution factor.

2.8 Determination of human primary cell culture infection by FACS

Uninfected cells were passed through the FACS to assess the side vs. forward scatter characteristics of the cells in each culture. The identity of the cells in each plot was confirmed by staining for cell surface markers (see section 2.9) mentioned in the text. For each infection 10,000 cells were recorded. Populations that reflect the morphology of cells positive for the appropriate cell markers were gated in the side vs. forward scatter plot of each infection and the cells in this gate were assessed for eGFP expression and/or the presence of a cell surface marker.

2.9 Detection of cell surface markers on primary human cells by FACS

All antibodies for cell surface markers were obtained from Harlan, UK, with their appropriate isotype controls. Monocyte-derived macrophage were detached in versine (GibCoBRL), and any adherent dendritic cells or T lymphocytes were detached with gentle pipetting. $5 \times 10^4$ cells were washed and resuspended in 200µl FACS Buffer (PBS + 3%BSA + 0.1% Sodium Azide). The required volume of antibody as stipulated by the company was diluted in FACS buffer and added to the cells. Cells
were incubated on ice for 1 hour with several agitations. The cells were washed three times in FACS buffer and resuspended in 200 µl of cold PBS. The cells were then passed through a FACScan (Beckton Dickinson). Data was analysed by the CELL QUEST software.

CD1a was detected using 1/20 dilution of CyChrome conjugated mouse-anti-human CD1a (PharMingen). The same dilution of the isotype control, CyChrome conjugated mouse-anti-human IgG (PharMingen), was used in parallel. CD3 was detected using 1/100 dilution of FITC conjugated mouse-anti-human CD3 (Insight Biotech). The same dilution of the isotype control, FITC conjugated mouse-anti-human IgG (Serotech), was used in parallel.

2.10 Quantification of p24 by ELISA

Capture of p24 was performed using High Bind White plates coated 24 hours beforehand with 100 µl D7320 anti-p24 (Aalto Bioreagents, Dublin) diluted to 5 µg/ml in coating buffer (0.1M NaHCO₃ pH 8.5). Plates were washed twice with LBS and each well blocked with LBS+2% w/v milk powder for 1 hour at room temperature.

Each sample was prepared by adding 80 µl of p24 containing supernatant to 20 µl of PBS containing 5% of the detergent Empigen BB (Surfachem Ltd, UK) This was then diluted 1/20 with the addition of 10 µl of sample plus Empigen solution to 190 µl LBS. Each plate also contained a three-fold dilution series of p24 (MRC ADP620) diluted in 0.05% Empigen in LBS.

After blocking each well was washed once with LBS and 100 µl of each sample was added in duplicate to the plate with the p24 standard series. Plates were incubated at room temperature for 4 hours and then washed twice with LBS.

100 µl of the second layer was then added to each well and incubated at room temperature for 1 hour. The second layer contains the alkaline phosphatase conjugated anti-p24 antibody EH12E1-AP (MRC ADP452) diluted to 0.5 µg/ml in 4% w/v milk powder, 20% sheep sera, 0.5% Tween 20 in LBS.
After exposure to the second antibody plates were washed 5 times with LBS+0.5% Tween 20 and 100 μl of alkaline phosphatase substrate Lumi-Phos Plus (Aureon Biosystems) was added to each well. Plates were incubated for another hour uncovered on the bench at room temperature or 30 minutes in the dark at 37°C.

Luminescence of each sample was read using Stingray software (Dazdaq, UK) and a Lucy 1 luminometer (Athos-Labtech). This gave each sample a value of relative light units which was converted to pg p24/ml using the standard curve derived from the p24 standard dilution series.

2.11 Immunoblotting

Virus producer cells were lysed in 0.5ml of RIPA buffer (50mM TrisCl (pH 7.5), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Bohringer Manheim). Lysates were incubated for 30 min at 4°C and then centrifuged for 30 min at 3000 g to pellet the nuclei. 6x loading buffer (375 mM Tris-HCl (pH 6.8) containing 6% SDS, 30% 2-mercaptoethanol, 10% glycerol, and 0.06% bromophenol blue) was diluted to 1x in the resulting supernatant to a final volume of 0.3ml. Virus in supernatant (8ml) was pelleted for immunoblot analysis by ultracentrifugation in an SW41 Beckman Rotor (30,000 rpm, 1 h, 4°C). Pellets from the supernatants were resuspended in 30 μl of 6x loading buffer diluted in OptiMEM. Supernatant and lysate samples were boiled for 5 min and frozen at -20°C until further analysis.

Supernatant and lysate samples (15-25 μl) were then run on 10-14% polyacrylamide (SDS) gels. After protein transfer using semi-dry transfer apparatus and transfer buffer (39mM glycine, 48mM Tris base, 20% methanol) onto Hybond ECL nitrocellulose filters (Amersham Pharmacia Biotech) total protein was stained with Ponceau S to ensure equal loading. Blocking was performed in Tris Cl, pH 7.4, with 5% w/v milk powder and 0.1% Tween 20. Where applicable antibodies are diluted in this solution.
The TM subunit of MLV-A and GALV envelopes was detected using undiluted supernatant from the rat hybridoma 42/114 (Pinter et al., 1982). MLV-A SU was detected with goat polyclonal anti-Rauscher leukemia virus gp70 (Quality Biotech Inc., Camden, N.J.), diluted 1/1000. RD114 SU was detected with goat polyclonal anti-RD114 gp70 (Quality Biotech Inc., Camden, N.J.), diluted 1/5000. HIV-1 CA was detected with a 1:1 mixture of the murine monoclonal antibodies ADP365 and ADP366 (MRCARD) diluted 1/200. MLV capsid (CA) was detected with goat polyclonal anti-Rauscher leukemia virus gp70 (Quality Biotech Inc., Camden, N.J.), diluted 1/1000. Blots were developed with horseradish peroxidase-conjugated anti-immunoglobulin (DAKO), diluted 1/1000, and an enhanced chemiluminescence (ECL) kit (Amersham Life Science).
Chapter 3
Pseudotyping of HIV vectors with chimeric retroviral envelope glycoproteins

3.1 Introduction

The aim of the study in this chapter is to determine how efficiently HIV vectors can be pseudotyped with gammaretrovirus envelope glycoproteins.

3.1.1 Pseudotyping of HIV vectors with gammaretrovirus envelopes

The most common pseudotyping of HIV vectors involves the use of the G envelope protein of the rhabdovirus vesicular stomatitis virus (VSV-G). VSV-G pseudotyped vectors can be produced at high titre with HIV cores and have been used in a number of cases in the transduction of many clinically relevant human cell types \textit{ex vivo} (Follenzi et al., 2000; Naldini et al., 1996). VSV-G is, however, cytotoxic to producer cells, as it is highly fusogenic (Burns et al., 1993). It has been suggested that phosphatidylserine, a common component of cell membranes, is a receptor, or part of a receptor complex, for VSV-G (Mastromarino et al., 1987; Schlegel et al., 1983).

The low cytotoxic nature of gammaretroviral envelope glycoproteins has enabled the creation of stable gammaretroviral packaging cell lines based on MLV. Envelopes that have been used to pseudotype MLV virions in such cell lines include the envelope glycoproteins of amphotropic murine leukaemia virus (MLV-A), gibbon ape leukaemia virus (GALV) and the feline endogenous virus RD114 (Cosset et al., 1995; Miller et al., 1991). The cellular receptors for these envelopes (Table 3.1) are found on a wide variety of human cell types and have been used with MLV vectors for the transduction of several clinically relevant cell types such as lymphocytes and CD34\(^+\) progenitor cells both \textit{in vitro} (Demaison et al., 2002; Hanawa et al., 2002; Kelly et al., 2000; Uckert et al., 2000) and \textit{ex vivo} for the purposes of clinical trials (Abonour et al., 2000; Blaese et al., 1995; Bordingnon et al., 1995; Cavazzana-Calvo et al., 2000).
The MLV-A, GALV and RD114 envelope proteins are, therefore, excellent candidates for use in stable HIV packaging cell lines to produce virus for the transduction of human primary cell cultures.

Previous experiments using MLV pseudotypes have shown GALV and RD114 pseudotypes could mediate entry of MLV in lymphocytes with higher efficiency than MLV-A pseudotypes (Gladow et al., 2000; Porter et al., 1996; Uckert et al., 2000). Furthermore, it has been shown that MLV(MLV-A), MLV(GALV) and MLV(RD114) could infect melanoma and human bone marrow progenitor cells with equal efficiency (Porter et al., 1996). MLV(VSV-G) was not, however, tested in any of the aforementioned studies. Interestingly, no correlation can be made between the relative level of MLV-A and GALV receptor mRNA levels and susceptibility of the cell to MLV pseudotype infection (Uckert et al., 1998).

3.1.2 Synthesis of gammaretroviral envelope glycoproteins

The retrovirus envelope consists of a trimer of heterodimers. These heterodimers consist of the extraviral surface (SU) subunit and the membrane spanning transmembrane (TM) subunit.

Env is translated as a polyprotein that contains both subunits (Figure 3.1). Early in translation from ribosomes on the surface of the rough endoplasmic reticulum (RER), the hydrophobic signal peptide, or leader sequence, at the N-terminus of the polyprotein is inserted into the lipid bilayer of the RER. The nascent Env polyprotein is consequently co-translationally translocated into the lumen of the RER (Swanstrom and Wills, 1997). A hydrophobic region of approximately 30-60 amino acids from the C-terminus of TM remains anchored in the ER membrane. The region from the membrane spanning part of TM to the C-terminus is termed the cytoplasmic tail.

In the ER, the signal peptide is cleaved and Env trimers form while mannose chains are attached at N-glycosylation sites in SU. From the ER the trimeric form of the Env is then transported to the Golgi where O-linked carbohydrates are added to SU and the
Figure 3.1 The gammaretrovirus glycoprotein

Shown above is a schematic representation of the organisation of the gammaretrovirus envelope. The positions of the surface (SU) and transmembrane (TM) subunits are indicated. Also shown are the positions of the signal peptide or leader sequence (L) plus the receptor binding domain (RBD) and proline rich region (PRR) in SU and the ectodomain (Ecto), membrane spanning region (MS), cytoplasmic tail (Cyt) and R peptide (R) in TM. Sites of cellular and viral protease cleavage are also indicated.
N-glycosylated sugars are trimmed (Swanstrom and Wills, 1997). Also in the Golgi, cleavage of SU from TM occurs. SU and TM remain connected via several non-covalent interactions (Gliniak et al., 1991) and a labile disulphide bond that may have a role in the triggering of membrane fusion (Pinter et al., 1997). From the Golgi, SU-TM complexes move to the plasma membrane to be incorporated into virions along with other cell surface proteins present at the host cell membrane.

3.1.3 Gammaretroviral envelope glycoprotein maturation; R peptide cleavage

During maturation the envelope undergoes a final maturation step when PR cleaves a peptide from the transmembrane C-terminus, converting the envelope from its immature form, p15, to the mature form, p12. Green et al. (Green et al., 1981) were the first to show this using sequence specific monoclonal antibodies. The 16 amino acid sequence removed (p2) was recognised by antibody R and was subsequently termed the R peptide. It was confirmed that the removal of p2 is due to the action of the viral PR, as p2 is not removed when the viral PR is non-functional (Crawford and Goff, 1985).

It is proposed that this final processing step promotes a conformational change in the TM region, making it fusion competent, thus allowing envelope-receptor fusion only after the virus has exited the cell (Olsen and Andersen, 1999). A possible mechanism to activate the fusion capacity of the envelope has been proposed by Li et al. (Li, Yang, and Compans, 2001). Addition of a palmitic acid residue to the R peptide anchors the MLV envelope to the plasma membrane (Olsen and Andersen, 1999; Yang and Compans, 1996), specifically in lipid rafts (Li et al., 2002). Li and co-workers (Li, Yang, and Compans, 2001) and Olsen and co-workers (Olsen and Andersen, 1999) propose that this anchorage prevents the cytoplasmic helical domains of the TM trimer from interacting with each other, and cleavage of the R peptide during budding allows these domains to interact (i.e. spring together) and the envelope becomes fusion competent. The removal of a peptide from the envelope cytoplasmic tail is not limited to gammaretroviruses. Cleavage of the envelope TM by the viral protease during maturation has been observed in the betaretrovirus Mason-Pfizer monkey virus (MPMV) (Brody et al., 1992) and so far in only one lentiviral
envelope, that of equine infectious anaemia virus (EIAV) (Rice et al., 1990). Cleavage of the MPMV Env cytoplasmic tail activates fusion but it is unclear if cleavage of the EIAV envelope serves the same purpose.

3.1.4 Envelope-receptor interactions; binding and fusion

The attachment of gammaretrovirus particles to cells is both cell type and envelope independent (Pizzato et al., 1999). Which cellular molecules are involved in attachment is unclear (Walker et al., 2002). It is interesting to note, however, that in vitro modification of the mannose residues found on the gammaretrovirus envelope can promote interactions with DC-SIGN and DC-SIGNR that lead to enhanced infectivity (Lin et al., 2003). Whether gammaretroviral envelopes display DC-SIGN and DC-SIGNR binding forms of mannose in vivo is unknown.

When Env encounters its cellular receptor, it binds to this molecule with high affinity. This binding triggers a series of events leading to membrane fusion. The receptors of the gammaretrovirus envelopes used in this study, all multiple membrane spanning molecules, are shown in Table 3.1. It would appear that the receptor for MLV-A, GALV and RD114 are ubiquitously expressed on human tissues.

Two hypervariable regions within the receptor binding domain (RBD) of SU (Figure 3.1) are the major determinants of receptor binding specificity. Indeed, multiple specific interactions take place between sequences in the RBD and the receptor (Tailor, Nouri, and Kabat, 2000). Although it has been proposed that the receptor for polytropic and xenotropic MLV may be involved in G-protein coupled signal transduction (Battini, Rasko, and Miller, 1999), there is as yet no evidence that interactions of any other gammaretrovirus envelope with its cellular receptor stimulates intracellular signalling.

Once bound, it is thought that the structure of the RBD is modified, triggering a signal to activate fusion (Barnett and Cunningham, 2001; Barnett, Davey, and Cunningham, 2001; Lavillette et al., 1998). The "receptor-activated" RBD structure interacts with a
<table>
<thead>
<tr>
<th>Retrovirus</th>
<th>Cellular receptor</th>
<th>Alternate nomenclature</th>
<th>Function</th>
<th>Retrovirus utilising the same receptor</th>
</tr>
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<tbody>
<tr>
<td>MLV-A</td>
<td>Hu Pit2</td>
<td>Ram1, Glvr2</td>
<td>Na(^+)-dependent inorganic phosphate transporter</td>
<td>MLV-10A1</td>
</tr>
<tr>
<td>GALV</td>
<td>Hu Pit1</td>
<td>Glvr1</td>
<td>Na(^+)-dependent inorganic phosphate transporter</td>
<td>FeLV-B, SSAV</td>
</tr>
<tr>
<td>RD114</td>
<td>RDR</td>
<td>hATB(^0), SLC1A5, ASCT2</td>
<td>Neutral amino acid transporter</td>
<td>BaEV, MPMV, HERV-W, SRV-1,2</td>
</tr>
</tbody>
</table>

Table 3.1 Cellular receptors of the gammaretroviruses MLV-A, GALV and RD114

Shown above are the cellular receptors for the gammaretroviruses MLV-A, GALV, and RD114 (Kavanaugh et al., 1994; Miller, Edwards, and Miller, 1994; Olah et al., 1994; Rasko et al., 1999; Tailor et al., 1999; Zeijl et al., 1994). Also shown are their functions and examples of retrovirus that use the same receptor (Blond et al., 2000; Sommerfelt and Weiss, 1990). It should be noted that although RD114 is classified as a gammaretrovirus (Hunter et al., 2000) its envelope has some sequence similarity with betaretrovirus envelopes (Barker et al., 1986), hence RD114 and the betaretrovirus MPMV share the same receptor.

(MLV-10A1 - dualtropic MLV strain 10A1, FeLV-B - feline leukaemia virus subgroup B, SSAV - simian sarcoma associated virus, BaEV - baboon endogenous virus, MPMV- Mason-Pfizer monkey virus, HERV-W - human endogenous retrovirus W, SRV-1,2 - simian retrovirus type1 and type 2).
conserved disulphide loop in the C-terminus of SU (Lavillette et al., 2001). This interaction, essential for fusion, is mediated by conformational changes in a proline rich region between the RBD and the C-terminus (Lavillette et al., 1998). These interactions in the SU subunit are thought to expose the cellular membrane to a six-helix bundle in TM with a hydrophobic fusion peptide at its N-terminus. The interactions between the fusion peptide and the cell membrane are thought to initiate viral and cellular membrane mixing (Jones and Risser, 1993; Russell and Cosset, 1999).

3.1.5 pH dependency of retroviral entry

In general terms, there are two possible mechanisms of actual retrovirus entry into the cell; fusion of viral and cellular membranes following pH-dependent endocytosis of the cellular receptor(s) and the attached virus, or pH-independent direct virus-cell fusion. Viruses that use direct virus-cell membrane fusion include HIV, MLV-A and RD114, while ecotropic MLV (MLV-E) and vesicular stomatitis virus (VSV) are endocytosed (McClure, Marsh, and Weiss, 1988; McClure et al., 1990).

The current classification of retrovirus entry into pH-dependent or pH-independent groups may, however, prove to be inaccurate. Firstly, in a study by Mothes et al. (Mothes et al., 2000) it was shown that avian leukosis virus (ALV) infection, whose mechanism of cell entry was thought to be independent of pH (Gilbert et al., 1995; Gilbert, Mason, and White, 1990), is pH-dependent. Specifically, Mothes and co-workers demonstrated that the interaction between the envelope and its cellular receptor "primes" the virus for entry and only at low pH does actual fusion occur. During previous studies of ALV, pH-dependence was compared to that of influenza A (Gilbert et al., 1995; Gilbert, Mason, and White, 1990). As the fusion of ALV is slower than that of influenza A, treatment of ALV infected cells with lysosomotropic agents did not appear to affect infection, whereas these agents significantly inhibited influenza A infection. The now apparent pH-dependent step of ALV fusion was, therefore, overlooked. Secondly, data have been presented showing that MLV-A entry may be sensitive to pH and enter via endocytosis (Katen et al., 2001). The pH-dependency of retroviral infection may, therefore, require re-examination.
3.2 Results

3.2.1 Pseudotyping of HIV and MLV vectors with VSV-G and the envelope glycoproteins from MLV-A, GALV and RD114

The ability of envelope glycoproteins from VSV-G, MLV-A, GALV and RD114 to pseudotype vectors based on HIV and MLV was examined. Virus was produced by transient transfection of separate packaging, vector genome and envelope plasmids into 293T cells. HIV based vectors were produced using the packaging plasmid pHCMVΔR8.91 (Zufferey et al., 1997), which does not express any of the HIV accessory genes, and the vector genome plasmid pHR'SINcPPT-SE (Demaison et al., 2002). For MLV production the packaging and vector genome plasmids pHIT60 and pCNCG were used (Soneoka et al., 1995). Both HIV and MLV vector genomes contain an eGFP reporter gene. The VSV-G, MLV-A, GALV and RD114 expression plasmids pMD-G, phCMV-A, phCMV-GALVwt and phCMV-RD114wt respectively were used. Virus was harvested in the serum free media OptiMEM and plated onto 293T cells in the presence of polybrene, a polycation used to enhance virus infection (Toyoshima and Vogt, 1969). The percentage of cells expressing eGFP was assayed by FACS 48 hours after infection. Titre (infectious units/ml of viral supernatant) was derived from %-infected cells and the number of cells on the day of infection using the equation; (%-infected cells x no. of cells at infection/100) x dilution factor. The results are shown in Figure 3.2. As a negative control 293T were transfected with the HIV and MLV vector and packaging plasmids without an envelope expression plasmid. No infectious envelope-less virus could be detected by FACS (not shown).

