The evolution of novel subgroups of feline leukaemia virus

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Abstract

Feline leukaemia virus is a significant pathogen of domestic cats which causes a range of proliferative and non-proliferative haematopoietic disorders. This virus has been extensively studied in the past, however advancements in molecular techniques now allow long-standing controversial topics to be revisited and reanalysed. Although FeLV-A is the only transmittable form of the virus, FeLV-B and –C may arise in infected cats if the initial virus escapes immune clearance and establishes a chronic infection. These studies aimed to investigate previously-unanswered questions regarding FeLV pathogenesis, specifically pertaining to the ability of FeLV-A to evolve into the novel subgroups B and C.

These results indicate that strains of FeLV-A possessing residues D83 and D91 in their envelope glycoprotein display increased rates of viral replication, mediated by an enhanced interaction with their cognate receptor, THTR1. Evidence is provided that these viral proteins are also able to bind efficiently to the FeLV-C receptor, FLVCR1, and that these mutations represent the first in a step-wise accumulation of mutations which eventually result in a FeLV-C viral variant emerging within the host. Subsequent studies aimed to elucidate the respective roles of the acquired immune response (neutralising antibodies) and receptor availability in driving this evolutionary process; however a definitive conclusion regarding FeLV-C selection pressures was not reached due to limitations of the model.

These studies also describe the first isolation of novel FeLV-B field isolates which present without a FeLV-A co-infection. Characterisation of these strains revealed they possessed recombinant genomes, composed of exogenous LTRs and mostly endogenously-derived *env* genes. Further investigations into the potential functionality of endogenous FeLV elements within the domestic cat genome revealed numerous intact *env* genes, the proviruses of which may be restricted from exogenous transmission by their inability to form homodimeric RNA genomes with functional secondary structures. Although this suggestion requires experimental validation, this represents a novel mechanism of endogenous retroviral restriction.

This thesis is dedicated to my parents, Ian and Joy,
for their unwavering support.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
BFU-E	Burst Forming Units - Erythroid
bp	base pairs
CA	Capsid Protein
cDNA	complementary deoxyribonucleic acid
CFU-E	Colony Forming Units – Erythroid
CFU-GM	Colony Forming Units – Granulocyte-Macrophage
CPE	Cytopathic Effect
CTL	Cytotoxic T-lymphocytes
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
enFeLV	endogenous feline leukaemia virus
env	Envelope-encoding gene
Env	Envelope glycoprotein
ER	Endoplasmic Reticulum
ERV	Endogenous Retrovirus
FcEV	*Felis catus* endogenous virus
FeFV	Feline foamy virus
FeLV	Feline leukaemia virus
FeLV-A	Feline leukaemia virus, subgroup A
FeLV-B	Feline leukaemia virus, subgroup B
FeLV-C	Feline leukaemia virus, subgroup C
FeLV-FAIDS	Feline leukaemia virus-Feline AIDS
FeLV-T	Feline leukaemia virus, subgroup T
FeLIX	Feline leukaemia virus Infectivity Accessory Protein
FeSV	Feline Sarcoma Virus
FIV	Feline Immunodeficiency Virus
FLVCR	Feline Leukaemia Virus Subgroup C Receptor protein
FOCMA	Feline Oncornavirus-Associated Cell Membrane Antigen
gag	Group-specific antigen-encoding gene

Gag	Group-specific antigen protein
HA	Haemagluttinin
HIV	Human Immunodeficiency Virus
IgG	Immunoglobulin class G
IN	Integrase
kB	kilobases
kDa	kilodaltons
LTR	Long Terminal Repeat
MA	Matrix Protein
MAb	Monoclonal Antibody
mRNA	Messenger Ribonucleic Acid
MLV	Murine Leukaemia Virus
NC	Nucleocapsid Protein
ORF	Open Reading Frame
PBS	Primer Binding Site
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pit	Sodium-Phosphate Symporter
PRCA	Pure Red Cell Aplasia
Pro	Protease
pro-pol	Protease-integrase-polymerase- encoding gene
PRR	Proline Rich Region
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SU	Surface Unit domain
THTR1	Thiamine Transporter
TM	Transmembrane domain
USA	United States of America
VNA	Virus Neutralising Antibodies

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Authors Declaration

I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:

Printed name: Hazel Stewart

1. Introduction and Literature Review

Retroviruses are enveloped, single-stranded RNA viruses which, upon infection of the host cell, use a virally-encoded RNA-dependent DNA polymerase to produce a double-stranded DNA copy of their genome. This is integrated into the host DNA, forming the provirus. The provirus is then transcribed and translated as a standard host gene, producing both viral proteins and genomic RNA for viral replication.