This experiment (Figure 3.2) demonstrated that, firstly, the VSV-G and MLV-A glycoproteins could efficiently pseudotype both HIV and MLV vectors. Secondly, no infectious HIV(GALVwt) virus above the level of detection by FACS (2 x 10^3 iu/ml) could be detected. MLV, however, could be efficiently pseudotyped with the GALV envelope indicating the envelope construct can be expressed in 293T cells and is functional in the context of MLV pseudotyping. Finally, HIV can be pseudotyped with the RD114wt envelope but vector titre is significantly less than HIV(MLV-A).
The lack of HIV(GALVwt) and HIV(RD114wt) titre made it necessary to seek an alternative strategy to allow vector entry through the GALV and RD114 receptors.

Rather than explore pseudotyping with envelopes from other members of the retrovirus family that use the same receptors as GALV and RD114 (Sommerfelt and Weiss, 1990) it was decided to explore strategies exploiting the efficient pseudotyping of HIV with the MLV-A envelope.

Although HIV could be efficiently pseudotyped with the MLV-A envelope, modification of the MLV-A receptor binding domain to target virus to the GALV and RD114 receptors may not be possible as the functional interactions between retrovirus envelopes and their receptors involve multiple regions of the envelope SU subunit (Tailor, Nouri, and Kabat, 2000). Furthermore, it is unknown how this strategy would affect the structure and, therefore, function of SU.

It was decided to modify the GALV and RD114 envelopes to increase HIV vector titre. These modifications were based on two possible reasons why HIV(GALVwt) and HIV(RD114wt) titre was poor.
Figure 3.2 Pseudotyping of HIV and MLV vectors with VSV-G and the envelope glycoproteins from MLV-A, GALV and RD114

Pseudotyped HIV and MLV vectors carrying the eGFP reporter gene were generated by transient transfection of 293T cells using a panel of retroviral envelopes. Pseudotypes were plated onto 293T cells. Target cells were assayed for eGFP expression by FACS 48 hours after infection. Titre was derived from the percentage of eGFP expressing cells. The minimum level of detection by FACS (2 x 10^3 iu/ml) is indicated with an arrow. Data shown is representative of three experiments.
Firstly, it was speculated that GALVwt and RD114wt envelope incorporation into HIV virions is inefficient. Since Env interacts with the virion via the cytoplasmic tail (Januszeski et al., 1997) it was decided to modify this portion of the GALVwt and RD114wt envelopes, replacing the cytoplasmic tails of GALVwt and RD114wt with that of the MLV-A Env used above. Secondly, it was speculated that production of infectious HIV(GALVwt) and HIV(RD114wt) virions could be dependent on cleavage of the R peptide.

Studies of MLV envelope incorporation have been carried out using a HIV mutant that contains mutations in MA (Kieman and Freed, 1998). This mutant replicated with near wild-type kinetics but was uninfectious when pseudotyped with the MLV Env but not an HIV Env or VSV-G. Compared to the parental virus, MLV Env incorporation was not affected, however the R peptide was not cleaved from the MLV Env in the mutant virus. Kieman and Freed were then able to show that infectivity of the mutant could be restored with the use of an R-less MLV Env and the HIV PR cleaves the R peptide from the MLV envelope in a wild-type virus. In conclusion, R peptide cleavage was not absolutely necessary for incorporation of the MLV envelope into the HIV virion but was necessary for the pseudotyped virion to be infectious (Kieman and Freed, 1998).

It is unknown if the HIV PR can cleave the R peptide from the GALVwt or RD114wt envelopes. GALVwt and RD114wt envelopes could, therefore, be incorporated into the HIV virion but not efficiently processed; hence the pseudotyped virions are uninfected. It was therefore decided to introduce mutations predicted to enhance R cleavage by the HIV PR into the GALVwt and RD114wt envelopes. Complete removal of the R peptide was considered, however R-less envelopes are highly fusogenic and their expression in virus producing cells can cause the formation of syncitia. This in turn can lead to cell death (Diaz et al., 2000; Higuchi et al., 2000; Januszeski et al., 1997).
Figure 3.3 Schematic of chimeric retroviral envelopes

All envelopes sequences are found in the phCMV expression cassette (Figure 2.2). For MLV-A and RD114wt envelopes this cassette is found in the backbone pIRES2-eGFP. The GALV Env expression cassettes are found in the backbone pcDNA3.1. GALV Env sequences are shown in green, MLV-A or MLV in white and RD114 in blue. The position of the HIV PR recognition sequence in RD114-PR is shown in red.
Chimeric GALV and RD114 envelopes incorporating the above modifications were constructed in collaboration with the laboratory of F-L Cosset (Figure 3.3). All RD114 envelope expression constructs were produced by F-L Cosset, as was one of the GALV envelope expression plasmids, phCMV-GALV/MLV. The other two GALV envelope expression constructs, phCMV-GALVwt and phCMV-GALV-KQ, were produced by BLS for this study. Details of each envelope are provided in the sections that follow.

3.2.2 Pseudotyping of HIV and MLV vectors with chimeric GALV envelope glycoproteins

The GALV envelope sequence in phCMV-GALV/MLV differs from the GALV envelope as the cytoplasmic tail is substituted for that of MLV. phCMV-GALVwt and phCMV-GALV-KQ were constructed as follows using the plasmid pMD-pCEII which contains the GALV envelope sequence. pMD-CEII was also supplied by F-L Cosset.

Using pMD-pCEII as a template the mutation K618Q was introduced into the GALVwt envelope by PCR site-directed mutagenesis. This construct was termed pMD-GALV-KQ. This substitution occurs in the GALV envelope R peptide cleavage site, removing a positively charged residue, lysine and replacing it with glutamine, found in this position in the MLV envelope (Figure 3.4.A). It was hypothesised that the removal of a charged residue from the cleavage site may enhance enzyme/substrate interactions. The sequences of the wild type GALV and GALV-KQ cytoplasmic tails were then substituted for the MLV sequence in phCMV-GALV/MLV to create phCMV-GALVwt and phCMV-GALV-KQ.

As before pseudotyped HIV and MLV vectors were produced via transient transfection of 293T cells in 24-well plates using the packaging and vector plasmids mentioned above.
As shown in Figure 3.5.A the titre of the HIV(GALVwt) pseudotype did not exceed the threshold level detectable in this assay (2 x 10^3 iu/ml). Significant HIV vector titre, comparable to HIV(MLV-A), could be observed with the GALV-KQ and GALV/MLV envelopes. In repeated experiments there was less than a two-fold difference in titre on 293T cells between the two pseudotypes.

These results would indicate that the cytoplasmic tail of the envelope plays a role in the production of infectious HIV virions. However, with this data alone it could not be stated if the observed increase in titre was due to efficient envelope incorporation and/or R peptide cleavage.

Modifications to the GALVwt envelope did not appear to have a significant effect on MLV titre. In the case of pseudotyping with GALV/MLV this is perhaps unsurprising, as an MLV virion should certainly be able to interact with the cytoplasmic tail from an MLV envelope. At this point it was speculated that the mutation K618Q appeared not to be inhibitory to either envelope incorporation into the MLV virion or R peptide cleavage by the MLV PR.

3.2.3 Pseudotyping of HIV and MLV vectors with chimeric RD114 envelope glycoproteins

Pseudotyping of HIV and MLV vectors with the chimeric RD114 glycoproteins RD114/MLV and RD114-PR was then assessed.

Firstly, the RD114wt cleavage site was replaced with an HIV PR substrate from HIV Gag, rather than introduce specific mutations. Again, it was speculated that the removal of a +ve charged residue from the HIV PR substrate could enhance enzyme/substrate interactions. The RD114wt cleavage site has an overall charge of +1. The MA-CA cleavage site is the only site in Gag with no overall charge and was elected for introduction into the RD114 envelope to create HIV-PR (Figure 3.4.B). In the RD114/MLV envelope, like GALV/MLV, the RD114wt cytoplasmic tail is substituted with that of the MLV-A Env used above.
Transfections and infections were carried out as above. The resulting titers are shown in Figure 3.5.B. In comparison to HIV(RD114wt) titre, an increase in titre can be observed with the substitution of the cytoplasmic tail for that of MLV. This increase is not, however, as great as that seen using the RD114-PR envelope. Indeed, using the RD114-PR envelope vector titre is at least as high as that of HIV(MLV-A).

As before, this data would indicate that the envelopes cytoplasmic tail plays a role in the production of infectious HIV virions. Again, with this data alone it is, however, not clear if the observed increase in titre was due to efficient envelope incorporation and/or R peptide cleavage.

As with the GALV envelope chimeras modification of the cytoplasmic tail appears to have no significant effect on MLV titre. In the case of pseudotyping with the RD114-PR envelope it was speculated that the MLV PR could recognise the HIV MA-CA site as a substrate as there is some homology between this site and the MLV p12-CA cleavage site (Figure 3.4.B). Furthermore, in the absence of either MLV or HIV PR, expression of the RD114-PR Env in 293T cells causes cell-cell fusion (Figure 3.6). This would indicate RD114-PR is constitutively fusogenic in these cells. This observation suggests that the cytoplasmic tail of RD114-PR may be removed in 293T cells and could, in part, explain the efficient pseudotyping of HIV and MLV pseudotyping with this envelope. Neither wtRD114 nor RD114/MLV appear to be constitutively fusogenic in 293T cells.
Figure 3.4. Retroviral envelope C-terminus region and protease substrate sequences

(A) The amino acid sequence of the cytoplasmic tails of the transmembrane subunits of the gammaretroviral envelopes MLV-A, GALV and RD114. Points at which the amino acid sequences differ from the MLV-A sequence are indicated in red. As noted in the Materials and Methods section the MLV-A Env used here contains the Friend MLV cytoplasmic tail hence the Leucine residue at codon 628 (shown in blue). Codon 618 in the GALV envelope sequence where the mutation K-Q is made in the GALV-KQ is marked *. The numbering of each Env is from the mature protein after the removal of the signal peptide. (B) Viral protease substrate sequences in HIV and MLV Gag. HIV sequences are taken from NCBI entry AF033819. MLV sequences are taken from MLV amphotropic strain 4070A. The HIV MA-CA sequence in RD114-PR is marked ″. PR cleaves polyprotein between P1 and P1′.
Figure 3.5 Titration of HIV and MLV pseudotypes bearing chimeric GALV (A) and RD114 (B) envelopes

Pseudotyped HIV and MLV vectors carrying the eGFP reporter gene were generated by transient transfection of 293T cells using a panel of retroviral envelopes. Pseudotypes were plated onto 293T cells and titre was derived from the percentage of eGFP expressing cells. Target cells were assayed for eGFP expression by FACS 48 hours after infection. 293T cells were also transfected with the respective HIV and MLV vector and packaging plasmids in the absence of an envelope expression plasmid. No infection of envelope-less virus could be seen above the level of detection by FACS. The minimum level of detection by FACS (2 x 10^3 iu/ml) is indicated with an arrow.

Each bar represents the mean of two experiments and the error bars show the actual data points.
Figure 3.6 Fusion of 293T cells expressing the RD114-PR envelope

293T cells were transfected with plasmids expressing the envelopes RD114wt, RD114/MLV and RD114-PR with or without the HIV packaging plasmid pHCMVΔR8.91. After 72 hours incubation cells were fixed and stained with Giemsa stain. Few multinucleated bodies could be seen in the cells transfected with the RD114wt or RD114/MLV expression plasmids with or without the presence of HIV PR. Many multinucleated bodies could be seen in cells expressing RD114-PR. The number of RD114-PR induced syncitia does not increase with expression of HIV PR. Cells transfected with pHCMVΔR8.91 and the RD114wt and RD114/MLV expression vectors are not shown.
3.2.4 The relationship between virus dose and FACS measurement of % infected cells

Accurate determination of titre ((% -infected cells x number of cells at infection / 100) x dilution factor) relies on the figure of % -infected cells being in the linear range of titration, i.e. cells expressing eGFP are the products of a single not multiple infections. As stated in Materials and Methods (section 2.8) to determine titre of the viral supernatant values between 0.1% and 10%-infected cells are utilised. It is believed that these values are in the linear range of titration. The reasoning behind this statement is as follows.

It is reasonable to assume in titration experiments using established cell lines as target cells all cells within a given population have the same probability of infection regardless of if they are already infected or not. It can, therefore, be said that infection falls into a Poisson distribution where cells are either not infected or are infected once, twice, three times and so on. From the Poisson distribution a statistical relationship between multiplicity of infection (MOI) and % -infected cells, MOI = (-log P)/0.4343, (where P is the proportion of uninfected cells) can be derived (Porter et al., 1998). This formula indicates that to achieve 10% infected cells requires an MOI of 0.105. In contrast, 50%-infected cells corresponds to an MOI of 0.69, i.e. there are 690 infection events between 500 cells (50% of 1000 cells). Acknowledging the possibility there is a negligible number of cells multiply infected in a population of 10% infected cells, it is thought values of 10% or less infected cells are mostly the products of single infections. Using values of 10% infected cells or less safeguards that determination of viral titre is accurate.

To experimentally determine if values under 10%-infected cells are in the range of linear dose-dependent infection the relationship between infection and virus dose was examined by titration experiments.

HIV pseudotyped with VSV-G, MLV-A, GALV/MLV and RD114-PR were produced from 6-well plates and diluted with OptiMEM in a 2-fold dilution series. Equivalent volumes of supernatant from each point in this series were titred on 293T cells as before or assayed for quantity of HIV CA (p24) by ELISA.
As a negative control for both the infection assay and p24 ELISA, 293T cells were transfected with only the HIV vector plasmid, pHRSINcPPT-SE, and the VSV-G expression plasmid pMD-G. No eGFP expressing cells could be observed by FACS when supernatant from these cells was plated onto 293T cells. ELISA readings from this supernatant did not exceed background levels.

The values of µl supernatant versus %-infected cells were plotted for each pseudotype (Figure 3.7). Where infection with respect to virus dose is linear the gradient of the curve is 1. With regard to the retroviral envelope pseudotypes, Figure 3.7 indicates that between the values of 10% to 0.1%-infected cells the gradient of each curve is 1. Therefore, infection between the values of 10% to 0.1%-infected cells is linear with respect to virus dose, thus a significant population of cells were not multiply infected. It must be noted that in this experiment the % infected cells measured for the VSV-G pseudotype did not fall below 1%. The gradient of the HIV(VSV-G) curve is, however, approximately 1 between 5% and 25% infected cells. In subsequent titration experiments HIV(VSV-G) titrated down in a linear fashion between 10% and 0.1% infected cells (not shown).

Analysis of µl viral supernatant versus pg p24 (Figure 3.8) shows that all four pseudotypes had similar levels of p24. Retroviral pseudotypes titrated linearly showing a gradient of 1 in a log-log plot. It was, however, noted that HIV(VSV-G) had a slightly greater gradient, indicating some abnormality in this particular experiment. Why this should be the case is unknown.

The relationship between p24 in viral supernatant and %-infected cells is shown in Figure 3.9. Here p24 values that fall within the ELISA standard curve have been plotted. With regard to the retroviral envelope pseudotypes between the values of 10% to 1%-infected cells the gradient of the curves is approximately 1, therefore infection is linear with respect to virus dose. A caveat applies for HIV(VSV-G) and HIV(GALV/MLV), however. No values in Figure 3.9 for either HIV(VSV-G) or HIV(GALV/MLV) fall below 10%-infected cells as no points in this titration below 10%-infected cells fall in the ELISA standard curve.
From Figure 3.9 it is clear that HIV(VSV-G) had a much higher titre to p24 ratio. The ratio of titre to p24 or infectivity (iu/pg) was calculated from the titre (iu/ml) determined in Figure 3.7 and pg p24/ml determined in Figure 3.8. The values for each pseudotype can be seen in Table 3.2.

In conclusion, using the relationship between multiplicity of infection and %-infected cells derived in Porter et al. (Porter et al., 1998) it was hypothesised that values of less than 10% infected cells were the products of single infections and could be used to accurately calculate virus titre. Here it was shown using titration experiments that infection of less than 10%-infected cells is linear with respect to virus dose and, therefore, values below 10%-infected cells are most likely the products of single infections and appropriate for calculation of viral titre.
Figure 3.7 Dilution series of HIV pseudotypes; μl viral supernatant versus %-infected cells

Each pseudotype was produced by three plasmid transfection of 293T cells. The resulting supernatant was diluted in a 2-fold dilution series and titred on 293T cells in the presence of polybrene. The black bar indicates a gradient of 1.
Figure 3.8 Dilution series of HIV pseudotypes; µl viral supernatant versus pg p24

Each virus was produced by three plasmid transfection of 293T cells. The resulting supernatant was diluted in a 2-fold dilution series and each point in the series was assayed for level of HIVCA (p24). The coloured points represent the log10 values of the actual data points. The solid coloured lines represent the trendline obtained by a least-square fitting method of each titration. The black bar represents a gradient of 1.
Figure 3.9 Dilution series of HIV pseudotypes; p24 versus %-infected cells

Each virus was produced by three plasmid transfection of 293T cells. The resulting supernatant was diluted in a 2-fold dilution series and each point in the series was assayed for level of HIVCA (p24) and %-infected 293T cells. The black bar represents a gradient of 1.
3.2.5 Infectivity per particle of HIV(VSV-G) and gammaretrovirus pseudotypes

It was speculated that, although HIV pseudotyped with different envelopes differs in their infectious titre, an equivalent number of virus particles might be produced. This point must be considered when standardizing pseudotyped virus for infection as inputting the same amount of HIV CA (p24) for each pseudotype would mean exposing target cells to significantly different levels of infectious virus. In order to investigate pseudotype infectivity the amount of p24 in the viral supernatant versus virus titre on 293T cells was examined.

HIV pseudotyped with VSV-G, MLV-A, GALV/MLV and RD114-PR was produced from 6-well plates and diluted with OptiMEM in a 2-fold dilution series. Equivalent volumes of supernatant from each point in this series were titred on 293T cells in the presence of polybrene as before or assayed for quantity of p24 by ELISA.

As a negative control for both the infection assay and the p24 ELISA assay, 293T cells were transfected with only the HIV vector plasmid, pHR'SINcPPT-SE, and the VSV-G expression plasmid pMD-G. No eGFP expressing cells could be observed by FACS analysis when supernatant from these cells was plated onto 293T cells. ELISA readings of this supernatant did not significantly exceed background levels.

For each virus, the titre (iu/ml) was determined using the mean of two points that fall with 10% to 0.1%-infected cells. Total quantity (pg/ml) of p24 in each supernatant was determined using the mean of the two highest points in the dilution series that fall within the ELISA standard curve. From these values the infectivity, infectious units per pg of p24 (iu/pg), was determined (Table 3.2).

The titre of VSV-G pseudotyped HIV on 293T cells was at least 12x greater than that of the retroviral pseudotypes. The level of p24 in the HIV(VSV-G) containing supernatant, however, was found to be less than half of HIV(MLV-A) and HIV(RD114-PR) and 1.7x times higher than HIV(GALV/MLV) in this experiment. Thus, the infectivity (iu/pg) of HIV(VSV-G) compared to the most infectious retroviral pseudotype (HIV(GALV/MLV)) was 7.3x greater. The infectivity of the retroviral pseudotypes varies in this experiment. This experiment will require
repetition to determine if this variation is envelope-dependent or perhaps a facet of virus production by transient transfection.