Early evidence of retroviral associations with cancer led to their description as *RNA tumour viruses* or *oncoviruses*, however the majority of retroviruses are not oncogenic and this nomenclature is no longer commonly used. Retroviruses were also classified according to their morphology, which led to the A-, B-, C- and D-type particle descriptions found in early studies (Bernhard, 1960). The members of the Retroviridae family are now classified into two subfamilies, the orthoretrovirinae and the spumaretrovirinae. The latter subfamily contains a single genus; the spumaretroviruses (see Section 1.11.2). However there are six genera within the orthoretrovirinae, including the alpha-, beta-, gamma-, delta- and epsilon-retroviruses and lentiviruses. Most of the orthoretroviruses are termed “simple” retroviruses as their genomes contain only essential genes required for viral replication. By contrast, lentiviruses and spumaretroviruses are “complex” retroviruses which contain accessory genes that may allow avoidance of the host innate immune system.

Gammaretroviruses are able to infect an unusually broad range of vertebrates (Gifford & Tristem, 2003) and are associated with leukaemia and lymphoma in cats, pigs, mice, and marsupials. These pathogenic viruses, especially the prototype murine leukaemia virus (MLV), have been widely utilised as models of human retroviral infection and have contributed to the understanding of numerous diseases, including the events leading to cancer progression. The gammaretrovirus feline leukaemia virus (FeLV) was the first feline retrovirus to be described (W. F. Jarrett, Crawford, Martin, & Davie, 1964) and was quickly recognised to be an important pathogen of both domestic and wild cats. Discovered in 1964, it was initially described as C-type viral particles, isolated

from the plasma of cats with both spontaneous and experimentally-transmitted leukaemia (W. F. Jarrett, et al., 1964). It is now known to consist of three major subgroups, FeLV-A, -B, and -C and a fourth, rare variant FeLV–T which differ in their *in vitro* behaviour and pathogenic consequences for the host. Almost fifty years after its discovery, FeLV research has produced both vaccines and treatment for infected cats; it has also been a useful model for human disease research. However there are still many questions regarding FeLV infection, replication and pathogenesis that remain unanswered. This introductory chapter will provide an overview of the current understanding of FeLV infection and disease manifestation, and highlight the areas in which further research is required.

1.1 Introduction to FeLV

1.1.1. Prevalence

FeLV displays a worldwide distribution, although its prevalence varies significantly across geographical regions. On a local scale the rate of infection depends highly upon the size of the feral cat population and the degree of interaction between domestic and feral cats; for example recent studies found 9% of cats in central Italy are infected (Bandecchi, Dell'Omodarme, Magi, Palamidessi, & Prati, 2006) compared to 4% in Germany where the cats studied were mainly indoor pets in urban areas (Gleich, Krieger, & Hartmann, 2009). Early epidemiological studies found 5% of healthy cats and 18% of sick cats within the UK were FeLV-positive (Hosie, Robertson, & Jarrett, 1989), which were similar statistics to those determined in the USA (J. K. Levy, Scott, Lachtara, & Crawford, 2006; Shelton, Waltier, Connor, & Grant, 1989). In Australia 25% of clinically ill domestic cats were found to be FeLV-positive, a proportion which has not been observed elsewhere (Sabine, Michelsen, Thomas, & Zheng, 1988). In mainland Europe infection rates ranged between 1 to 10% (Moraillon, 1990; Sukura, Salminen, & Lindberg, 1992; Ueland & Lutz, 1992).

Recent studies from both Canada (Little, Sears, Lachtara, & Bienzle, 2009) and Central America (Guatemala and Costa Rica) (Blanco, Prendas, Cortes, Jimenez,

& Dolz, 2009; Coelho et al., 2008; Lickey, Kennedy, Patton, & Ramsay, 2005) indicate this rate of infection has remained constant in some areas. However its European prevalence has drastically decreased in recent years as a result of wide-spread vaccination and pet-owner awareness schemes (J. Levy et al., 2008), decreasing to as low as 1% of healthy cats in some regions (Juvet, Brennan, & Mooney, 2011). The positive effects these programs have are apparent when comparing these infection rates to countries where vaccines are not commercially available; for example Iran where 15% of cats remain positive for FeLV (Akhtardanesh, Ziaali, Sharifi, & Rezaei, 2010).

FeLV is also a major threat to both the highly endangered Iberian lynx (*Lynx pardinus*) and Florida panthers (*Puma concolor coryi*), as it causes severe clinical symptoms and high mortality in these felids (M. A. Brown et al., 2008; Meli et al., 2010; Meli et al., 2009). Infection has also been found in ocelot (*Leopardus pardalis)*, puma (*Puma concolor*) and oncilla (*Leopardus tigrinus*) in Brazil although clinical symptoms were not observed in these species (Guimaraes et al., 2009). Within the UK, approximately 10% of Scottish wildcats (a threatened species) are infected with FeLV (Daniels, Golder, Jarrett, & MacDonald, 1999). Therefore FeLV remains a significant threat to both domestic and wild cats despite the decrease in prevalence since its initial discovery.

1.1.2. Transmission

Initial FeLV infection occurs in the oropharynx, with viral replication occurring mainly in the local lymph nodes and circulating lymphocytes and monocytes (Hofmann-Lehmann et al., 2008; Rojko, Hoover, Mathes, Olsen, & Schaller, 1979). This initial systemic lymphatic viraemia may be successfully cleared in a minority of cases; more commonly a successful infection of the bone marrow follows (Lutz, Pedersen, & Theilen, 1983). This is the major site of viral replication. A secondary viraemia is then observed during which the virus spreads via the peripheral vasculature system to peripheral organs and epithelial tissues. This cycle generally occurs over 3 weeks and viral RNA is detectable in plasma approximately one week after FeLV exposure. Infectious virus is shed in the saliva approximately four weeks after the initial infection (O. Jarrett, 1999).

The main FeLV transmission route is oronasal, therefore both fighting and mutual grooming are high risk activities between infected and naïve cats. Infectious virus is shed through saliva, nasal secretions, milk, faeces and urine (Gomes-Keller et al., 2009; W. D. Hardy, Jr. et al., 1976; Pacitti, Jarrett, & Hay, 1986), although saliva represents the highest potential for virus transmission. As FeLV does not survive outside the host for extended periods of time (Francis, Essex, & Gayzagian, 1979) the risk factor presented by faeces and urine was presumed to be negligible, however recent evidence indicates transmission may occur through these routes (Gomes-Keller, et al., 2009). There is also preliminary evidence that fleas may be able to transmit infectious virus between hosts through their saliva and faeces (Vobis, D'Haese, Mehlhorn, & Mencke, 2005). FeLV is transmissible trans-placentally (Rojko, Hoover, Quackenbush, & Olsen, 1982), although usually viraemic queens are unable to sustain pregnancy (Lutz et al., 2009). Transmission has also been observed through blood transfusions between latently infected and naïve cats; although reactivation of latently infected cells is rare (Chen et al., 1998).

1.1.3. Detection and diagnosis

FeLV infection is often suspected following assessment of clinical signs and haematology. The initial diagnosis is then confirmed by screening for either viral proteins or genetic material. As vaccinated and infected cats are often serologically indistinguishable, antibody detection is not indicative of a current infection. Historically, virus culture (the isolation of infectious virus from clinical samples) was the gold standard for the diagnosis of FeLV (O. Jarrett, 1980), however due to the time and level of scientific equipment required, this was not a feasible option for most diagnostic clinics.