These results demonstrate that standardizing infection of these different pseudotypes by the level of p24 in the supernatant would mean exposing target cells to variable levels of infectious virus. Standardising virus input by both multiplicity of infection derived from infectious titre on a standard cell line such as 293T and level of p24 in viral supernatant would, therefore, be potentially useful in various *in vitro* and *in vivo* experiments.
Table 3.2 Infectivity of HIV(VSV-G) and retroviral pseudotypes on 293T cells

<table>
<thead>
<tr>
<th>HIV pseudotype</th>
<th>Titre on 293T cells (iu/ml)</th>
<th>Level of p24 in supernatant (pg/ml)</th>
<th>Infectivity on 293T cells (iu/pg)</th>
</tr>
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<tbody>
<tr>
<td>VSV-G</td>
<td>$2.7 \times 10^7$</td>
<td>$3.5 \times 10^6$</td>
<td>7.7</td>
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<tr>
<td>MLV-A</td>
<td>$3.2 \times 10^5$</td>
<td>$7.3 \times 10^6$</td>
<td>0.04</td>
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<tr>
<td>GALV/MLV</td>
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<td>$2.0 \times 10^6$</td>
<td>1.05</td>
</tr>
<tr>
<td>RD114-PR</td>
<td>$5.3 \times 10^5$</td>
<td>$7.3 \times 10^6$</td>
<td>0.07</td>
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</table>

Table 3.2 Infectivity of HIV(VSV-G) and retroviral pseudotypes on 293T cells

Titre (iu/ml) of HIV pseudotypes was determined on 293T cells with polybrene and the level of HIV CA (p24) in the supernatant (pg/ml) was measured by ELISA. The infectivity of each pseudotype in this experiment is expressed as infectious units per pg of p24 (iu/ pg).
3.2.6 Expression and incorporation into HIV and MLV virions of chimeric retroviral envelope glycoproteins

Pseudotyping of HIV with chimeric GALV and RD114 envelopes indicated that the cytoplasmic tail of Env plays a role in the production of infectious virions. It was, however, unknown if the higher titres observed were due to more efficient envelope incorporation and/or R cleavage. GALV and RD114 pseudotypes were generated by transient transfection in 10cm plates and analysis by western blotting of envelope incorporated into virions and R peptide cleavage, both in virus pelleted from supernatants and in producer cell lysate, was attempted.

GALV envelope samples were probed with antibodies against HIV CA (p24), MLV CA (p30) and supernatant from the rat hybridoma 42/411. The antibody in this supernatant was raised against the ectodomain of the MLV envelope transmembrane subunit (Pinter and Honnen, 1983; Pinter and Honnen, 1984). No antibodies raised against the GALV Env were available during this study but it has been shown that antibodies raised against MLV envelopes can recognise the GALV envelope (Duisit et al., 1999).

Although considerable pseudotype titres were achieved, detection of the GALV envelopes in the HIV supernatants was problematic compared to the MLV samples. While GALV envelopes could be readily detected in MLV supernatants only the GALV/MLV envelope could be detected in the HIV supernatant samples at the highest sensitivity. Figure 3.10 illustrates this point. Shown are the western blots of GALV/MLV in both HIV and MLV samples. In the MLV supernatant sample (lane 4) mature Env (p12) can be clearly seen as well as some immature Env (p15). In the HIV sample (lane 3) only minimal amounts of p15 can be seen after at least 5x longer exposure of the membrane to film. Why there is a difference in Env processing and accumulation in the supernatant is unclear. It was not possible to detect the GALV envelopes in the cell lysate from HIV or MLV producer cells.
Figure 3.10. Western blot analysis GALV pseudotype supernatants

Supernatant was titred on 293T cells and then concentrated by ultracentrifugation and the pellet resuspended in 30µl of loading buffer and 15µl of each sample were used in this experiment. Proteins were separated using 14% polyacrylamide gels. After transfer membranes were probed with supernatant from the hybridoma 42/411, a 1:1 mixture of murine monoclonal antibodies ADP365 and ADP366 raised against HIV CA (p24) and a goat polyclonal serum raised against Rauscher MLV CA (p30). The positions of protein markers (in kDa) are shown.

Lanes 1 and 2 show HIV and MLV envelope-less transfections respectively. Lanes 3 and 4 show HIV(GALV/MLV) and MLV(GALV/MLV) transfections. The titre of these viruses on 293T cells was $1.3 \times 10^6$ iu/ml and $6.5 \times 10^6$ iu/ml respectively. Titre of envelope-less virus on 293T cells did not achieve the level of detection by FACS ($4.0 \times 10^2$ iu/ml). Note that in Lane 4 of the supernatant samples probed with supernatant from 42/411 cells membrane exposure to film of 5 seconds is shown in contrast to 25-30 seconds exposure seen in lanes 1, 2 and 3.
Examination of p24 in the lysates of the RD114 samples (Figure 3.11.A) indicates that transfection efficiency in the HIV samples appears to be equal. However, RD114-PR expression in the producer cells (Figure 3.11.A, Lane 4) is higher than RD114wt and RD114/MLV. Why there should be this difference in expression is unknown. This does raise the possibility that the observed increase in HIV titre with the RD114-PR envelope could be due to increased incorporation that may not be due solely to modification of the envelope cytoplasmic tail but also increased availability of envelope free to be incorporated into virions.

Furthermore, RD114wt was incorporated less inefficiently compared to RD114/MLV, therefore it remains possible that titre could be linked to envelope incorporation.

The anti-RD114 SU serum used here did not, as expected, recognise the RD114 TM subunit at any detectable level. To date no other antibodies that recognise the RD114 envelope have been reported in published literature. It is, therefore, not possible at present to determine the effect, if any, modifications made to the RD114 cytoplasmic tail have on R peptide cleavage. Data from the western blotting experiments presented here was, therefore, inconclusive with regard to the link between HIV vector titre and the incorporation and R cleavage of envelopes with modified cytoplasmic tails.

Based on the data presented in this chapter recommendations regarding which envelopes were most suitable for introduction into the novel HIV packaging cell line STAR were sought. Although RD114-PR provides the highest titre with HIV, the fusogenic nature of this envelope may make the cloning of producer cells based on 293T cells problematic. Both the RD114-PR and RD114/MLV envelopes should, therefore, be used to derive stable HIV packaging cell lines producing RD114 pseudotyped HIV.

Although the titre of the GALV-KQ and GALV/MLV envelopes with HIV are similar, GALV/MLV was nominated for use in pseudotype production from stable HIV packaging cells as data from a direct comparison between GALV/MLV and RD114/MLV pseudotyped virus may be of interest in future experiments.
Figure 3.11. Western blot analysis of RD114 pseudotype and producer cell lysates (A) and supernatants (B)

6 x 10^6 of each producer cell were lysed in 500μl of RIPA buffer. 15μl of each sample was used in this experiment. Supernatant was titred on 293T cells and then concentrated by ultracentrifugation, and the pellet resuspended in 30μl of loading buffer. 15μl of each sample was used in this experiment. Proteins were separated on 10% polyacrylamide gels. After transfer membranes were probed with a goat polyclonal serum raised against the RD114 Env SU (gp70), a 1:1 mixture of the murine monoclonal antibodies ADP365 and ADP366 raised against HIV CA (p24) and a goat polyclonal serum raised against Rauscher MLV CA (p30). The positions of protein markers (in kDa) are shown. Also shown is the titre of virus on 293T cells in the presence of polybrene.
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<td>HIV CA</td>
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1 - HIV/No Env  
2 - HIV/RD114wt  
3 - HIV/RD114/MLV  
4 - HIV/RD114-PR  
5 - MLV/No Env  
6 - MLV/RD114wt  
7 - MLV/RD114/MLV  
8 - MLV/RD114-PR

(below 4.0 x 10^2)
(1.0 x 10^4)
(1.8 x 10^3)
(1.8 x 10^3)
(below 4.0 x 10^2)
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B  

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3.3 Discussion

In this study, pseudotyping of HIV with gammaretrovirus envelope glycoproteins was examined. Using a transient method of pseudotype production, the ability of chimeric retroviral envelopes to increase HIV vector titre was investigated. It was shown that modification of the cytoplasmic tails of the GALV and RD114 envelopes, thought to aid Env incorporation into the virion or R peptide cleavage from the envelope, increased HIV vector titre but had no significant effect on MLV vector titre.

3.3.1 Modification of the cytoplasmic tail of the GALV and RD114 envelopes transmembrane subunit increases HIV vector titre

Initial experiments indicated that the titre of HIV pseudotyped with the GALV envelope was below the level of detection in the assay used here (Figure 3.2). Modifications thought to aid Env incorporation into the virion (GALV/MLV) or R peptide cleavage from the envelope (GALV-KQ) increased HIV vector titre at least 20-fold (Figure 3.5).

In subsequent experiments, attempts were made to examine levels of chimeric GALV envelope incorporation and R peptide cleavage (Figure 3.7). Reproducible and quantifiable detection with supernatant from the 42/411 hybridoma could only be achieved with MLV supernatant samples (Figure 3.7). No comment could be made regarding efficiency of envelope incorporation into HIV virions and R peptide cleavage.

While this work was underway two similar investigations were published. The results from both studies were consistent with those presented in this report. Firstly, Stitz and co-workers (Stitz et al., 2000) show the replacement of the GALV cytoplasmic tail for that of MLV significantly increases HIV vector titre. In the second study Christodoulopoulos and Cannon (Christodoulopoulos and Cannon, 2001) produced a range of chimeric GALV and MLV-A envelopes to pseudotype HIV vectors, 13 in total. These included the substitution of the GALV cytoplasmic tail for that of MLV-A, but unlike the GALV-KQ mutant used here they created an envelope where two
substitutions K618Q and I619A were made in the GALV cytoplasmic tail. Substitution of the GALV cytoplasmic tail for that of MLV significantly increased HIV vector titre as did the introduction of mutations into the R peptide cleavage site.

These authors further investigated GALV Env incorporation and R peptide cleavage using antibodies from the 42/411 hybridoma that is also used in this study. In contrast to the results presented here, the GALV envelopes could be detected with equal efficiency in both HIV and MLV samples. Their results indicate that the modifications to the cytoplasmic tail mentioned above improve GALV envelope incorporation into the HIV virion. In addition, from the results presented by Christodouloupolous and Cannon it is unclear if R peptide cleavage correlates with titre.

Overall, modification of the GALV cytoplasmic tail can significantly increase HIV vector titre. With such modifications Env incorporation can be improved, but it is as yet unclear how important the role of the R peptide is in the formation of infectious HIV(GALV) pseudotypes (Christodouloupolous and Cannon, 2001).

In contrast to the initial observations made with the GALV envelope, HIV could be pseudotyped with the RD114 envelope to some degree (Figure 3.2), although, as a practical matter, methods to increase vector titre were sought. Like the GALV Env, modifications thought to aid Env incorporation into the virion (RD114/MLV) or R peptide cleavage from the envelope (RD114-PR) were performed. Using either of these chimeric envelopes an increase in vector titre was observed, although not as dramatic as that seen with the chimeric GALV envelopes (Figure 3.5). This work is the first instance where modification of the RD114 envelope cytoplasmic tail has been carried out to increase HIV vector titre.

What effects both envelope incorporation and R peptide cleavage have on RD114 pseudotype titre are as yet unclear. In this study, measurements of both were attempted by western blotting (Figure 3.8), although, as described in the text, these experiments were incomplete. Different levels of Env were seen in the supernatant but it is not clear if this is solely responsible for the observed increase in titre, as different levels of Env were seen in the producer cell lysate and the efficiency of R peptide
cleavage could not be measured. In conclusion, while the increase in HIV(RD114/MLV) titre could be due to modification of the RD114 cytoplasmic tail, the observed increase in RD114-PR titre could be due to both modification of the RD114 cytoplasmic tail or increased availability of Env to be incorporated into virions.

Incorporation of RD114 into SIV vectors was recently examined (Sandrin et al., 2002). Consistent with the results above (Figure 3.5), substitution of the RD114 cytoplasmic tail for that of MLV increased SIV vector titre. Improved envelope incorporation was observed, but expression of the different envelopes in the producer cells was not examined. In light of the results presented in Figure 3.8 it may be presumptuous to assign the increase in Env incorporation solely to modification of the RD114 cytoplasmic tail. Sandrin and co-workers also could not offer any data concerning the correlation, if any, between R peptide cleavage and Env incorporation or vector titre.

Altogether, modification of the RD114 cytoplasmic tail can significantly increase HIV vector titre. With such modifications Env incorporation can be improved (Sandrin et al., 2002). From the data to hand it is unknown what role, if any, the R peptide has in the formation of infectious HIV(RD114) pseudotypes.

3.1.2. Cleavage of the gammaretrovirus envelope and R peptide and incorporation into the HIV virion

It is generally agreed that incorporation of viral and cellular cell surface molecules into the retrovirus virions as a whole occurs by two possible mechanisms (Christodouloupolos and Cannon, 2001; Pickl, Pimentel-Muinos, and Seed, 2001; Sandrin et al., 2002; Swanstrom and Wills, 1997). Firstly, by a non-obligatory mechanism where incorporation is possible if sufficient levels of envelope are present at the site of budding and there is no steric hindrance to incorporation caused by the envelopes cytoplasmic tail, and, secondly, where specific interactions between the constituents proteins of the virions core and the envelope actively govern incorporation of the envelope into the virion.
Given the relative lack of data presented in this study, and elsewhere, regarding cleavage of the R peptide and the link, if any, between cleavage and envelope incorporation into the virion, it cannot be said by which of the aforementioned mechanisms gammaretrovirus envelopes are incorporated into HIV virions. While it is possible the cleavage of the R peptide is a specific interaction between the envelope and virion required for incorporation of the envelope into the virion, it is equally likely at this point that cleavage of the R peptide is a mechanism to remove a structure capable of hindering envelope incorporation and/or keep high levels of the envelope at the site of budding.

Indeed, it is possible that removal of the R peptide could regulate the level of envelope at the cell surface. Downstream of the R peptide cleavage site all gammaretrovirus envelopes harbour the motif YxxΦ (see Figure 3.4), where Y is Tyrosine, x is any amino acid and Φ is an amino acid with a “bulky” hydrophobic side chain. This motif is found in the cytoplasmic tails of all lentiviruses and has been extensively studied in this context. Binding of the YxxΦ motif to μ2 chains of AP2 adapter complexes recruits cell surface proteins into clathrin-coated pits, therefore, the envelope is constitutively endocytosed from the plasma membrane (Berlioz-Torrent et al., 1999; Boge et al., 1998). Therefore, removal of the R peptide from the cytoplasmic tail of gammaretrovirus envelopes would potentially prevent endocytosis of the envelope leaving the glycoprotein available for incorporation into virions at the cell surface. Furthermore, it has been demonstrated that the expression of HIV Gag can alter the rate of endocytosis of the HIV envelope from the plasma membrane, removing excess envelope from the cell surface if Env is overexpressed in the cell (Egan et al., 1996). If HIV Gag has the same effect on gammaretrovirus envelope endocytosis, it is possible in the studies presented here that the lack of GALV and RD114 envelope seen in cells transfected with the HIV packaging construct is due to endocytosis of uncleaved envelope and subsequent degradation of the glycoprotein in late endosomes.

Given the data presented by Egan and co-workers (Egan et al., 1996) it would be interesting to examine gammaretrovirus expression and availability of at the cell surface in the presence or absence of HIV Gag, including mutation of the R peptide cleavage site to prevent cleavage and mutation of the YxxΦ motif.
Chapter 4
Characterisation of HIV vectors pseudotyped with chimeric retroviral envelopes produced from stable HIV packaging cells

4.1 Envelope dependent properties of the virion

To assess their utility for future ex vivo and in vivo use, different pseudotypes should be examined to determine certain characteristics such as resistance to inactivation by human sera, thermo-stability and the ability of infectious particles to be concentrated as these properties are dictated, in part, by the envelope borne by the virion.

Resistance to inactivation by human complement is an especially important characteristic for in vivo applications and is dictated by two factors. (α 1-3) Galactose carbohydrates are expressed by most mammals, but are absent in humans, who lack a functional (α 1-3) galactosyltransferase gene. Anti-(α 1-3) Galactose antibodies inactivate retroviruses produced from animal cells by way of complement in human sera (Takeuchi et al., 1996). This data indicates that production of vectors from human cells is advantageous, however, the envelope the virion displays also dictates virion sensitivity to complement, therefore, both the viral envelope and producer cell affect virion resistance to human complement (Takeuchi et al., 1994).

As mentioned, the envelope SU and TM subunits remain connected via several non-covalent interactions (Gliniak et al., 1991) and a labile disulphide bond (Pinter et al., 1997). Given the relative weakness of these interactions, thermo-stability of infectious particles and their ability to be concentrated, are, in part, dictated by the envelope, as dissociation of the surface and transmembrane envelope subunits inactivates the virus.
4.2 Results

4.2.1 Expression of retroviral envelopes in the stable HIV packaging cell line STAR

According to recommendations made from the transient pseudotyping data above and data from similar studies reported while this work was on-going (Christodouloupolous and Cannon, 2001; Sandrin et al., 2002; Stitz et al., 2000), expression vectors for MLV-A, GALV/MLV, RD114/MLV and RD114-PR were introduced into STAR cells, a novel stable HIV packaging cell line based on 293T (Ikeda et al., 2003). STAR cells producing HIV pseudotyped with the envelopes mentioned above were generated by Y. Ikeda in our laboratory. These cell lines were produced as described in Ikeda et al. (Ikeda et al., 2003) and section 1.5. Briefly, envelope constructs were stably transfected and clonal cell lines for envelope expression were obtained. These clones were infected with HIV(VSV-G) carrying the vector HV, which contains the reporter gene eGFP, and the envelope clones producing highest titre virus were selected.

After receiving these cell lines it was necessary to confirm envelope expression and correct receptor usage.

Firstly, correct envelope expression was confirmed by western blotting of STAR cell supernatants (Figure 4.1). Proteins were separated using a 10% polyacrylamide gel and after transfer the membrane was probed with antibodies specific for HIV CA (p24) and the RD114 envelope surface subunit.

In the supernatant samples HIV CA can be visualised (Figure 4.1). Antibodies raised against the RD114 SU recognised the RD114 gp70 in the supernatant from STAR cells expressing RD114/MLV or RD114-PR envelopes (Figure 4.1, lanes 5 and 6), although the two cannot be differentiated in this experiment.
Figure 4.1 Western blot analysis of STAR cell supernatant

Supernatants were concentrated by ultracentrifugation and the pellet resuspended in 30μl of loading buffer. 15μl of each sample was run on 10% or 14% polyacrylamide gels. After transfer membranes were probed with a goat polyclonal serum raised against RD114 SU, a 1:1 mixture of the murine monoclonal antibodies ADP365 and ADP366 raised against HIV CA, rat monoclonal antibodies 42/411 raised against MLV TM or a goat polyclonal serum raised against Rauscher MLV SU. The position of protein markers (in kDa) are shown. Also shown is the titre of each virus on 293T cells in the presence of polybrene.
Samples were also separated on a 14% polyacrylamide gel and after transfer probed with the supernatant from the rat hybridoma 42/411. Both p15 and p12 of the MLV-A and GALV/MLV envelopes could be observed (Figure 4.1, lanes 3 and 4). This is in sharp contrast to the results presented in Figure 3.7. It was speculated that the envelopes are expressed at much higher levels in STAR cells compared to expression in transient transfection experiments, therefore the GALV/MLV envelope could be clearly seen. In order to differentiate between the GALV/MLV and MLV-A envelopes, the membrane was probed with a polyclonal goat serum raised against an MLV envelope SU. It has been shown that these antibodies strongly recognise the MLV-A SU but show only weak cross-reaction with the GALV SU (Duisit et al., 1999). Using this serum the MLV-A but not GALV SU could be observed (Figure 3.9, lanes 3 and 4). Taken together these results demonstrate correct envelope expression.

4.2.2 Receptor usage of virus from STAR cells

Next, it was demonstrated by receptor interference that the retroviral pseudotypes produced from STAR cells have the correct receptor usage. Cells chronically infected with gammaretrovirus exhibit superinfection resistance (or receptor interference) by preventing superinfecting virus that uses the same receptor from interacting with that receptor. The mechanism by which this occurs in infected cells probably involves direct interaction between the envelope and its cellular receptor within the cytoplasm of the infected cell, preventing the receptor from reaching the cellular membrane and competition for the receptor at the cell surface between progeny virus and superinfecting virus (Hunter, 1997). Plating of a pseudotyped virus onto a panel of chronically infected cells can, therefore, indicate what receptor the pseudotype uses to gain entry into the cell.

TE671 cells chronically infected with MLV-A, GALV, and RD114 viruses were provided by Y. Takeuchi. Virus from STAR cells expressing the envelopes of MLV-A, GALV/MLV, RD114/MLV and RD114-PR was harvested in serum free media (OptiMEM) and plated on these cells and on parental TE671 cells. Also tested was HIV pseudotyped with VSV-G. This was produced by transient transfection of 293T cells with the HIV packaging plasmid pHCMVΔR8.91, the VSV-G expression
plasmid pMD-G and the HIV vector plasmid pHV. The results from this experiment are shown in Figure 4.2. HIV(VSV-G) could infect each cell line with similar efficiency. TE671 cells producing MLV-A were resistant to HIV(MLV-A) infection, those infected with GALV were resistant to HIV(GALV/MLV) infection and TE671 producing RD114 virus were resistant to both HIV(RD114/MLV) and HIV(RD114-PR) infection. No cross interference was observed. These data indicate viruses produced from the STAR cells do show the expected receptor usage.

4.2.3 Resistance to inactivation in human sera of HIV(VSV-G) and gammaretrovirus pseudotypes

Important properties of a pseudotyped virus for gene therapy purposes in vivo include the ability to resist inactivation by human sera and stability at 37°C. The capacity to withstand freeze/thaw cycles would also increase the usefulness of the pseudotype. Using the data from these experiments it will be possible to compare the properties of HIV(VSV-G) pseudotypes compared to retroviral pseudotypes and also evaluate the suitability of virus produced from STAR cells for future ex vivo and in vivo applications. Furthermore, it has yet to be investigated what effect, if any, the media a pseudotype is harvested in effects the characteristics of the virus. Therefore, in these experiments virus was harvested in both DMEM+10% FCS and the serum free media OptiMEM.

In the following experiments retroviral pseudotypes from STAR cells and VSV-G pseudotyped HIV (produced as in the sections above) were harvested in either OptiMEM or DMEM + 10% FCS from 10cm plates. All infections were performed on 293T cells in the presence of polybrene. Envelope-less virus from STAR cells was also harvested in each experiment. At no time did the titre of envelope-less virus exceed the level of detection by FACS (4 x 10^4 iu/ml).
Figure 4.2 Receptor interference assay of virus produced from STAR cells

TE671 cells or TE671 cells chronically infected with either MLV-A, GALV or RD114 (T/MLV-A, T/GALV and T/RD114 respectively) were infected with viruses produced from STAR cells and HIV(VSV-G) produced by transient transfection. Infections were performed using polybrene. The minimum level of detection by FACS in this assay ($2 \times 10^3$ iu/ml) is indicated with an arrow.
The ability of virus produced from STAR cells to resist inactivation in human sera was investigated. Inactivation of gammaretrovirus pseudotypes in human sera has been extensively studied. Retrovirus can be inactivated on exposure to human sera via the classical pathway of complement (Takeuchi et al., 1996) which limits its potential in vivo use. Furthermore, differential resistance to inactivation between different retrovirus pseudotypes is controlled by retroviral envelope (Takeuchi et al., 1994).

Human sera were harvested from clotted peripheral blood and kindly provided by Y. Ikeda. Aliquots of sera were frozen at -80°C until further use or subjected to incubation at 56°C for 45 minutes, thus inactivating heat-labile components of the complement cascade, and then frozen at -80°C. Each virus, in either OptiMEM or DMEM + 10% FCS, was incubated at 37°C for 1 hour with an equal volume of freshly frozen or heat inactivated serum. To quantitatively assess stability in human sera, titre was represented as a percentage of the viral titre after incubation with fresh frozen serum in relation to incubation with the corresponding heat inactivated serum (Figure 4.3).

All retroviral pseudotypes exhibited, in general, good stability when exposed to human sera. HIV virions pseudotyped with the MLV-A envelope lost at most 8% of titre while those bearing the GAVL/MLV envelope showed a loss of titre (27%) in one instance. The highest reduction in retroviral pseudotype titre (the RD114-PR virus harvested in DMEM + 10% FCS exposed to Sera 1) is no greater than 40%. HIV(VSV-G) virus, in contrast, appears to be sensitive to inactivation by fresh human sera, possibly due to the presence of human complement. Exposure to Serum 1 causes a loss of up to 80% of titre, whereas incubation with Serum 2 caused a drop in titre of this virus to below the threshold level of detection by FACS.

The ability of any pseudotype tested here to resist inactivation in human sera did not appear to be influenced by the media in which it was harvested and subsequently incubated.
Figure 4.3 Stability of pseudotyped HIV vectors in fresh human sera

Pseudotyped HIV vectors harvested from STAR cells in either OptiMEM or DMEM + 10% FCS was exposed to human sera. Titre (on 293T cells) is represented as a percentage of the viral titre after incubation with fresh frozen serum in relation to incubation with corresponding heat inactivated serum. Values shown are the mean of two experiments. In the two experiments viral titre did not vary by greater than 2x. Values below 6% of the control titre (titre of virus incubated in heat inactivated sera) could not be detected by FACS.
To exclude the possibility that HIV(VSV-G) instability in human sera was due to a non heat-labile component of the sera, HIV(VSV-G) and HIV(MLV-A) were incubated with either heat inactivated human serum or heat inactivated FCS. Any difference in titre between the FCS and human serum incubated virus should indicate that a non heat-labile component of human sera is indeed responsible for the drop in HIV(VSV-G) titre upon incubation. The results are shown in Figure 4.4. There was no significant difference in HIV(VSV-G) and HIV(MLV-A) titre between virus incubated in the two different sera. Taken together with the data in Figure 4.3 these data would indicate that HIV(VSV-G) instability in fresh human sera is controlled by a heat labile component(s) of human serum such as the components of the complement cascade.
Figure 4.4 Stability of HIV(VSV-G) and HIV(MLV-A) in heat inactivated human sera

Virus harvested in OptiMEM was incubated with either OptiMEM, heat inactivated (HI) FCS or heat inactivated human sera and plated onto 293T cells in the presence of polybrene. The results from two experiments are shown above, shown as a percentage of the titre of the virus incubated with OptiMEM.
4.2.4 Stability at 37°C of HIV(VSV-G) and gammaretrovirus pseudotypes

Another consideration for applications that require *in vivo* gene delivery is the stability of a pseudotyped virus at 37°C.

Aliquots of each virus in OptiMEM or DMEM + 10% FCS were incubated at 37°C and titrated on 293T cells 2 and 6 hours after the start of incubation. The results of these titrations are shown in Figure 4.5 where titre is presented as a percentage of the viral titre before incubation. In contrast to stability in human sera, the HIV(VSV-G) pseudotype was less sensitive to incubation at 37°C than the retroviral pseudotypes tested.

After two hours of incubation the titre of the HIV(VSV-G) virus dropped by less than 15% whereas the titre of HIV(MLV-A) and HIV(RD114-PR) fell below 50% of the original titre indicating that the half-life of these pseudotypes at 37°C was less than 2 hours. HIV bearing either the GALV/MLV or RD114/MLV envelopes appeared to be slightly more stable over 2 hours incubation. There was less than a 2-fold difference between these pseudotypes and HIV(MLV-A) and HIV(RD114-PR).

After 6 hours incubation all retroviral pseudotypes fell to less than 50% of their original titre. The half-life of the GALV/MLV and RD114/MLV pseudotypes was, therefore, between 2 and 6 hours. The HIV(VSV-G) virus, meanwhile, had lost only up to 40% of its original titre after 6 hours incubation at 37°C. Virus titration after 24 hours incubation at 37°C showed that HIV(VSV-G) titre has fallen by 90% (not shown). The half-life of this virus at 37°C was, therefore, not in excess of 24 hours. Further experimentation is required to accurately determine the half-life of VSV-G. No significant difference in stability at 37°C between virus incubated in OptiMEM versus that incubated in DMEM + 10% FCS could be observed.
Figure 4.5 Stability of pseudotyped HIV vectors at 37°C

Pseudotyped HIV vectors harvested from STAR cells in either OptiMEM or DMEM + 10% FCS were incubated at 37°C and titrated at different time points. The titre (on 293T cells) is presented as a percentage of the viral titre before incubation. Values shown are the mean of two experiments and the error bars show the actual data points. Control titre is pseudotype titre before incubation.
4.2.5 Resistance of HIV and gammaretrovirus pseudotypes to freeze/thaw cycling

The ability of virus to resist freeze/thaw cycles was then examined by titrating virus on 293T cells after cycling between -80°C and 37°C. The titre is presented as a percentage of titre before commencing the first cycle (Figure 4.6). In general, all gammaretrovirus pseudotypes showed some resistance to freeze/thaw after one cycle. After one cycle MLV-A pseudotyped virus lost less than 5% of its original titre in either media. All other retroviral pseudotypes appeared to be less resistant to freeze/thaw, although after three cycles no retroviral pseudotype lost greater than 50% of its original titre. With regard to the retroviral envelope pseudotypes, there was no clear answer as to which media provided the most protection to freeze/thaw cycling. This, however, was not the case for the HIV(VSV-G) virus. After one cycle the virus harvested in OptiMEM lost up to 65% of its original titre, while virus titre in DMEM + 10% FCS appeared to be stable. Moreover, after three cycles virus in OptiMEM lost 90% of its original titre while that in DMEM + 10% FCS lost less than 10%. Why there should be this difference is unclear as yet and requires further experimentation to determine if the difference in media or the presence of FCS in the media plays a role in the protective effect observed.

4.2.6 Concentration of STAR cell derived gammaretrovirus pseudotypes by three different methods

Efficient concentration of vector stocks is a practical necessity for in vitro and in vivo applications that require infection at high multiplicity of infection. It is known that the VSV-G pseudotyped HIV can be efficiently concentrated by ultracentrifugation (Reiser, 2000) but there is, as yet, no clear consensus as to how HIV vectors pseudotyped with retroviral envelopes can be efficiently concentrated.

Virus was harvested in OptiMEM from STAR cells or from 293T cells transfected to produce HIV(VSV-G) virus in 10cm plates. Three plates were used to harvest each virus and the supernatant was pooled. This supernatant was then aliquoted and concentrated by three methods – ultracentrifugation, low-speed centrifugation or filtration using a filter with a cut-off of 100kDa.
Figure 4.6 Resistance of pseudotyped HIV virions to freeze/thaw cycles

Pseudotyped HIV vectors harvested from STAR cells in either OptiMEM or DMEM + 10% FCS were frozen and thawed (between -80°C and 37°C) up to three times. Titre (on 293T) is presented as a percentage of the control titre: viral titre before initiating the first cycle. Values shown are the mean of two experiments and the error bars indicate the actual data points. The number of cycles is indicated on the x-axis.
Before and after concentration virus was titrated on 293T cells in the presence of polybrene (Table 4.1).

Each pseudotype appeared to be amenable to concentration by ultracentrifugation and low-speed centrifugation. Unexpectedly, however, the fold-concentration values exceed the fold volume reduction values in a number of cases. Why this should be is unknown but possible explanations for this observation are discussed below (section 4.3.1). Ultracentrifugation is not, however, a practical method for concentrating large volumes. Low-speed centrifugation could, therefore, be used in future applications that require the preparation of concentrated virus stocks.

Further experimentation is required to determine if the presence of serum in the media during concentration has any effect on the efficiency of the method used to concentrate virus.
Table 4.1 Concentration of HIV vectors harvested from STAR cells

Virus produced was concentrated by ultracentrifugation, centrifugation or filtration. Virus was titred on 293T cells with polybrene and eGFP expression assayed by FACS.
4.2.7 Enhancement of infection of tissue culture cell lines using polybrene and spinoculation

Thus far it had been determined that HIV pseudotyped with gammaretrovirus envelopes, produced from the novel packaging cell line STAR possessed characteristics that were advantageous for use \textit{ex vivo} and \textit{in vivo}. However, in only one area, resistance to inactivation by human sera, did retrovirally pseudotyped virus significantly outperform HIV(VSV-G).

It was then necessary to investigate how pseudotype titre might be enhanced with a view to the \textit{ex vivo} transduction of primary cell cultures including DCs. Here the use of polybrene and centrifugal inoculation or "spinoculation" was examined.

The polycation polybrene, added directly to the media during infection, neutralises negative charges on the cell surface and is widely used to enhance virus infection. It is unknown, however, if polybrene has a differential effect between pseudotypes.

Spinoculation has been shown to increase HIV titre by directly sedimenting virus onto cells. Spinoculation of virus onto cells was carried out as previously described (O'Doherty, Swiggard, and Malim, 2000). Briefly, viral supernatant is plated onto target cells and the plate(s) spun at 1,200g for 2 hours at 25°C. Plates are then immediately placed at 37°C and incubated overnight. The media is then changed and the percentage of eGFP positive cells was determined by FACS as before. What effect the presence of polybrene during spinoculation has on HIV pseudotype titre was unknown at that point.

Virus from STAR cells expressing the envelopes MLV-A, GALV/MLV, RD114/MLV and RD114-PR and the vector pHV were harvested in serum free media (OptiMEM) from 6-well plates. VSV-G pseudotyped HIV was produced as before by transient transfection of 293T cells with the HIV packaging plasmid pCMVΔR8.91, the VSV-G expression plasmid pMD-G and the HIV vector plasmid pHV.
In each of the following experiments supernatant from STAR cells that contain pHV but no envelope expression cassette was titrated in parallel. In no experiment could viral titre above the level of detection by FACS (4x10^4 IU/ml on 293T cells) be detected from these cells. Each pseudotype was titrated onto 293T cells in the presence of the polybren, with the use of spinoculation or both. The results from these experiments are shown in Figure 4.7.

With regard to the titres of the retroviral pseudotypes, polybren enhances infection on average 5-6x whereas spinoculation enhances infection on average 4x. The presence of polybren does not appear to enhance HIV(VSV-G) pseudotype titre by greater than 2x. In only one instance did spinoculation increase HIV(VSV-G) titre by greater than 2x. When polybren and spinoculation were used together their effect in all cases is additive. Their use together would, therefore, appear to be an efficient method of increasing titre of all pseudotypes on tissue culture cell lines and it can be proposed that the use of polybren and spinoculation should be used to optimise \textit{ex vivo} transduction of primary cells.

As mentioned earlier it will be necessary to standardise infection using multiplicity of infection determined on a tissue culture cell line. Thus far 293T have been used exclusively to titre pseudotypes but it is possible that another tissue culture cell line is significantly more susceptible to infection.

To test this each pseudotype was titrated onto a panel of tissue culture cell lines including the human cell lines 293T, TE671, HeLa and HT1080 and the murine cell line NIH 3T3 in the presence or absence of polybren. In the presence of polybren, however, there was less than 2x difference in titre for each pseudotype on each of the human cell lines tested (Figure 4.8). The effect polybren had on pseudotype titre varied between cell lines (Figure 4.9). As before the presence of polybren did not raise the titre of the VSV-G pseudotyped virus by more than 2x. This was true of each cell line examined. The effect of polybren appeared to be most significant when used on 293T or TE671 cells, while its effect is less dramatic on HeLa or HT1080 cells. The infection enhancement of polybren on NIH 3T3 cells appears to be negligible. (NIH 3T3 do not bear the receptors for GALV or RD114 (Sommerfelt, 1999).
Accordingly, HIV pseudotyped with GALV/MLV or chimeric RD114 envelopes could not infect these cells at detectable levels).

If titration is required on more than one cell line without the use of polybrene to standardise infection the data presented here should be taken into account. Furthermore, given the differential effect of polybrene between HIV(VSV-G) and gammaretrovirus pseudotypes, titration in the absence of polybrene on tissue culture cells favours HIV(VSV-G) titre, therefore, the number of infectious retroviral pseudotypes could be underestimated. Moreover, polybrene may have little effect on infection of primary cell cultures or, more importantly, may harm primary cells. The use of polybrene for infection of primary cell cultures requires further study.
Figure 4.7 Enhancement of retroviral pseudotype titre using polybrene or spinoculation

Virus from STAR cells was titred on 293T cells and spun down at 1200g for 2 hours at 25°C or titred on 293T in the presence of polybrene (8 μg/ml). eGFP expression was assayed for by FACS. Values from two experiments are shown. Control titre signifies titre of virus on 293T cells both in the absence of polybrene and without spinoculation.
Figure 4.8 Titre of virus harvested from STAR cells on a panel of tissue culture cell lines in the presence of polybrene

Target cells were assayed for eGFP expression by FACS. The minimum level of detection by FACS in this assay was $4 \times 10^4$ infectious units/ml of viral supernatant. Values shown are the mean from two experiments and the error bars indicate actual data points.
Figure 4.9 Enhancement of retroviral pseudotype titre on a panel of tissue culture cell lines using polybrene

Virus from STAR cells was titrated on 293T cells in the presence or absence of polybrene. Shown above is the ratio of infection without polybrene to infection with polybrene. Arrows indicate no difference in titre. As NIH 3T3 cells do not display receptors for GALV or RD114 no infection with GALV/MLV, RD114/MLV or RD114-PR pseudotypes above the level of detection by FACS (4 x 10^4 iu/ml) was observed.
4.3 Discussion

In this chapter, the characteristics conveyed to the virion by the retroviral envelopes they incorporate were investigated. These viruses were produced from a novel stable HIV packaging cell line, STAR (Ikeda et al., 2003). Thus, it was possible to assess the suitability of virions from this cell line for future ex vivo and in vivo use. The findings, in brief, from this part of the investigation are as follows. Retroviral pseudotypes from STAR cells show good resistance to inactivation in human sera and fair stability at 37°C. These viruses showed good resistance to inactivation during freeze/thaw cycling and could be efficiently concentrated by both low-speed centrifugation and ultracentrifugation. Also, use of polybrene and spinoculation to enhance HIV titre were examined with a view to enhancing infection conditions for ex vivo transduction.

It was found that the effect of polybrene and spinoculation on retroviral pseudotype infection was significant and additive for pseudotypes bearing gammaretroviral envelopes, but neither of these methods significantly improved HIV(VSV-G) infection.