Enzyme Linked Immunosorbent Assays (ELISAs) for the detection of the core capsid protein (CA, or p27) in whole blood is the most common method of diagnosis in veterinary clinics worldwide (Lutz, Pedersen, Durbin, & Theilen, 1983) and displays very high sensitivity and specificity against all other methods tested to date (Hartmann, Werner, Egberink, & Jarrett, 2001; Hofmann-Lehmann et al., 2001). Although tears and saliva may also be used in some ELISAs, whole blood is the most accurate predictor of clinical status (Hawkins, 1991; Hawkins,

Johnson, Pedersen, & Winston, 1986). Currently the leading ELISA-based diagnostic in veterinary clinics is the SNAP FIV/FeLV Combo kit (Idexx Laboratories), which detects simultaneously FeLV antigen and feline immunodeficiency virus (FIV) antibodies in serum samples.

Immunochromatography diagnosis kits have also been developed and display similar sensitivity and specificity parameters to ELISAs, however these are less economical and more laborious for commercial clinics (Hartmann et al., 2007; Robinson et al., 1998). Immunofluorescence-based tests, although available, are not commonly conducted due to their irreproducibility and ambiguity in the interpretation of results (Floyd, Suter, & Lutz, 1983). A Polymerase Chain Reaction (PCR) to detect proviral DNA in either whole blood or saliva has also been developed (Gomes-Keller, Gonczi, et al., 2006; Gomes-Keller, Tandon, et al., 2006). This method is more sensitive than ELISAs and allows detection at an earlier stage of infection, as the initial presence of infectious virus in plasma coincides with the detection of proviral DNA in PCR assays (Hofmann-Lehmann et al., 2006). However detection of provirus will not distinguish between active infection and recovered cats and thus confirmatory testing is usually required. Real-time PCR may be conducted to detect and/or quantify the presence of low levels of viral RNA or proviral DNA within a sample.

1.1.4. Treatment

Due to the range of clinical symptoms associated with FeLV infection, there are multiple options for treatment of the infected cat. However these are merely therapeutic; there is no known method of clearing infection and most treatments will only relieve symptoms for a short duration.

Although initial studies were not encouraging (Hartmann et al., 1998), immunomodulators are now used to relieve the immunosuppression caused by FeLV infection and allow the immune system to attempt to clear the infection (McCaw et al., 2001). The antiviral recombinant protein feline interferon-ω (Virbac Animal Health) is used widely, as this is naturally produced from leukocytes during FeLV infection. This treatment correlates to a moderate increase in survival rates (de Mari, Maynard, Sanquer, Lebreux, & Eun, 2004),

although it does not decrease viraemia. Human interferon-α (Alfa Wasserman, Italy) has been used to treat cats infected simultaneously with both FeLV and FIV; however this is administered infrequently (Pedretti et al., 2006).

Alternatively anti-retroviral drugs may be used; however the only compound commercially available for treatment of cats is the nucleoside analogue zidovudine. This reduces plasma viral load and improves clinical status, however significant side effects may develop at higher dosage concentrations (Hartmann et al., 1992). Retroviral integrase inhibitors have also displayed promising results in *in vitro* assays but are not commercially available as treatment (Cattori et al., 2011). Treatment of FeLV-C-infected anaemic cats with reverse transcriptase-inhibitors allows the recurrence of erythropoiesis (Abkowitz, 1991), but must be continued through the cats life to prevent viral reactivation. Similarly, leukopaenia may be temporarily relieved by administration of granulocyte colony-stimulating factor to stimulate haematopoiesis (Fulton, Gasper, Ogilvie, Boone, & Dornsife, 1991). FeLV-induced lymphomas may be treated with chemotherapy, although this will not prevent future viraemia (Ettinger, 2003).

It is obvious, therefore, that despite the decrease in FeLV prevalence suitable treatments remain unavailable for those cats presenting with active infections. This highlights the necessity of further research into the pathogenic mechanisms of FeLV infection and the subsequent identification of potential drug targets.