4.3.1 Characterisation of virus gammaretrovirus pseudotypes produced from STAR cells

Based on published literature (Christodoulopoulos and Cannon, 2001; Sandrin et al., 2002; Stitz et al., 2000) and the data presented here recommendations regarding which envelopes were most suitable for introduction into the novel HIV packaging cell line STAR were sought. The MLV-A, GALV/MLV, RD114/MLV and RD114-PR envelopes were elected. As mentioned, STAR cells producing HIV pseudotyped with these envelopes were produced by Y. Ikeda in our laboratory. The virus from these cells was then characterised with regard to its suitability for use in future ex vivo and in vivo experiments.

Consistent with published data indicating that HIV(VSV-G) pseudotyped virus is readily inactivated in human serum by complement (DePolo et al., 2000), HIV(VSV-G) virus showed poor stability in human sera. All retroviral pseudotypes produced from STAR cells, did, however, show good resistance to inactivation by complement
(Figure 4.3). It has been established that in vivo retroviral vector half-life correlates with in vitro resistance to complement (DePolo et al., 1999), therefore, retrovirally pseudotyped virus from STAR cells may be suitable for gene therapy applications that require in vivo gene delivery.

Why there should be a difference in sensitivity between VSV-G and retroviral pseudotypes remains unclear. It would be of interest to ascertain if resistance to inactivation is equivalent to the levels of complement modulating molecules such as CD46, CD55 and CD59 incorporated into each of the different pseudotypes. Furthermore, it is unknown how VSV-G pseudotyped HIV is inactivated by human complement. DePolo and co-workers (DePolo et al., 2000) speculate that specific, nonneutralising antibodies against VSV-G activate the classical pathway of complement against HIV(VSV-G). It should be possible to ascertain if the classical pathway is indeed involved in HIV(VSV-G) inactivation by measuring inactivation of HIV(VSV-G) in human sera deficient in components or co-factors of the classical complement cascade.

Several other characteristics of virus from STAR cells were also tested. The stability of STAR cell produced virus at 37°C is notably poorer than that of transiently produced HIV(VSV-G) (Figure 4.5). This may limit the use of these vectors for in vivo systemic delivery of therapeutic genes. Murine tissue can be efficiently infected in vivo by systemic injection of transiently produced HIV(MLV-A) (Peng et al., 2001). A comparison of the stability at 37°C of transiently produced HIV(MLV-A) versus that from STAR cells may, therefore, be insightful.

Two other characteristics were tested: resistance to freeze/thaw cycling and virus concentration. Consistent with findings reported by Watson et al. (Watson et al., 2002), resistance of gammaretrovirus pseudotypes to freeze/thaw cycling compared to VSV-G was good (Figure 4.6). Among then HIV virions pseudotyped with the MLV-A envelope showed good resistance to freeze/thaw cycling. Furthermore, it was interesting to note that harvesting virus in DMEM+10% FCS had a protective effect against freezing for VSV-G but not retroviral pseudotypes. Why this should be so remains unclear and it will be of interest to examine if this is true for other non-retroviral HIV pseudotypes such as HIV pseudotyped with the envelope glycoproteins.
of Ebola, Mokola and lymphocytic choriomeningitis virus (LCMV) (Watson et al., 2002).

All pseudotypes could be efficiently concentrated by ultracentrifugation and centrifugation (Table 4.1). In the concentration experiment performed here, greater than 100% recovery was observed. Why this should be is as yet unclear. Slingsby and co-workers (Slingsby et al., 2000) have, however, reported that MLV(MLV-A) packaging cells FLYA13 (Cosset et al., 1995) expel large quantities of envelope protein into the harvest media and this acts as an inhibitor of infection, competing with virion associated envelope for its cellular receptor. It is possible that STAR cells also expel into the harvest media significant amounts of retroviral envelope, or indeed another as yet unidentified inhibitory factor, and that the concentration methods used here removed soluble inhibitory envelope/factor from the media. Although the SU and TM subunits of the retroviral envelopes are not covalently linked it is possible they may dissociate during ultracentrifugation, thus inactivating the virus (Pham et al., 2001). This does not appear to be the case. From a practical perspective it is possible, though unlikely, that the ultracentrifuge conditions used here were optimal, whereas those used by other authors were too harsh for optimal recovery. It is also possible that the resistance to inactivation at high speed observed here is a facet of virus produced from STAR cells. To test this supposition it will be necessary to investigate the concentration characteristics of transiently produced retroviral pseudotypes versus those from STAR cells.

4.3.2 Infection enhancement using polybrene and spinoculation

With a view to ex vivo transduction, different methods of enhancing pseudotype titre were studied.

Firstly, the effects of polybrene and spinoculation on the different pseudotypes on 293T cells were tested. It was found that the effect of polybrene and spinoculation on the infectious titre of the retroviral pseudotypes was cumulative, however neither the presence of polybrene nor spinoculation appeared to significantly enhance VSV-G pseudotyped virus infection (Figure 4.7).
It is proposed that spinoculation enhances infection by depositing virions on the surface of target cells (O'Doherty, Swiggard, and Malim, 2000) and the polycation polybrene neutralises electrostatic repulsion between cell membranes and the virion allowing enhanced attachment (Toyoshima and Vogt, 1969). This electrostatic repulsion is in part due to the presence of negatively charged GAGs in the cellular membrane (Zhang et al., 2002). As they appear to enhance infection by two different methods is it not surprising that their effect is additive but it is interesting that VSV-G pseudotype binding may not require neutralisation of electrostatic repulsion.

The infection enhancement effect of polybrene on retroviral pseudotypes varied between the cell lines tested (Figure 4.9). The effect was most noticeable on 293T and TE671 cells. Why titration onto HeLa, HT1080 or NIH 3T3 cells did not show the same level of effect is unknown. One possibility is that the electrostatic repulsion of the cells to virus is greater in these cell lines than that of 293T or TE671 cells. Differences in repulsion could be due to differing levels of negatively charged GAGs on each cell lines and it may be interesting to increase the concentration of polybrene during infection to determine if transduction efficiency can be increased.

Polybrene has been used in the transduction of DCs with VSV-G pseudotyped SIV vectors and its presence does not appear to affect the maturation or stimulatory capacity of the cells (Mangeot et al., 2002). While polybrene may possibly be dispensed with for VSV-G pseudotype infection, its use in vitro may be a valid method for increasing the efficiency of retrovirally pseudotyped HIV transduction without adversely affecting the cell culture.

It is, therefore, proposed that optimal conditions for primary cell transduction with HIV bearing retroviral envelopes include the use of polybrene and spinoculation. It will, of course, be necessary to test the use of polybrene and spinoculation to ascertain the optimal conditions for ex vivo transduction of primary cell cultures.

In conclusion, chimeric retroviral envelopes of GALV and RD114 incorporating mutations thought to increase Env incorporation or enhance R peptide cleavage significantly increased HIV vector titre but had no significant effect on MLV vector titre. Furthermore, these chimeric envelopes could be efficiently expressed in stable HIV packaging cells to constitutively produce high-titre virus.
These viruses are resistant to inactivation in human sera and have the potential to be used in *in vivo* applications. Enhancing the infection of the pseudotypes used here was studied on tissue culture cell lines and it was concluded that the use of polybrene and spinoculation should be used in order to find optimal conditions for the transduction of human primary cell cultures with HIV(VSV-G) and gammaretrovirus pseudotypes.
Chapter 5
Production and characterisation of HIV vectors pseudotyped with alphavirus envelopes

5.1 Introduction

It may be useful to pseudotype HIV vectors with envelopes other than VSV-G or those from gammaretroviruses.

In order to assess the efficiency of pseudotyped HIV vector transgene delivery in pre-clinical studies, it will be necessary to assess pseudotyped vector infection in animal models such as mice. Murine cells do not, however, display receptors that can be recognized by the GALV or RD114 envelopes (Overbaugh, Miller, and Eiden, 2001). The inability to test pseudotyped vectors in mice would be a drawback to the clinical development of HIV based vectors. As yet there are no transgenic murine models whose tissues display the GALV or RD114 receptors.

VSV-G pseudotypes can infect a wide range of mammalian cell types (Dirks and Miller, 2001; Towers et al., 2000). HIV(VSV-G) pseudotypes are not, however, ideal for use in a clinical setting as, due to the inherent toxicity of the protein, it is not as yet possible to produce VSV-G pseudotypes from stable packaging cells (Burns et al., 1993).

Pseudotyping HIV vectors with glycoproteins from viruses that infect clinically relevant human cells in vivo may be a more efficient method of mediating vector entry than using either gammaretroviral envelopes or VSV-G. Ideally these envelopes would not be cytotoxic and, therefore, could be expressed in stable HIV packaging cells.

With the points raised above in mind, the pseudotyping of HIV vectors with envelope glycoproteins from alphaviruses was examined. These are arthropod-borne enveloped viruses such as Ross River virus (RRV), Semliki Forest virus (SFV) and Sindbis virus
(SIN) that replicate in many different tissues and hosts, including mammals (including humans and rodents), avians and insects.

Previously, it had been shown that the envelope from RRV could pseudotype MLV vectors as part of a stable packaging system (Sharkey et al., 2001). Furthermore, Morizono and colleagues have shown that HIV vectors can be pseudotyped with the envelope glycoprotein from Sindbis virus (Morizono et al., 2001). Also, Kang and co-workers (Kang et al., 2002) demonstrated that this envelope could pseudotype FIV vectors and that these pseudotypes can infect various murine tissues in vivo upon systemic injection and direct injection into the brain and skeletal muscle. It is interesting to note that these authors were able to show that systemic injection of FIV(RRV) virus into mice was less cytotoxic to murine cells than administration of FIV(VSV-G).

It was, therefore, speculated that it would be possible to produce HIV pseudotyped with alphavirus envelopes from the novel HIV packaging cell line STAR (Ikeda et al., 2003) and that these pseudotypes might efficiently transduce clinically relevant cell types in both humans and animal models.

5.1.1 The Alphaviridae

Alphaviruses are members of the Togaviridae and as such are lipid-enveloped icosahedral particles containing a single positive stranded RNA genome of approximately 11-12 kilobases in length (Griffin, 2001), which assemble under and bud through the plasma membrane (Acheson and Tamm, 1967).

Members of the alphavirus family, their hosts and the human disease states they cause are listed in Table 5.1. Viruses are grouped into the alphavirus family on the basis of genomic homology. At present, this family contains 25 members. Based on the amino acid sequence of one of the alphavirus envelope subunits, E1, alphaviruses are currently subdivided into six clades by serologic cross-reactivity (see Table 5.1). With regard to amino acid sequence, members of the alphavirus family share a minimum identity of 60% in the nonstructural proteins (those which aid virus replication) and
<table>
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<th>Virus (Abbreviation)</th>
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<th>Principal vertebrate host</th>
<th>Symptoms of human disease</th>
<th>Susceptible animals</th>
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<td>Avians</td>
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<td>Mammals</td>
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<td>Horse</td>
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<td>Fever, arthritis, rash</td>
<td></td>
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<td>Fever, encephalitis</td>
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<td>Fever, encephalitis</td>
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<td>Whataroa (WHA)</td>
<td>WEE</td>
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</table>

Table 5.1 Members of the alphavirus family. Table adapted from (Griffin, 2001).
40% in the structural proteins (components of the virion, such as the envelope proteins).

Alphaviruses replicate in arthropod, avian and mammalian cells. The primary mode of transmission to humans is via mosquito biting. Infection of Langerhans cells, immature dendritic cells found in the epidermis, is thought to be a common mode of initiating natural infection in humans (Griffin, 2001). Macrophage may also play a role here as they too can support infection (Linn, Aaskov, and Suhrbier, 1996). Lymphoid tissue then becomes infected as infected antigen presenting cells migrate to lymphoid organs (Griffin, 2001). The virus then spreads and replicates in a number of cell types and tissues, most notably synovial tissue (Journeaux, Brown, and Aaskov, 1987), neurons and glial cells and striated and smooth muscle cells (Strauss and Strauss, 1994). The wide tissue tropism of alphaviruses is highlighted by the symptoms displayed by infected individuals. These include rash, arthritis and fever. In the most serious cases of alphavirus infection, neurovirulent strains can evolve in the infected individual and cause fatal encephalitis (Griffin, 2001; Strauss and Strauss, 1994) (see Table 5.1).

Alphavirus infection is not limited to humans. Alphaviruses can also be transmitted to horses via insect bites and these are generally considered to be dead-end infections. Furthermore, alphaviruses can be transmitted between avian species (see Table 5.1).

Murine models have been used extensively to study alphavirus infection, especially that which leads to neurological disease. In a similar fashion to human infection, upon injection of virus murine antigen presenting cells become infected and as the disease progresses viral antigens can be found in a wide range of tissues including lymphoid and neuronal tissue (Griffin, 2001).
5.1.2 Synthesis of Alphavirus envelope glycoproteins and incorporation into virions

The mature alphavirus envelope glycoprotein, sometimes referred to as a spike, consists of a trimer of heterodimers. Each heterodimer is composed of two transmembrane proteins, E1 and E2, which are derived from the polyprotein C-E3-E2-6K-E1. This polyprotein is transcribed from a subgenomic RNA, 26S (Strauss and Strauss, 1994).

The maturation of the alphavirus envelope has been extensively studied using SFV and SIN. Given the homology between alphavirus envelope glycoproteins it is thought that maturation of other alphavirus envelope glycoproteins occurs in a similar fashion.

The polyprotein C-E3-E2-6K-E1 is translated into the rough endoplasmic reticulum (RER). The capsid protein (C) has a protease function that removes C from the polyprotein shortly after translation. E3-E2-6K-E1 is proteolytically processed into individual subunits by cellular proteases during maturation (Strauss and Strauss, 1994).

The envelope polyprotein is anchored in the RER membrane via a motif in E2. In the RER cellular proteases, which have yet to be identified, cleave E3-E2 from 6K-E1. 6K is then removed from E1 (Yao, Strauss, and Strauss, 1996). E1 remains in the membrane as it is anchored by a sequence in its carboxyl-terminal domain. E3-E2 (also known as pro-E2 or PE2) forms heterodimers with E1 (Barth, Wahlberg, and Garoff, 1995) that are then transported to the Golgi where E3 is removed from E2 by a furin-like cellular protease (Strauss and Strauss, 1994). E1 has several potential N-linked glycosylation sites and sugars attached in the RER are trimmed in the Golgi (Knipfer and Brown, 1989). The mature heterodimers then move to the plasma membrane to be incorporated into nascent virions. Either before reaching the plasma membrane or during budding three E1/E2 heterodimers assemble to make a spike (Strauss and Strauss, 1994). The alphavirus envelope glycoprotein is anchored at the plasma membrane by the addition of palmitic acid residues to the cytoplasmic tail of E2 (Zhao et al., 1994). Here, the cytoplasmic tail of E2 interacts specifically with the alphaviral nucleocapsid protein to mediate its incorporation into the virion at the
plasma membrane (Cheng et al., 1995; Lopez et al., 1994). It is thought that budding through the plasma membrane does not take place in lipid rafts. Analysis of the lipid content of alphavirus virions indicates that there are few detergent-insoluble complexes (Scheiffele et al., 1999). Such complexes are characteristically found in the lipid bilayers of viruses that bud from lipid rafts.

5.1.3 Attachment and fusion

Within the trimer of hetrodimers displayed on the mature virion, E2 mediates virus attachment at neutral pH. Which molecules mediate alphavirus attachment is the source of much discussion. The presence of heparan sulfate, a glycosaminoglycan (GAG) molecule, on the cells surface is important for initial binding of many enveloped viruses. The requirement of heparin sulfate for virus attachment to target cells appears to differ between members of the alphavirus family. For example, the presence of heparan sulfate is important for the initial binding to target cells of SIN, but not RRV (Byrnes and Griffin, 1998; Heil et al., 2001).

Actual alphavirus envelope/cell fusion is pH-dependent and virus entry into the cell occurs by endocytosis (Corver et al., 1995; Sharkey et al., 2001; White and Helenius, 1980). At low pH, E2 and E1 dissociate within the trimer of hetrodimers. The E1 subunits then form a homotrimer, which is thought to be the complex that actually promotes envelope/cell fusion (Fuller et al., 1995; Wahlberg et al., 1992; Wahlberg and Garoff, 1992).

5.1.4 Alphavirus cellular receptors

None of the cell surface molecules identified as alphavirus receptors thus far are used exclusively. For example, major histocompatibility complex class 1 (MHC I) molecules were identified as cellular receptors for SFV on human and murine cells (Helenius et al., 1978) but they are not absolutely required for infection as cells lacking these molecules can also be infected (Oldstone et al., 1980). The wide host range and cellular tropism displayed by members of the alphavirus family has lead to speculation that several receptors or several receptor-coreceptor combinations are involved in mediating virus entry.
Studies of SIN and SFV cell entry have implicated lipid molecules in the cell surface membrane as being important for alphavirus cell entry. Although the cellular adhesion glycoprotein laminin had been identified as the cellular receptor for SIN (Wang et al., 1992), it was found that SIN virus could fuse with protein free cholesterol and sphingolipid containing liposomes (Smit, Bittman, and Wilschut, 1999). Furthermore, it appeared that the presence of cholesterol was essential for the fusion process in SFV infection, and that sphingolipid molecules in the cell membrane acted as co-factors for envelope/cell fusion (Corver et al., 1995; Nieva et al., 1994). In these experiments sphingolipids had been proposed only as co-factors for the fusion process as efficient fusion is possible with only minimal concentrations of sphingolipids in the cell membrane (Nieva et al., 1994). Elucidation of the identity of receptor molecules used in vivo may, however, be complicated by the use of tissue culture adapted virus strains in receptor screening experiments (Klimstra, Ryman, and Johnston, 1998). An excellent analogy can be found in data concerning the cellular receptor for measles virus. The complement receptor CD46 was described as a receptor for the vaccine strain (Edmonston strain) of the measles virus (Dorig et al., 1993). The Edmonston strain is, however, highly tissue culture adapted and it was subsequently shown that clinical isolates of the measles virus cannot use CD46 as a receptor but rather utilise CDw150 or SLAM (signalling lymphocyte-activation molecule)(Tatsuo et al., 2000).

Apoptosis can occur in the infected cell, triggered early in the virus life cycle, most likely during virus entry into the cell (Scallan, Allsopp, and Fazakerley, 1997). This is most commonly observed in neurovirulent strains that arise in advanced infection. This phenomenon has been extensively studied using Sindbis virus. In infected neuronal cells the product of the cellular oncogene bcl-2 can block apoptosis (Levine et al., 1993). Neurovirulent strains of Sindbis virus possess a single point mutation in the amino terminus of E2 that can circumvent this block to apoptosis (Ubol et al., 1994). Bcl-2 can also inhibit apoptosis in SFV infected cells (Scallan, Allsopp, and Fazakerley, 1997) but it is unknown what mutations in the envelope glycoproteins of other neurovirulent alphaviruses can cause them to become apoptotic triggers during advanced infection. It was subsequently shown that Bax, a member of the Bcl-2 family, can also inhibit Sindbis mediated apoptosis (Lewis et al., 1999) but it is unclear what mutations, if any, in the viral envelope of neurovirulent Sindbis might circumvent Bax mediated inhibition of apoptosis.
5.2 Results

5.2.1 Transient production of HIV and MLV vectors pseudotyped with Alphavirus envelope glycoproteins

The RRV, SFV and SIN envelope glycoproteins were tested for their ability to pseudotype HIV and MLV vectors. Expression vectors containing the RRV, SFV and SIN virus envelope coding sequences were a gift from D. Sanders (Indiana, USA). In each case the E3-E2-6K-E1 coding region had been inserted into the mammalian expression vector pcDNA3.1/Zeo(+) (Invitrogen). The resulting plasmids were named pRRV-E2EIA, pSFV-E2EIA, and pSIN-E2EIA. In these expression vectors the CMV promoter controls envelope glycoprotein expression.