1.1.5. FeLV Vaccines

FeLV was the first retrovirus for which a vaccine was successfully developed, which at the time was viewed as a promising indication that it was possible to induce protection against other retroviruses including HIV. Early studies tried various antigenic preparations, however whole killed virus and envelope-based subunit vaccines were found to be ineffective (Hunsmann, Pedersen, Theilen, & Bayer, 1983; R. G. Olsen, Hoover, Schaller, Mathes, & Wolff, 1977; Salerno, Lehman, Larson, & Hilleman, 1978; Yohn et al., 1976). There were also reports of enhanced infection occurring during challenge of cats vaccinated with the early whole inactivated virus preparations (N. C. Pedersen, Johnson, Birch, & Theilen, 1986).The first commercially available FeLV vaccine was introduced in

the USA in 1984, and was prepared from purified FeLV antigens (Lewis, Mathes, & Olsen, 1981). However this vaccine has now been withdrawn from the market due to poor efficacy (Hoover, Mullins, Chu, & Wasmoen, 1995; Legendre, Mitchener, & Potgieter, 1990; Lutz, et al., 2009; N. C. Pedersen, et al., 1986). There were also subsequent investigations into the utilisation of a canarypox-based virus vector, which expresses retroviral proteins in the host cell upon infection (Tartaglia, Jarrett, Neil, Desmettre, & Paoletti, 1993). This vaccine is currently licenced in Europe (Purevax, Merial) but not USA. However this method does not induce virus-neutralising antibodies (VNA) and the acquired immunity is mediated through the priming and stimulation of B and T lymphocytes. This is in direct conflict to the commonly-held view that the vaccination event must induce VNA to ensure protective immunity (Sparkes, 2003). Currently there are conflicting opinions as to whether VNAs and/or CTL responses are required for a protective response against FeLV.

Numerous vaccines are currently available (Sparkes, 2003). The most commonly used formulation is FeL-O-Vax (Fort Dodge Animal Health), a multivalent vaccine with a preparation containing antigens from feline herpes virus, calicivirus, parvovirus and chlamydia. A monovalent vaccine consisting of whole inactivated FeLV is also available from Fort Dodge Animal Health; this induces high titres of VNAs associated with high levels of protection (Hoover, et al., 1995). Other currently available vaccines include Fevaxyn FeLV, a whole inactivated vaccine available from Schering-Plough Animal Health, and Leukocell, a preparation of subunit proteins from Pfizer Animal Health. Current recommendations for kittens are an initial vaccination at 8 weeks of age followed by a booster inoculation 3 – 4 weeks later (J. Levy, et al., 2008). All vaccines are recommended to be re-administered annually, as the duration of immunity has not been precisely defined (Harbour et al., 2002; Hoover, Mullins, Chu, & Wasmoen, 1996).

Multiple studies of vaccine efficacy have been conducted and there is no correlation between any one vaccine and the induction of protective, sterilising immunity (Sparkes, 2003). Therefore, although vaccination provides a degree of protection, initial infection may still occur. Indeed, cats displaying low level RNA viraemia and persistent detection of proviral DNA, without any development of clinical signs, are often regarded as protected (Lutz, et al., 2009). Recent

investigations into the viral DNA and RNA loads in vaccinated cats has revealed that some may possess circulating viral RNA at levels detectable by qPCR, despite not being antigenaemic (Torres, Mathiason, & Hoover, 2005; Torres, O'Halloran, Larson, Schultz, & Hoover, 2010). It is possible that these nucleic acids correlate to non-infectious virions. It was also found that those cats which successfully controlled infection upon challenge did not always contain high VNA titres, in direct contrast to the commonly-held assumption that these were essential for FeLV protection (Torres, et al., 2010). This had been suggested previously (Haffer, Koertje, Derr, & Beckenhauer, 1990; Hofmann-Lehmann, et al., 2006).

In recent years attempts at developing more efficient or safer FeLV vaccines have been rare, however there has been promising research towards utilisation of the TM of Env as an immunogen (Langhammer, Fiebig, Kurth, & Denner, 2011; Langhammer, Hubner, Jarrett, Kurth, & Denner, 2011). Despite the initial promise FeLV vaccines offered for retroviral immunology, the induction of sterilising immunity has still not been achieved and continues to be regarded as the ultimate aim of a retroviral vaccine.

1.2 Genetics and proteins of FeLV

The members of the gammaretroviridae genus have a highly conserved genomic structure, containing three open reading frames (ORFs); *gag* (group-specific antigen), *pro-pol* (protease-polymerase) and *env* (envelope glycoprotein). The ORFs encode numerous structural and enzymatic proteins (Figure 1.1). Within the provirus, the ORFs are bracketed by long terminal repeats (LTRs). All proteins are translated from the genomic mRNA; however a subgenomic transcript is also produced via splicing of whole transcripts. The majority of Env proteins are produced from this RNA.

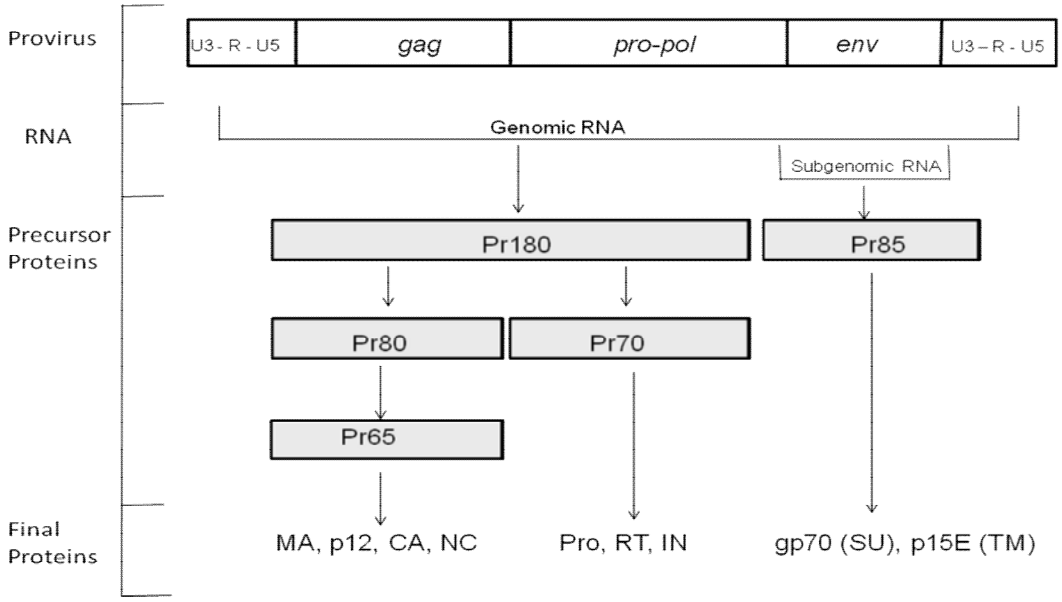


Figure 1.1: The genes and proteins of FeLV.

1.2.1. Non-coding RNA structures

The LTRs of retroviral genomes are non-coding regions at the termini of the provirus. Each proviral LTR consists of three regions, being U3 (unique 3' region), R (repeat region) and U5 (unique 5' region) (5' to 3', respectively). Comparatively, viral genomic RNA contains the R and U5 regions at the 5' terminus, and the U3 and R regions at the 3' terminus (Figure 1.2). Duplication of the U3 and U5 regions occurs during reverse transcription of the viral genome (see Section 1.3). Although the R and U5 regions are highly conserved, the U3 region differs significantly between endogenous FeLV elements (see Section 1.5) and exogenous FeLV genomes (Berry, Ghosh, Kumar, Spodick, & Roy-Burman, 1988; Casey et al., 1981; Okabe, DuBuy, Gilden, & Gardner, 1978). For this reason LTR analysis is often utilised to determine the proviral origin of FeLV strains (Tandon, Cattori, Willi, Lutz, & Hofmann-Lehmann, 2008).

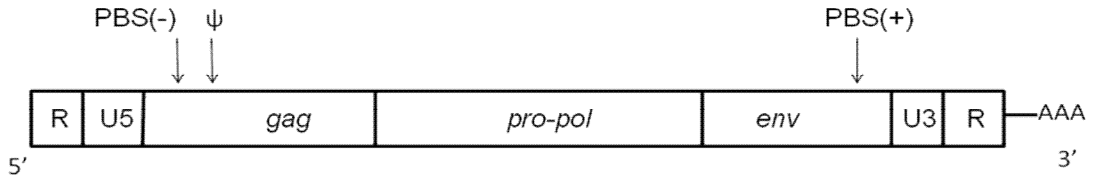


Figure 1.2: The FeLV RNA genome.