HIV and MLV pseudotypes were produced by three-plasmid transfection of the envelope glycoprotein expression vectors into 293T cells with the HIV packaging and vector plasmids pHCMVAR8.91, pHR'SINcPPT-SE or the MLV packaging and vector plasmids pHIT60 and pCNCG. HIV(VSV-G) and MLV(VSV-G) pseudotypes were also produced by the transfection of the VSV-G expression vector pMD-G with the HIV or MLV packaging and vector plasmids. The HIV and MLV packaging and vector constructs were also transfected without an envelope expression vector.

Transfections were performed in 24-well plates. 48 hours post transfection virus was harvested in OptiMEM. The viral supernatant was then filtered through a 0.45 μm filter, diluted and plated onto 293T cells in the presence of polybrene.

While both the RRV and SFV envelope glycoproteins could pseudotype both the HIV and MLV vectors to some degree, no HIV(SIN) or MLV(SIN) virus could be observed above the level of detection by FACS (2 x 10^3 iu/ml) (Figure 5.1). As before both HIV and MLV vectors could be efficiently pseudotyped with the VSV-G glycoprotein (Figure 3.5). No infection above the level of detection by FACS was
Figure 5.1 Pseudotyping of HIV and MLV vectors with the envelopes of RRV and SFV

Pseudotypes were produced by three-plasmid transfection of the relevant envelope glycoprotein expression plasmid into 293T cells with the HIV packaging and vector plasmids pHCMVΔR8.91 and pHRSINcPPT-SE or the MLV packaging and vector plasmids pHIT60 and pCNCG. This was performed in 24-well plates. Supernatant was filtered, diluted and plated onto 293T cells. Titre (iu/ml) was determined from the percentage of GFP positive cells as assayed by FACS. Results from virus production with the SIN expression plasmid or no envelope expression plasmid are not shown. The minimum level of detection by FACS (2x10³ iu/ml) is indicated with an arrow.
observed when supernatant from the cells transfected without an envelope expression
vector was plated onto 293T cells (not shown).

The supernatant and producer cell lysate from virus producing 293T cells was then
examined by western blotting. 293T cells were transfected in 10cm plates with the
same plasmids as before. Each viral supernatant was titred on 293T cells as before
and then subjected to ultracentrifugation to pellet any virus particles. The producer
cells, meanwhile, were lysed in RIPA buffer. Both cell lysate and supernatant samples
were probed with rabbit antiserum raised against the SFV polyprotein C-E3-E2-6K-
E1 (Marsh and Bron, 1997). This reagent was a gift from M. Marsh (London, England).

The RRV and SFV envelopes could be observed in both the cell lysate and
supernatant samples (Figures 5.2). It is unknown what envelope subunits the rabbit
antiserum recognizes, therefore, it cannot be accurately said what subunits are shown
in Figure 5.2. It must be noted, however, it was not possible to differentiate between
the RRV and SFV envelopes using the anti-SFV serum.

Under these experimental conditions no SIN envelope glycoprotein could be detected
in the either the lysate or supernatant samples. Given the lack of homology between
the envelope sequences of SFV and SIN (45%) and RRV and SIN (47%) it is possible
that the SIN envelope glycoprotein cannot be detected with the anti-SFV sera or the
SIN envelope glycoprotein expression plasmid is at fault and the envelope
glycoprotein is not expressed. During the course of this study antisera raised against
the RRV or SIN envelope glycoproteins was unavailable.

As the RRV and SFV envelope glycoproteins pseudotype HIV virions these envelope
glycoproteins were elected for introduction into the stable HIV packaging cell line
STAR.
Figure 5.2 Expression of alphavirus envelopes during transient vector production

Expression of alphavirus envelopes was examined by western blot. Pseudotypes were produced by transient transfection in 10cm plates. Supernatant was titred on 293T cells. Vector particles were then concentrated by ultracentrifugation and the pellet resuspended in 30μl of loading buffer. 15μl of each sample were used in this experiment. 6 x 10⁶ of each producer cell was lysed in 500μl of RIPA buffer. 15μl of each sample was analysed. Proteins were separated using 10% polyacrylamide gels. After transfer, membranes were probed with a rabbit polyclonal serum raised against the SFV envelope, a 1:1 mixture of murine monoclonal antibodies ADP365 and ADP366 raised against HIV CA (p24) and a goat polyclonal serum raised against Rauscher MLV CA (p30). The positions of protein markers (in kDa) are shown. The titre of each pseudotype on 293T cells is also indicated.
Supernatant

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</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
<td>HIV/SFV (1.3x10^6)</td>
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<tr>
<td>4</td>
<td>HIV/SIN (less than 2x10^3)</td>
</tr>
<tr>
<td>5</td>
<td>MLV/No envelope (less than 2x10^3)</td>
</tr>
<tr>
<td>6</td>
<td>MLV/RRV (6.0x10^2)</td>
</tr>
<tr>
<td>7</td>
<td>MLV/SFV (2.9x10^2)</td>
</tr>
<tr>
<td>8</td>
<td>MLV/SIN (less than 2x10^3)</td>
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</table>

Cell Lysate

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<tr>
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<td>HIV/SIN (less than 2x10^3)</td>
</tr>
<tr>
<td>5</td>
<td>MLV/No envelope (less than 2x10^3)</td>
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<tr>
<td>6</td>
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<td>8</td>
<td>MLV/SIN (less than 2x10^3)</td>
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5.2.2 Introduction of RRV and SFV envelope glycoproteins into STAR cells

The RRV and SFV expression plasmids pRRV-E2E1A and pSFV-E2E1A, which contain the zeocin resistance gene Sh ble were transfected into the HIV pre-packaging cell line, STAR (Ikeda et al., 2003). Transfected cells were cultured in zeocin for 2 weeks and zeocin resistant colonies, 12 from pRRV-E2E1A transfected cells and 12 from pSFV-E2E1A transfected cells, were isolated. These clonal cells were then tested for virus production by introducing into them the HIV vector HV (Ikeda et al., 2003). The HV vector was introduced into each clone by infection with HIV(VSV-G) virus carrying HV at an MOI of 30 as determined by infection of 293T cells. After insertion of the HV genome the clones were cultured for 48 hours in DMEM + 10% FCS. The clonal cells were then replated and incubated for a further 48 hours in OptiMEM. Viral titre was determined by titration on 293T cells as above.

Two clones from the pRRV-E2E1A transfected cells (clones 2 and 10) were found to produce virus above the level of detection by FACS (4 x 10^2 iu/ml). In this experiment both clones 2 and 10 produced virus at a titre of 2 x 10^2 iu/ml on 293T cells. None of the clones from the pSFV-E2E1A transfected cells produced virus above the level of detection by FACS (4 x 10^2 iu/ml). The two HIV(RRV) producing clones were named STAR-RRVc2 and STAR-RRVc10.

STAR-RRV cell lysates were tested for envelope glycoprotein expression by western blot using the anti-SFV sera mentioned earlier. The lysates of three zeocin resistant clones from the cells transfected with pSFV-E2E1A were also tested (Figure 5.3). Envelope glycoprotein expression could be observed in both STAR-RRV clones (lanes 5 and 6) but envelope glycoprotein expression could not be observed in the lysates of clones 3, 4 and 5 from pSFV-E2E1A transfected cells (lanes 1, 2 and 3). It is, therefore, possible that few or no HIV(SFV) pseudotypes were produced from STAR cells due to little or no SFV envelope expression. Why this should be is unknown.

As it was not possible to confirm envelope identity with the anti-SFV serum, primers specific to the RRV envelope sequence were designed to confirm by sequencing the presence of the RRV envelope sequence in the STAR-RRV cells. Lysates of both
Figure 5.3 Expression of alphavirus envelopes in STAR cells

2 x 10^6 of each STAR-RRV clone was lysed in 500 µl of RIPA buffer, as were three SFV clones. 15 µl of each sample was analysed by western blot. Proteins were separated using 10% polyacrylamide gels. After transfer membranes were probed with a goat polyclonal serum raised against the SFV envelope and a 1:1 mixture of murine monoclonal antibodies ADP365 and ADP366 raised against HIV CA (p24). The positions of protein markers (in kDa) are shown.
STAR-RRVc2 and STAR-RRVc10 were prepared and the PCR product obtained from each lysate was subcloned into pGEM-T Easy (Promega). Both inserts were sequenced in both directions. The nucleotide sequences of the PCR products were found to be complementary and the sequence products from both STAR-RRVc2 and STAR-RRVc10 were identical. Both sequence products were that of the RRV envelope.

5.2.3 Infection of tissue culture cell lines with virus from STAR-RRV cells

To determine what effect, if any, the use of centrifugal inoculation, or "spinoculation", and polybrene have on HIV(RRV) infection of tissue culture cell lines the titration of virus on 293T cells was carried out with or without spinoculation and in the presence and absence of polybrene. HIV(RRV) and HIV(MLV-A) pseudotypes were harvested from STAR cells in OptiMEM. HIV(VSV-G) carrying the HV vector was also included in this experiment. This pseudotype was produced, as above, by transient transfection of 293T cells. The results from this experiment are shown in Figure 5.4.

As before spinoculation had little effect on HIV(VSV-G) titre, while the effects of polybrene and spinoculation on HIV(MLV-A) titre appeared to be additive (Figure 4.7). The presence of polybrene enhanced HIV(RRV) infection on 293T cells 4-6 fold, whereas spinoculation had no effect on HIV(RRV) titre.

Titrations were then carried out on several tissue culture cell lines. Virus was harvested in OptiMEM from STAR-RRV cells plus STAR cells producing HIV(MLV-A) and titrated onto a panel of human tissue culture cell lines and the murine cell line NIH 3T3 in the presence of polybrene (Figure 5.5). HIV(RRV) could infect each cell line with similar efficiency.
Figure 5.4 Use of polybrene and spinoculation to increase HIV(RRV) titre

MLV-A and RRV pseudotypes were harvested from STAR cells in OptiMEM. HIV(VSV-G) was produced by transient transfection of 293T cells. Titration of the virus was carried out on 293T cells in the presence of polybrene or with spinoculation or both. Data is represented as the titre compared to titre of virus plated without polybrene or spinoculation.
Figure 5.5 Titration of HIV(RRV) on tissue culture cell lines

MLV-A and RRV pseudotypes were harvested from STAR cells in OptiMEM. Titrations were carried out in the presence of polybrene. The minimum level of detection by FACS (4x10^2 iu/ml) is indicated with an arrow.
5.2.4 Stability of HIV(RRV) pseudotypes in human sera

The ability of a pseudotyped virus to resist inactivation in human sera is a useful characteristic for \textit{in vivo} use. The ability of HIV(RRV) to resist inactivation in human sera was then examined. HIV(RRV) and HIV(MLV-A) virions were harvested from STAR cells in OptiMEM. HIV(VSV-G), also harvested in OptiMEM, was produced by three plasmid transfection of 293T cells as before. Each pseudotype was mixed at a ratio of 1:1 with heat inactivated FCS and fresh or heat inactivated human sera and incubated at 37°C for 1 hour. After incubation each virus was titred on 293T cells in the presence of polybrene. The results of this experiment, expressed as a percentage of virus titre upon incubation with heat inactivated FCS, are shown in Figure 4.6.

These data would indicate that a non-heat labile component of human sera reduces HIV(RRV) but not HIV(VSV-G) or HIV(MLV-A) titre, although there is little further inactivation of HIV(RRV) virions in fresh human sera. Consistent with previous observations (Figure 3.12), HIV pseudotyped with the VSV-G envelope glycoprotein shows poor stability in fresh human sera compared to HIV(MLV-A).

What non-heat labile component of human sera decreases HIV(RRV) titre has yet to be identified, however, given that there is little inactivation in fresh human sera it does not appear as if HIV(RRV) is susceptible to inactivation by heat labile components of human serum such as complement.
Figure 5.6 Inactivation of HIV(RRV) pseudotypes in fresh human sera

MLV-A and RRV pseudotypes were harvested from STAR cells in OptiMEM. HIV(VSV-G) was produced by transient transfection of 293T cells. Each virus was incubated (37°C for 1 hour) 1:1 with heat inactivated FCS and fresh or heat inactivated human sera. Titrations were carried out on 293T cells in the presence of polybrene. Titre after incubation with fresh or heat inactivated human sera is represented as a percentage of the viral titre after incubation with heat inactivated FCS.
5.2.5 Stability of HIV pseudotyped with the RRV envelope glycoprotein at 37°C

Another characteristic that is advantageous for the *ex vivo* and *in vivo* use of RRV pseudotyped HIV vectors is good stability at 37°C. HIV(RRV) and HIV(MLV-A) pseudotypes were harvested from STAR cells in OptiMEM or DMEM + 10% FCS. HIV(VSV-G) was produced by transient transfection as above and was also harvested in OptiMEM or DMEM + 10% FCS. Each pseudotype was incubated at 37°C for 1, 3 or 6 hours. Viral titre at these time points as well as virus titre before incubation are shown in Figure 5.7.

HIV(RRV) harvested in OptiMEM showed similar stability to HIV(VSV-G) and HIV(MLV-A). It was, however, notable that HIV(RRV) harvested in DMEM +10% FCS is highly unstable at 37°C compared to that harvested in OptiMEM. Furthermore, there was a significant difference in starting titre between the two HIV(RRV) harvests. The results from this experiment, therefore, indicate although HIV(RRV) harvested from STAR-RRV cells appears to be quite stable at 37°C, the media this pseudotype is harvested in significantly affects its stability.

If HIV(RRV) is unstable in the presence of FCS it may be of interest to repeat the experiment shown in Figure 5.6 showing virus titre relative to incubation in OptiMEM, therefore indicating loss of HIV(RRV) titre in FCS and human sera.

5.2.6 Ability of HIV pseudotyped with the RRV envelope glycoprotein to resist inactivation during freeze/thaw cycling

Virus stocks must be frozen until required, therefore, as a practical necessity it is important that a pseudotyped vector be able to resist inactivation during freezing and thawing. Again, HIV(RRV) and HIV(MLV-A) were harvested from STAR cells in OptiMEM or DMEM + 10% FCS. HIV(VSV-G) was produced by transient transfection and was also harvested in OptiMEM or DMEM + 10% FCS. Each pseudotype was subjected to three rounds of freeze/thaw cycling between −80°C and 37°C. The resulting titers from this treatment, expressed as a percentage of virus titre before freezing, are shown in Figure 5.8.
Figure 5.7 Stability of HIV(RRV) at 37 °C

MLV-A and RRV pseudotypes were harvested from STAR cells in either OptiMEM (O) or DMEM + 10% FCS (D+F). HIV(VSV-G) was produced by transient transfection of 293T cells. Each virus was incubated at 37 °C for the indicated time and then titrated on 293T cells in the presence of polybrene. The minimum level of detection by FACS (2x10^3 IU/ml) is indicated with an arrow and data points at this level represent virus titre under this limit.
MLV-A and RRV pseudotypes were harvested from STAR cells in either OptiMEM or DMEM + 10% FCS. HIV(VSV-G) was produced by transient transfection of 293T cells. Each virus was cycled between 37°C and −80°C. The number of cycles is indicated on the x-axis. Data is represented as a percentage of viral titre before the first cycle. Each titration was performed in the presence of polybrene. During the third cycle titre of HIV(RRV) harvested in DMEM + 10% FCS dropped below the level of detection by FACS (2x10^3 iu/ml). These data points are marked *. 

Figure 5.8 Ability of HIV(RRV) to resist inactivation by freeze/thaw cycling
The resistance of HIV(RRV) harvested in OptiMEM to freeze/thaw was similar to HIV(MLV-A). In this experiment the titre of these pseudotypes does not fall below 50% of the original titre until after the second cycle. Furthermore, the resistance to freeze/thaw of HIV(RRV) harvested in DMEM +10% FCS is poor compared to that harvested in OptiMEM. Therefore, in this experiment HIV(VSV-G) is unstable in OptiMEM, while HIV(RRV) is unstable in DMEM + 10% FCS.

In summary, HIV(RRV) virions show good resistance to inactivation in this experiment, but again which media these virions are harvested in is important for their stability.

5.2.7 Stability of pseudotype production from STAR-RRV cells

While STAR cells can stably produce HIV vectors pseudotyped with gamma-retroviral envelopes over several months (Ikeda et al., 2003), a decline in vector titre from both STAR-RRVc2 and STAR-RRVc10 cells was observed (Table 5.2). After two months in culture, no virus of titre above the level of detection by FACS (4 x10^2 iu/ml on 293T cells) was produced from either STAR-RRV cell clone.

To determine what might have caused the observed loss of titre, envelope and HIV capsid (p24) levels in the STAR-RRV cells kept in culture over two months were compared to the samples shown in Figure 5.3 (Figure 5.9).

The levels of RRV envelope in both clones had decreased significantly. This loss of envelope alone could, therefore, be responsible for the observed drop in titre. Furthermore, in the STAR-RRVc2 samples similar amounts of p24 were observed but in the STAR-RRVc10 samples the level of p24 had significantly decreased. Why this should be is unknown.
<table>
<thead>
<tr>
<th>Days in culture</th>
<th>STAR-RRVc2 titre</th>
<th>STAR-RRVc10 titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>$3.3 \times 10^5$</td>
<td>$5.8 \times 10^5$</td>
</tr>
<tr>
<td>24</td>
<td>$4.2 \times 10^5$</td>
<td>$2.0 \times 10^5$</td>
</tr>
<tr>
<td>57</td>
<td>$2.5 \times 10^3$</td>
<td>ND</td>
</tr>
<tr>
<td>68</td>
<td>Below $4 \times 10^2$</td>
<td>Below $4 \times 10^2$</td>
</tr>
</tbody>
</table>

Table 5.2 Loss of titre from STAR-RRV cells

STAR cells transfected with the plasmid pRRV-E1E2A were selected in Zeocin. Zeocin resistant clones were selected (Day 0) and expanded. The titre of virus produced from clones STAR-RRVc2 and STAR-RRVc10 was assayed at certain time points by titration of virus supernatant on 293T cells in the presence of polybrene from cells seeded in a 6 well dish.
Figure 5.9 Western blot analysis of RRV envelope expression

After two months in culture no detectable virus could be harvested from either of the STAR cell clones. 2 x 10^6 cells of each clone were lysed in RIPA buffer and run on a 10% polyacrylamide gel with the samples shown in Figure 4.3. Equal loading was confirmed by staining the membrane with Ponceau S. After transfer membranes were probed with a rabbit polyclonal serum raised against the SFV envelope and a 1:1 mixture of murine monoclonal antibodies ADP365 and ADP366 raised against HIV CA (p24). The positions of protein markers (in kDa) are shown.
5.3 Discussion

In this study pseudotyping of HIV based vectors with envelope glycoproteins from members of the alphavirus family was examined. Transient virus production experiments indicated that it was possible to pseudotype, to some degree, both HIV and MLV virions with the envelope glycoproteins from Ross River virus and Semliki Forest virus. However, in the experiments presented here no pseudotyping at detectable levels of either HIV or MLV virions was observed using the plasmid for the envelope glycoprotein from Sindbis virus. Why this should be remains unknown.

The RRV envelope was then introduced into STAR cells and the HIV(RRV) virions produced from these cells were then characterized with regard to their usefulness in future ex vivo and in vivo experiments. Virus production from these cells was possible only in the short term. It was thought that the loss of RRV envelope expression observed over time in STAR-RRV cells was responsible for the loss of pseudotype production from these cells.

5.3.1 Pseudotyping of HIV and MLV virions with alphavirus envelope glycoproteins

Transient virus production experiments carried out in this study indicate that both HIV and MLV virions can be pseudotyped with the envelope glycoproteins from RRV and SFV although these pseudotypes were of lower titre compared to HIV(VSV-G) (Figure 5.1). If indeed alphavirus do not predominantly bud from lipid rafts (Scheiffele et al., 1999) this data would suggest that either over expression of the envelope during pseudotype production allows significant amounts of the envelope to accumulate in lipid rafts for incorporation into virions or HIV and MLV vectors bud from areas other than lipid rafts. Analysis of the lipid content of the pseudotyped virions such as that preformed by Scheiffele and co-workers (Scheiffele et al., 1999) would confirm if the pseudotypes virions are indeed budding from lipid rafts.

As noted above, there is no data to show that the Sindbis envelope is actually expressed during transient virus production, therefore, little comment can be made
concerning the lack of observable HIV or MLV pseudotyping with the Sindbis envelope. It must be noted that these results are different from those of Morizono and co-workers (Morizono et al., 2001) who show that pseudotyping of HIV with the Sindbis envelope is possible. In this study HIV(SIN) titre on 293T cells was repeatably in excess of $10^5$ iu/ml. The envelope sequence used in this study was taken from the Sindbis virus infectious clone TOTO2000, a variant of TOTO1000 (Rice et al., 1987). It has not been possible to obtain the envelope sequence of TOTO2000, therefore, it has not been possible to deduce what change or changes in amino acid sequence might prohibit the pseudotyping of HIV and MLV vectors with the envelope used here.

5.3.2 Characterisation of HIV(RRV) pseudotypes

As outlined above, HIV(RRV) pseudotypes display a number of characteristics which would be advantageous in future *ex vivo* and *in vivo* experiments. In comparison to HIV(VSV-G) and HIV(MLV-A) virions HIV(RRV) pseudotypes appear to be resistant to inactivation by heat-labile components in human sera and exhibit good stability at 37°C (Figures 5.6 and 5.7). Furthermore, this pseudotype shows good resistance to inactivation during freeze/thaw cycling (Figure 5.8).

Analysis of the ability of HIV(RRV) to resist inactivation in human sera reveals that incubation with heat-inactivated human sera lowers HIV(RRV) titre to a degree. This would indicate that a non-heat labile component or components of the human sera are responsible for the observed decrease in titre. This raises the possibility that some neutralizing antibody response is in operation.

Furthermore, there was a notable drop in titre when HIV(RRV) was harvested from STAR-RRV cells in DMEM + 10% FCS compared to harvesting in OptiMEM. Why this should be is unknown but this question could be first addressed by harvesting HIV(RRV) from STAR-RRV in DMEM only or incubating HIV(RRV) harvested in OptiMEM with increasing concentrations of FCS. It is equally possible that this observation is the result of harvesting HIV(RRV) pseudotypes from STAR cells. Comparing the titre of transiently produced HIV(RRV) harvested in DMEM + 10% FCS versus that from STAR-RRV cells should resolve this issue.
5.3.3 Loss of pseudotype production from STAR-RRV cells

Although HIV(RRV) virions produced from STAR-RRV cells do show characteristics that would be advantageous in future *ex vivo* and *in vivo* experiments, it was not possible in these experiments to produce pseudotypes from stable HIV packaging cells in the long term.

After a period of two months in culture a considerable drop in pseudotype production, attributable to loss of RRV envelope expression, was observed (Figure 5.9). Why this should be is unclear. The plasmid RRV envelope expression plasmid pRRV-E2E1A has been used previously in gammaretrovirus packaging cells to produce MLV(RRV) pseudotypes (Sharkey *et al.*, 2001). In this study it was stated that virus production remains constant for in excess of two months.

When establishing new STAR-RRV or STAR-SFV cells it would be wise to, firstly, screen more envelope-stable clones and, secondly, consider the subcloning of the alphaviral envelopes into the expression vectors described in Ikeda *et al.* (Ikeda *et al.*, 2003) that have been successfully used for the long term expression of gammaretrovirus envelopes in STAR cells. Also, it may be advantageous to attempt the establishment of HIV(RRV) producer cells with HT-STAR cells. Moreover, after selection of zeocin resistant colonies STAR-RRV cells were not maintained with zeocin. Culturing STAR-RRV cells with zeocin in future experiments may prevent the loss of titre observed here.

In summary, HIV and MLV virions can be pseudotyped with the envelope glycoproteins from RRV and SFV. Establishment of stable HIV packaging lines producing HIV(RRV) appears to be possible in the short-term at least. Importantly though HIV(RRV) virions derived from STAR cells display characteristics, such as resistance to inactivation in fresh human sera and good stability at 37°C, that would be advantageous in future *ex vivo* and *in vivo* experiments.
Chapter 6
Transduction of primary human cell cultures with pseudotyped HIV vectors

6.1 Introduction

It is widely agreed that modification of one cell type in particular, the dendritic cell (DC), is the key to the development of many novel therapeutic strategies for conditions such as cancer, allograft rejection, autoimmune disease and infectious disease.

Dendritic cells are a group of migratory bone-marrow derived leukocytes that specialise in the uptake, transport, processing and presentation of antigens to T lymphocytes. Briefly, the mechanism of antigen presentation involves phagocytosis of antigens from the extracellular environment, processing of antigens into peptides, loading of antigen peptides onto MHC molecules and, finally, display of the peptide loaded MHC molecules at the cell surface. In addition to phagocytosed endogenous antigens, molecules produced in DCs themselves can be presented as antigens (endogenous antigens). During processing and presentation DC undergo maturation, changing from so-called immature to mature cells. In general, DCs phagocytosing and processing antigens are termed immature and DCs presenting antigens are mature cells.

Exogenous antigens phagocytosed into the cell are usually presented on MHC class II (MHC II) molecules while endogenous antigens (from self-components or viral infection) are presented on MHC class I (MHC I) molecules. MHC I molecules present antigens to T cells bearing CD8 to stimulate a cytotoxic T cell response, while MHC II molecules present antigen to CD4 T cells, which in turn stimulate antibody producing B cells (den Haan, Lehar, and Bevan, 2000; Steinman, 1991). DCs also have the capability to “cross-present” exogenous antigens on MHC I molecules, thus generating cytotoxic T cell responses to viruses that do not infect antigen presenting cells (den Haan, Lehar, and Bevan, 2000). Therefore, depending on the antigen and
cellular microenvironment, DCs can promote the development of either cell-mediated (Th1) or antibody-dependent (Th2) immune responses. Modified DC presenting specific antigens to T cells, therefore, have the potential to be used in any number of gene therapy protocols that rely on modulation of the immune system.

Excellent examples of the potential of modified DC to promote an immune response can be found in cancer gene therapy. Commonly used approaches to "loading" DCs with tumour antigens involve incubation of DCs with synthetic peptides (Celluzzi et al., 1996; Nestle et al., 1998), purified protein (Paglia et al., 1996) or tumour cell lysate (Nair et al., 1997; Nestle et al., 1998). Using such methods, therapeutic anti-tumour immune responses can be generated and several clinical trials are now underway to test the safety and efficacy of these approaches.

Perhaps a more efficient approach to presenting tumour specific antigens on DCs would be expression of genes encoding tumour specific antigens in DCs. Delivery of sequences encoding tumour specific antigens into DCs can be achieved using a viral vector.

Induction of therapeutic anti-tumour immunity has been possible in a mouse models using an MLV vector carrying a model antigen (De Veerman et al., 1999; Specht et al., 1997). In this case, haematopoietic progenitor cells (CD34+ cells) were stimulated to divide, transduced with MLV and then differentiated into DCs. Such progenitor cells are not, however, found in large quantities in peripheral blood. Obtaining sufficient quantities of these cells for ex vivo transduction is, therefore, problematic. Monocytes, found in some quantity in peripheral blood, can be differentiated in dendritic cells. Monocytes and monocyte-derived DCs are non-dividing and, therefore, refractory to MLV infection. Vectors based on HIV can infect human monocyte-derived DCs (Chinnasamy et al., 2000b; Neil et al., 2001). Moreover, it has been shown that HIV-carrying genomes encoding model antigens can transduce murine DC, which in turn can elicit potent anti-tumour immune responses in vivo (Firat et al., 2002; Metharom et al., 2001).
In contrast to strategies that require the modification of DCs to promote potent immune responses to antigens, there is also much interest in the modification of DCs to prevent an immune response, tolerising the immune system to prevent allograft rejection and autoimmune disease.

Tolerance is a fundamental property of the immune system and dictates selective unresponsiveness to self-antigens. It is thought that induction of tolerance can be brought about with the down-regulation from the DC cell surface of either MHC molecules or molecules that are required for the stimulation of naive T cells during interactions with MHC molecules. A second approach is to engineer DC to produce immunosuppressive molecules.

This area of gene therapy is still very much in its infancy and there are, as yet, no examples of gene transfer to induce DC-mediated tolerance using a viral vector. Like therapeutic strategies that require the induction of immune responses to specific antigens, the potential use of this concept will rely on the efficiency with which viral vectors can transduce DCs. Gene transfer into DC could, of course, also be used for the eradication of intracellular parasites that replicate in DC. Examples of such organisms include HIV (Patterson and Knight, 1987), HSV-1 (Coffin et al., 1998) and mycobacterium tuberculosis (Tailleux et al., 2003).

To date, the VSV-G glycoprotein has been used almost exclusively to pseudotype HIV vectors for the transduction of monocyte-derived DCs. It remains unknown if any other HIV pseudotype transduces monocyte-derived DC with greater efficiency. The primary goal, therefore, of this study is to transduce human primary cell cultures of DC with a range of HIV vector pseudotypes to determine which is optimal in this regard.
6.2 Results

6.2.1 Infection of monocyte-derived CD1a positive dendritic cells with HIV(VSV-G)

Accurate analysis of pseudotype infection of primary cell cultures relies on the ability to differentiate certain cell types from others in a heterogeneous population. Standard in vitro preparations of human dendritic cells are routinely contaminated with high numbers of B and T lymphocytes. B and T lymphocytes are, therefore, removed from these cultures using antibodies recognising the pan-lymphocyte markers CD2 and CD3 and the pan-B cell marker CD19. During analysis of DC infection by FACS, cells expressing eGFP can be stained with antibodies against common surface markers conjugated to chromophores with a different florescence emission to eGFP.

Unlike many other human primary cell cultures populations of DCs are themselves highly heterogeneous, therefore, in the past identification of true DCs using one cell surface marker alone can be difficult. Indeed, by strict definition cells are said to be dendritic cells on the basis of (i) morphology (large, dendritic or irregular in shape, non-adherent), (ii) function (ability to stimulate T proliferation), (iii) the presence of specific cell surface markers (such as MHC molecules and the co-stimulatory molecules CD40 and CD86) and (iv) the absence of specific cell surface markers (CD2, CD3, CD14 and CD19). It is now well established that peripheral blood monocytes stimulated to differentiate without proliferation in the presence of IL-4 and GM-CSF become immature DCs expressing the cell surface marker CD1a (Sallusto and Lanzavecchia, 1994). Identification of HIV vector transduced monocyte-derived dendritic cells using CD1a has been successful in previous experiments by other authors (Granelli-Piperno et al., 2000; Neil et al., 2001), therefore it was decided to use CD1a to identify monocyte-derived dendritic cells in this study.

To ascertain that infection of CD1a positive cells was possible under the experimental conditions used here human monocytes were separated from peripheral blood, cultured with IL-4 and GM-CSF, purified of T and B cells as detailed above and finally infected with HIV(VSV-G) virus. HIV(VSV-G) was produced by transient
transfection of 293T cells with the plasmids pHCMVΔR8.91, pHR'SINcPPT-SE and pMD-G and harvested in OptiMEM. After concentration by ultracentrifugation virus was titrated on 293T cells and plated onto the purified preparation of dendritic cells at a MOI of 10. After infection both infected and uninfected cell cultures were stained with anti-CD1a antibodies and the appropriate isotype control antibody.

Shown in Figure 6.1 is the methodology used to identify CD1a positive cells. Panel A in the Figure shows the populations of cells in a dendritic cell culture as a FSC/SSC FACS dot plot. In Panel C cells stained with the anti-CD1a antibody shift to the right compared to their counterparts stained with the isotype control in Panel B. The region where cells deemed positive for CD1a was gated (the region R1 in panels B and C). The position of this gated population in a FACS scatter plot is shown in panel D. Note that compared with Panel E there are distinct CD1a positive and CD1a negative populations in the cell culture. The CD1a negative population is most likely contaminating T and B cells and/or dead and dying CD1a positive cells. CD1a staining using this methodology can identify where DCs are found in a FSC/SSC plot (Panel E). All the cells that reflect the morphology of CD1a positive cells in a FSC/SSC plot were then analysed for expression of eGFP (Figure 6.2).
Figure 6.1 Determination of CD1a positive cells in a dendritic cell culture

See section 6.2.1 for details.
Gating as in Figure 6.1.E indicates that at least 80% of cells in this population are DCs expressing CD1a (Figure 6.2). Furthermore, analysis of the population that reflect the morphology of CD1a positive cells indicates that while infection of CD1a positive cells is possible under these experimental conditions, increased viral MOI may be necessary to infect a significant number of cells (Figure 6.2). Moreover, 92% of all cells infected in the analysed population were CD1a positive. In future experiments it may, therefore, be as informative to analyse infection without staining infected cells for the presence of CD1a.

6.2.2 Infection of primary cell cultures with virus harvested from STAR cells

In order to assess what HIV pseudotype is optimal for the infection of primary cell cultures of DCs, DCs were prepared from the peripheral blood of three donors and exposed to HIV pseudotyped with either VSV-G or a gammaretroviral envelope. Virus from STAR cells was used as the titre of pseudotypes produced by transient transfection was found to be highly variable between preparations. T lymphocyte cultures from the same donors were infected in parallel. The T lymphocytes from each donor were stimulated with Concanavalin A. This is necessary as progression through the cell cycle is required for efficient HIV reverse transcription in T lymphocytes (Korin and Zack, 1998; Korin and Zack, 1999).

HIV(VSV-G) pseudotypes were generated by transfection of the VSV-G expression plasmid pMD-G into STAR cells containing the vector genome HV. Gammaretroviral pseudotypes were harvested from STAR cells as before. After harvest in OptiMEM, each pseudotype was concentrated by ultracentrifugation and the concentration of HIV CA (p24) in each virus preparation was assayed by ELISA. The concentration of p24 in each preparation was corrected to 10 μg/ml with OptiMEM and the DC and T lymphocytes from each donor were inoculated with 1 μg of p24 from each virus preparation.
Figure 6.2 Infection of CD1a positive cells with HIV(VSV-G)

Human monocytes were obtained from peripheral blood and cultured for 5 days with IL-4 and GM-CSF. After removal of the culture of cells expressing the markers CD2, CD3 and CD19, DCs were infected with virus harvested in OptiMEM and cultured for a further 7 days. As a negative control DCs were exposed to OptiMEM containing no virus. These are termed uninfected. Cells were then stained with the indicated antibodies and the appropriate isotype control and passed through a FACS.

The population examined for infection was determined by analysis of anti-CD1a staining of uninfected cells and can be seen in Figure 6.1, Panel E. Panel A in Figure 6.2 indicates the population of cells analysed with the isotype control. Panel B shows uninfected cells stained for the CD1a antigen and Panel C shows infected cells stained for the CD1a antigen.

The percentage of cells found in each quadrant of the FACS dot plot is indicated to the right of each panel.
Uninfected/Isotype stained

Uninfected/CD1a stained

HIV(VSV-G) infected (MOI=10)/CD1a stained
Table 6.1 Titration of pseudotypes harvested from STAR cells on 293T and TE671 cells in the presence and absence of polybrene

Virus was harvested in OptiMEM and concentrated by ultracentrifugation. The concentration of HIV CA (p24) in each stock was corrected to 10 µg/ml. Virus was titrated in parallel onto 293T and TE671 cells in the presence and absence of 8 µg/ml polybrene (PB). The same stocks were used for infection of primary cell cultures (Tables 6.2 and 6.3).

<table>
<thead>
<tr>
<th>Pseudotype</th>
<th>293T (+ PB)</th>
<th>293T (- PB)</th>
<th>TE671 (+ PB)</th>
<th>TE671 (- PB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV-G</td>
<td>17.8</td>
<td>13.9</td>
<td>7.1</td>
<td>4.7</td>
</tr>
<tr>
<td>MLV-A</td>
<td>25.8</td>
<td>5.7</td>
<td>16.4</td>
<td>2.1</td>
</tr>
<tr>
<td>GALV/MLV</td>
<td>29.9</td>
<td>9.8</td>
<td>13.7</td>
<td>3.3</td>
</tr>
<tr>
<td>RD114/MLV</td>
<td>42.7</td>
<td>22.9</td>
<td>21.7</td>
<td>6.3</td>
</tr>
<tr>
<td>RD114-PR</td>
<td>51.9</td>
<td>24.9</td>
<td>28.5</td>
<td>11.7</td>
</tr>
</tbody>
</table>
Each virus stock was titrated in parallel on 293T and TE671 cells with or without polybrene. The results from these titrations can be seen in Table 6.1.

Infected DC were stained with the anti-CD1a and isotype control antibodies as before and analysed by FACS for the percentage of cells in each culture positive for CD1a and expressing eGFP. The results from this experiment are shown in Table 6.2. In no cell culture could significant pseudotype infection be found.

With regard to the infection of T lymphocytes, uninfected T lymphocyte cultures were stained with anti-CD3 antibodies and the appropriate isotype control. The population of cells that reflect the morphology of CD3 positive lymphocytes was analysed in each experiment. As shown in Figure 6.3 in each population analysed contains mostly CD3 positive cells. Analysis of the infected T cell cultures reveals that, although there are significant differences in infection between the different pseudotypes there is little consistency in infection between donors (Table 6.3). Representative FACS data from this experiment is shown in Figure 6.4.

The results from these experiments were unexpected. Efficient infection of DC under the experimental conditions used here has been reported with HIV(VSV-G) virus (Neil et al., 2001). Even allowing for differences in donors it would be expected that infection with HIV(VSV-G) would be as efficient as the level of infection seen in Figure 6.2. Furthermore, although the T lymphocyte cultures could be infected to some degree several reports state that more efficient infection of T lymphocytes is possible with HIV(VSV-G) under similar experimental conditions (Chinnasamy et al., 2000a; Douglas et al., 1999; Ducrey-Rundquist, Guyader, and Trono, 2002). Indeed, it is possible to transduce unstimulated T lymphocytes using HIV(VSV-G) with higher efficiency than that seen here (Costello et al., 2000).
Table 6.2 Percentage of CD1a positive cells infected by pseudotypes harvested from STAR cells

<table>
<thead>
<tr>
<th>Pseudotype</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>0.34</td>
<td>0.52</td>
<td>0.07</td>
</tr>
<tr>
<td>VSV-G</td>
<td>0.62</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>MLV-A</td>
<td>0.20</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>GALV/MLV</td>
<td>0.21</td>
<td>0.36</td>
<td>0.07</td>
</tr>
<tr>
<td>RD114/MLV</td>
<td>0.49</td>
<td>0.18</td>
<td>0.09</td>
</tr>
<tr>
<td>RD114-PR</td>
<td>0.41</td>
<td>0.45</td>
<td>0.08</td>
</tr>
</tbody>
</table>

5 x 10^4 dendritic cells from three donors were infected with 1 µg of HIV CA (p24) from each virus preparation. Determination of virus titre on 293T cells (Table 6.1) indicates that the DCs were exposed to an MOI of at least 11 in each infection. After 7 days cells infected and uninfected (exposed to OptiMEM only) cells were stained for CD1a with the appropriate isotype controls. Cells were analysed by FACS as in Figures 6.1 and 6.2 to assess the percentage of CD1a positive cells in each infection expressing eGFP. The percentage of cells considered positive for eGFP and CD1a are shown.
Donor 1

85% CD3 positive

Donor 2

96% CD3 positive

Donor 3

90% CD3 positive

Figure 6.3 Analysis of T lymphocyte cultures for CD3 positive cells

Uninfected T lymphocyte cultures were stained for CD3. The FACS profile of CD3 positive cells was ascertained and this population in each culture was analysed for the presence of cells bearing the CD3 antigen. In each of the above histograms the population of cells stained with an isotype control is shown in grey. CD3 positive cells are indicated in region M1. The percentage of cells in M1 is indicated.
Table 6.3 Infection of human T lymphocyte cultures with pseudotypes harvested from STAR cells

5 x 10^4 T lymphocytes from three donors were infected with 1 μg of HIV CA (p24). After 7 days cells infected and uninfected (exposed to OptiMEM only) cells were stained for eGFP expression. Cells were then analysed by FACS as in Figures 6.3. The percentage of eGFP positive cells in each infection minus the percentage of those from analysis of uninfected cells (approximately 0.1%, see Figure 6.4) is shown in the table.

<table>
<thead>
<tr>
<th>Pseudotype</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV-G</td>
<td>5.19</td>
<td>2.94</td>
<td>5.10</td>
</tr>
<tr>
<td>MLV-A</td>
<td>7.18</td>
<td>1.10</td>
<td>5.07</td>
</tr>
<tr>
<td>GALV/MLV</td>
<td>5.06</td>
<td>11.40</td>
<td>12.60</td>
</tr>
<tr>
<td>RD114-PR</td>
<td>3.22</td>
<td>14.73</td>
<td>9.46</td>
</tr>
</tbody>
</table>

5 x 10⁴ T lymphocytes from three donors were infected with 1 μg of HIV CA (p24). After 7 days cells infected and uninfected (exposed to OptiMEM only) cells were stained for eGFP expression. Cells were then analysed by FACS as in Figures 6.3. The percentage of eGFP positive cells in each infection minus the percentage of those from analysis of uninfected cells (approximately 0.1%, see Figure 6.4) is shown in the table.
Figure 6.4 FACS analyses of infected T lymphocyte cultures

Panel A indicates the population of cells, predominantly CD3 positive, determined by staining of uninfected cells for CD3, using the same methodology for CD1a staining of DCs as described in Figure 6.1. Panel B shows analysis for green florescence of the population taken from panel A. Panel C shows infected cells. All data is taken from infection of cells from Donor 2.
Taken together, these results indicate that the experimental protocol used in these experiments requires optimisation. Given the successful transduction of CD1a positive cells in the preliminary experiment shown in Figure 6.2, it was hypothesised that some component of the virus used in these experiments was at fault. It was speculated that the observed lack of infection was attributable to some problem with the packaging construct, the lack of one or all HIV accessory proteins, or expression of the reporter gene eGFP. It was decided to first assess the expression of eGFP in dendritic cells using a number of different HIV vector constructs.

6.2.3 Expression of eGFP in dendritic cells from different HIV vector genomes

STAR cells contain the HIV vector genome HV. In order to construct HV a reporter gene cassette was taken from pHR'SINcPPT-SE and inserted into the HIV vector genome pH7G, whose LTRs remain intact. The vector genome can, therefore, be repackaged after vector transduction (i.e. not self-inactivating). This reporter gene cassette contains a gene encoding eGFP under the control of a promoter sequence from the spleen focus forming virus (SFFV) LTR with the woodchuck hepatitis posttranscriptional regulatory element (WPRE) encoded in the 3' untranslated region of the RNA transcript encoding eGFP. As DCs expressing eGFP could be visualised in preliminary experiments presented in Figure 6.2 it was reasoned that there may not be a problem with the use of the SFFV promoter in DCs *per se* but rather a problem with the use of the pH7G plasmid backbone.

HIV(VSV-G) virions were transiently produced from 293T cells as before, carrying vector genomes encoded in pHR'SINcPPT-SE, pH7G or pHV. Again these pseudotypes were harvested in OptiMEM. DCs were prepared as before and these cell cultures were infected with each of the viruses. 293T cells were infected in parallel.

The results from these experiments are shown in Figure 6.5. eGFP positive cells can be clearly visualised in all cases, although the mean florescent intensity (MFI) of both 293T cells and dendritic cells infected with virus carrying the HV genome is

186
Dendritic cells and 293T cells were infected in parallel with transiently produced HIV(VSV-G) carrying different vector genomes. Each infection was assayed for eGFP expression by FACS. The percentage of cells infected is indicated, as is the mean florescent intensity (MFI) of those cells. The multiplicity of infection (MOI) of the DC infection, as determined by infection of 293T cells, is also indicated. In the DC infections the population of cells reflecting the morphology of DCs expressing CD1a were analysed.
Uninfected

293T

DC

0.07%
MFI-23.99

0%
MFI-0

HV

MOI-15
4.28%
MFI-367

MOI-7
3.54%
MFI-1884

H7G

7.89%
MFI-843.56

7.86%
MFI-2131.60

HR’SIN

11.76%
MFI-1882.01

MOI-15
7.90%
MFI-2246

MOI-75
25.70%
MFI-2748
noticeably less than that of cells infected with virus carrying either the genomes from pH7G or pHR'SINcPPT-SE. Furthermore, comparison of the mean fluorescent intensity of infected DCs carrying pHR'SINcPPT-SE in Figure 6.2 (MOI-10, 3.17% infected, MFI-40) with this experiment (MOI-15, 7.90% infected, MFI-2246) would indicate that there is considerable variation in transgene expression between donors.

While it would seem that the use of the HIV vector genome HV is perhaps not optimal for transgene expression, the observed infection of DCs, even at relatively low MOI in this experiment, would suggest the lack of infection seen in Table 6.2 is not mainly due to lack of reporter gene expression but lack of vector transduction. This would indicate a possible deficiency in a component of the packaging construct used in STAR cells.

6.2.4 Comparison of different HIV packaging constructs in primary cell cultures

In order to examine infection of primary human cell cultures with virus produced using different Gag-Pol constructs, HIV(VSV-G) was produced by transient transfection of 293T cells using the plasmids pH7G, pMD-G and either pHCMVAR8.91 or pSYNGP that encodes the codon-optimised used in STAR cells. It is necessary to use pH7G in these experiments, as Rev is not encoded in pSYNGP.

These viruses were plated on cultures of DCs and Concanavalin A stimulated T lymphocytes. EGFP expression was assayed by FACS. From this experiment there would appear to be a significant difference in the ability of the virus produced using the codon-optimised Gag-Pol to infect DCs compared to virus produced using the packaging construct pHCMVAR8.91 (Table 6.4.A). Both viruses could, however, infect T lymphocytes with equal efficiency.
Table 6.4 Infection of DCs with pseudotypes assembled using different packaging constructs

5 x 10⁴ DCs were infected with (A) HIV(VSV-G) assembled using either pHCMVA R8.91 (8.9) or pSYNGP (CO) and (B) HIV(MLV-A) from STAR-A or 100R26A cells (MOI indicated above).

The populations of cells that reflect the morphology of CD1a or CD3 expressing cells were analysed. The percentage of cells infected expressing eGFP is indicated. In Table 6.4.B the values indicated are those after the percentage of eGFP positive cells in an uninfected control have been deducted.
To confirm this difference in DC infection DCs were infected with HIV(MLV-A) pseudotypes produced from either STAR cells and a prototype HIV packaging line 100R26. 100R26 cells are also based on 293T and contain a packaging cassette CNC-GPRT (Ikeda et al., 2003) taken from pHCMVΔR8.91 and the vector genome HV. Again, DC infection of virus produced using wild type Gag-Pol was superior to virus produced using the codon-optimised Gag-Pol (Table 6.4.B).

This data would argue that the lack of infection seen in DCs with virus harvested from STAR cells is, in part, due to some facet of the codon-optimised packaging construct used in STAR cells. Furthermore, even allowing for differences in donors, the level of infection seen with the HIV(VSV-G) pseudotype transiently produced using the codon-optimised Gag-Pol (Table 6.4.A) is significantly different from that observed in previous experiments (Tables 6.2 and 6.3). Infection of DCs and T lymphocytes is in general 3-4 fold higher. This may suggest that another factor related to harvesting virus from STAR cells is inhibitory to infection of both DCs and T lymphocytes.
6.3 Discussion

In this study the transduction of primary human cell cultures, in particular dendritic cells, was attempted with different HIV pseudotypes harvested from the HIV packaging cell line STAR. While it was possible to transduce primary cell cultures of human T lymphocytes to some extent, transduction of human dendritic cell cultures with pseudotypes from STAR cells was limited. Ultimately, it was determined that one factor limiting the infection of dendritic cells was the packaging construct used in STAR cells.

6.3.1 Codon-optimisation of HIV Gag-Pol

Retrovirus genomes exhibit in their coding sequences a bias toward gene sequences that are rich in the bases adenine and thymine as opposed to human gene sequences that exhibit a codon-bias to make them rich in the bases guanine and cytosine. Why this should be is as yet unclear but it is speculated that viral systems use the “AT bias” to control translation of their genomes (Haas, Park, and Seed, 1996).

Codon-optimisation of Gag-Pol negates the need for Rev (Kotsopoulou et al., 2000). How the codon-optimised viral mRNA exits the nucleus has yet to be elucidated. A further consequence of codon-optimisation is an increased level of Gag-Pol expression. It is thought that this is due to the removal of AUUUA motifs in the viral mRNA that are known to promote mRNA instability that is countered by the presence of Rev (Mikaelian et al., 1996; Schwartz et al., 1992; Schwartz, Felber, and Pavlakis, 1992). Viral particles produced using codon-optimised Gag-Pol have been used to generate Gag and Pol specific cytotoxic T lymphocyte responses in mice and rhesus macaques (Huang, Kong, and Nabel, 2001; zur Megede et al., 2000). Analysis of virion production in these studies indicated that codon-optimised Gag-Pol could be efficiently processed to its constituents in human cells (Huang, Kong, and Nabel, 2001; zur Megede et al., 2000), plus virions produced from human cells exhibited the expected density in a sucrose gradient (zur Megede et al., 2000). Furthermore, virions synthesised from codon-optimised Gag-Pol in simian cells displayed the correct
Sequences encoding Gag-Pol have been codon-optimised in an HIV packaging construct for the production of pseudotyped vectors (Kotsopoulou et al., 2000). As mentioned, this negates the need for Rev, but compared to packaging constructs containing the wild-type Gag-Pol encoding sequences there was no significant increase in titre. It was, however, proposed that as there is little homology between the codon-optimised gag-pol sequence and any residual viral sequences in the vector construct, therefore there is less chance of recombination between the two that might lead to the production of replication competent virus (Ikeda et al., 2003; Kotsopoulou et al., 2000). In the aforementioned study, Kotsopoulou and co-workers (Kotsopoulou et al., 2000) were able to demonstrate that HIV(VSV-G) pseudotypes produced using codon-optimised Gag-Pol possessed the ability to transduce growth-arrested human tissue culture cell lines, however no human primary cell cultures were tested. Indeed, the data presented here represents the first time vector produced using codon-optimised Gag-Pol have been used for the transduction of primary human cell cultures.

With regard to STAR cells, codon-optimised gag-pol sequences were used firstly to negate the need for Rev but also because the use of this packaging construct increased viral titre significantly compared to the use of the wild-type sequence packaging sequence taken from pHCMVΔR8.91 (Ikeda et al., 2003).

Why virions produced using a codon-optimised packaging sequence should be less efficient in the transduction of human dendritic cells compared to virions produced using a wild type packaging sequence (Table 6.4) is unclear and further investigation is required.

It is possible that the observed difference in infection is due to some problem in the production of the vector, perhaps in some posttranscriptional or posttranslational modification of the codon-optimised gag-pol sequence. Furthermore, there exists the possibility that there is some form of accessory gene expression from the pHCMVΔR8.91 backbone. The strategy employed to remove the sequences encoding accessory
proteins from pHCMVAR8.2 (Naldini et al., 1996), the construct from which pHCMVAR8.91 is derived (Zufferey et al., 1997), leaves some sequence from the HIV accessory genes intact. It is, however, unknown if any accessory gene fragments are expressed and unclear how they might be expressed. Substituting the gag-pol sequence from pHCMVAR8.91 with that from pSYNGP and HXB2, the molecular clone from which the gag-pol sequence in pSYNGP is derived (Kotsopoulou et al., 2000), would be informative. This would indicate if there is some factor limiting DC transduction other than the use of the codon-optimisation of Gag-Pol.

6.3.2. Infection of primary cell culture sub-populations

Analyses of what DC subpopulations can be infected with HIV-based vectors may also help explain the relative inefficiency of HIV vector pseudotype infection seen here and elsewhere. For example, exposure of cells to HIV(VSV-G) lacking any HIV accessory proteins, at high MOI (MOI = 500 as determined by titration on 293T cells) results in the transduction of less than 30% of the cells (Schroers et al., 2000). If only certain subpopulations are susceptible to infection that may explain these data. Indeed, it has been shown that infection of dendritic cells by wild type HIV appears to dependent on the phenotype of the virus and the maturation state of the cells. Firstly, mature DCs are largely refractory to infection by wild type HIV virus as the virus cannot undergo reverse transcription in these cells, while only macrophage tropic HIV virus efficiently replicate well in immature DCs even though both CXCR4 and CCR5 are present on the cell surface (Granelli-Piperno et al., 1998). Secondly, data by Patterson and co-workers indicates that plasmacytoid DCs (found mostly in lymphoid tissue) are more susceptible to wild type HIV infection than myeloid DCs (found mostly in peripheral tissues) (Patterson et al., 2001). The maturity of the cells used in the study by Patterson et al. is, however, unknown (Patterson et al., 2001).

In order to optimise infection of human primary cell cultures with vectors based on HIV it should be informative to determine the maturation and/or activation state of the cells that can be transduced and assess whether different packaging constructs and the presence of accessory proteins alter what populations are susceptible to infection.
Furthermore, efficient HIV vector infection may be dependent on a specific mode of entry into the cytoplasm that can only be mediated by the HIV envelope. It would, therefore, be interesting to assess the ability of HIV vectors pseudotyped with HIV envelopes to transduce DCs compared to HIV(VSV-G) or any other pseudotype used in this thesis.
Chapter 7
Discussion

The aims of this thesis were to understand the mechanisms by which vectors based on HIV can be pseudotyped with heterologous viral envelope glycoproteins, to characterise the properties that these envelopes convey to the virion and examine how efficiently different HIV pseudotypes can transduce clinically relevant human primary cell cultures.

7.1 Pseudotyping of HIV vectors with heterologous viral envelope glycoproteins

While the envelope glycoprotein from the gammaretrovirus MLV-A can efficiently pseudotype HIV vectors, it was found that modifications to the cytoplasmic tails of the envelope glycoproteins from GALV and RD114, predicted to increase R peptide cleavage, must be made for the envelopes to efficiently pseudotype HIV virions. It was concluded that these modifications circumvent whatever elements in the cytoplasmic tails of the GALV and RD114 limit the functional incorporation of these envelopes into the virion. At present, however, the link, if any, between R peptide cleavage and incorporation into the retrovirus virion remains elusive, although the strategies employed here to increase vector titre, substitution of the envelopes cytoplasmic tail for that of MLV-A and modification of the envelopes R peptide cleavage site, should be applicable in other situations where pseudotyping of HIV or MLV vectors with other gammaretrovirus envelopes is inefficient.

Also, it was found that HIV and MLV vectors could be pseudotyped with envelope glycoproteins from members of the alphavirus family. It is, however, as yet unknown how alphavirus envelope glycoproteins are functionally incorporated into retrovirus virions.

As shown by the work presented in this thesis and elsewhere, retrovirus virions can be pseudotyped with glycoproteins from a wide range of virus families. Indeed, it might be possible to pseudotype HIV and MLV vectors with a wider range of envelope
glycoproteins than perhaps was first thought. Recently, it was reported that HIV and MLV virions could be pseudotyped with hepatitis C glycoproteins (Bartosch, Dubuisson, and Cosset, 2003). Unlike HIV and MLV that bud from the plasma membrane, hepatitis C virions bud through the membrane of the Golgi. It was, therefore, speculated that upon overexpression of the envelope glycoprotein, the endoplasmic reticulum becomes saturated and subsequently “leaky”, allowing significant quantities of envelope to travel to the plasma membrane for incorporation into retrovirus virions (Bartosch, Dubuisson, and Cosset, 2003). This work should herald studies using retrovirus pseudotypes bearing the envelope glycoproteins from other viruses that bud through the Golgi membrane, such as members of the Bunyaviridae family (ie Bunyamwera virus, Hantaan virus). These viruses are increasingly recognised as posing a threat to human health.

7.2 Characterisation of pseudotyped HIV vectors for ex vivo and in vivo use

In this thesis a number of experiments were conducted to determine how useful pseudotyped HIV vectors might be in future ex vivo and in vivo experiments. It was interesting to note that virions pseudotyped with envelope glycoproteins from both gammaretroviruses and alphaviruses fair well in human sera and at 37°C. These features alone may warrant the continued development of alphavirus envelope pseudotyped HIV vectors. Furthermore, it would be interesting to perform the same experiments on HIV virions pseudotyped with a more diverse range of envelope glycoproteins. These experiments, particularly incubation in human sera, might also provide valuable data on the in vivo behaviour of some human pathogens. Indeed, a study of MLV-based vectors produced from human cells in primate models has demonstrated that in vivo half-life correlates with in vitro resistance to complement (DePolo et al., 1999).

While it may be tempting to use the envelope glycoproteins from human pathogens for transduction of specific human cell types and tissues, a cautionary note must be added, as the presence of neutralising antibodies against envelope glycoproteins from human pathogens would limit the in vivo use of a vector pseudotyped with such an
envelope. Furthermore, use of a glycoprotein from a human pathogen may have unforeseen detrimental effects to the cells or tissues one might wish to transduce. For example, as mentioned in previous chapters, interaction of the HIV envelope with cell surface molecules can be an apoptotic trigger. Conversely, the use of retrovirus pseudotypes may allow the elucidation of determinants in viral envelope glycoproteins that act as apoptotic triggers and may help uncover cellular pathways activated in response to interactions between viral glycoproteins and cellular molecules at the cell surface.

7.3 Infection of human primary cell cultures with pseudotyped HIV virions

Transduction of human primary cell cultures with HIV pseudotypes generated with wild type Gag-Pol should lead to much data concerning how HIV pseudotype can be used in future pre-clinical and clinical studies. Another, perhaps more intriguing, avenue of research involves the transduction of primary cell cultures with pseudotypes generated using a codon-optimised Gag-Pol. The data presented in this thesis would indicate that the use of the codon-optimised Gag-Pol limits the transduction of human dendritic cells. At present it cannot be said if this is the result of some deficiency in virus assembly in 293T cells, or the infection process in dendritic cells. It will be interesting to investigate what stage of the HIV life cycle the use of codon-optimised Gag-Pol impacts upon. Indeed, experimentation with the codon-optimised Gag-Pol may shed new light on how the HIV lifecycle is viewed in certain cell types.
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