The 3' LTR often contributes significantly to the virulence and pathogenic potential of retroviruses, including FeLV. As promoter and enhancer-like elements including the CCAAT (Grosschedl & Birnstiel, 1980) and Goldberg-Hogness boxes (Corden et al., 1980; Proudfoot, 1979) are located within the U3 region, the binding of transcription factors to these elements may activate proto-oncogenes downstream of the site of insertion (Fan, 1997; L. S. Levy, Lobelle-Rich, & Overbaugh, 1993; Uren, Kool, Berns, & van Lohuizen, 2005). The availability of these transcription factors will vary between cell types; therefore the 3' LTR affects the pathogenic potential of the virus across various tissue types (Short, Okenquist, & Lenz, 1987). This insertional activation may also have long range effects if the U3 enhancer elements increase transcription from host promoters further downstream from the site of integration.

In addition to the activation of oncogenes, gammaretroviruses may interrupt host tumour-suppressor genes during proviral integration, thereby leading to transformation of the host cell. Collectively, these two oncogenic mechanisms were assumed to be the only two available to gammaretroviruses, as they do not encode any viral oncogenes which may directly induce transformation. However there is recent evidence that antisense transcription may occur in MLV, initiated from multiple positions within the 5' LTR. This produces chimaeric host-virus transcripts and therefore contributes to enhanced expression of host oncogenic proteins (M. H. Rasmussen et al., 2010). Thus the oncogenic mechanisms of gammaretroviruses may not be as fully explored as previously thought. Whether this occurs in FeLV infection has not been investigated.

In addition to the LTRs, the non-coding RNA regions of importance in the FeLV genome are the primer binding site (PBS) and the packaging signal (Ψ). These motifs are located across the 3' region of the 5' LTR and the initiation codon of the *gag* gene, and are both utilised during the retroviral replication cycle (see Section 1.3). The packaging signal also contains the splice donor site for the generation of the subgenomic *env* mRNA transcript. The packaging signal is a section of *cis*-acting RNA which forms complex secondary structures including multiple hairpin regions (Burns, Moser, Banks, Alderete, & Overbaugh, 1996; Konings, Nash, Maizel, & Arlinghaus, 1992). These structures allow RNA dimerisation and recognition of such dimers by the structural proteins, resulting

in packaging of the viral genome by core proteins. Both matrix and nucleocapsid domains of the Gag precursor protein recognise sections of FeLV-ψ (Linial & Miller, 1990; Wang, Norris, & Mansky, 2003). As MLV and FeLV are able to cross-package *in vitro* (Burns, et al., 1996) it is assumed that high structural conservation exists between FeLV-ψ and MLV-ψ, however the primary sequences of these genomic regions display low identity. The secondary structures required for FeLV RNA dimerisation and packaging have therefore not been extensively investigated. Such studies would allow comparisons between FeLV and other retroviruses and the identification of essential structures required for retroviral replication.

1.2.2. Structural proteins

The structural proteins which form the core of the FeLV virion do not contribute to pathogenicity, nor do they vary to a large degree between subgroups or strains of FeLV. Therefore they are not as thoroughly studied as the Env proteins or LTR sequences. The *gag* (Group-specific antigen) gene encodes a polyprotein, Pr65, which is translated from the *gag* ORF within the genomic mRNA transcript. This polyprotein is cleaved into four mature structural proteins, being the matrix (MA, or p15), capsid (CA, or p27), nucleocapsid (NC, or p10) and a small protein of unknown function termed p12.

The FeLV virion structure (Figure 1.3) is similar to that of other retroviruses. The outermost protein layer of the viral core (i.e., those which face the lipid bilayer) is composed of MA proteins. The association between these proteins and the membrane is essential for viral budding and maturation and is often aided by the presence of myristic acid groups on the protein (Copeland et al., 1988). The CA protein (p27) forms the inner core of the virion and is the most easily detectable antigen in FeLV-infected cells. Within the virion core are NC proteins, which bind to the genomic RNA dimer during production of the virion and remain associated with the RNA during subsequent cellular entry (Prats et al., 1990). NC proteins tend to be highly basic and often contain the zinc-finger structures characteristic of nucleic-acid binding proteins (Katz & Jentoft, 1989).

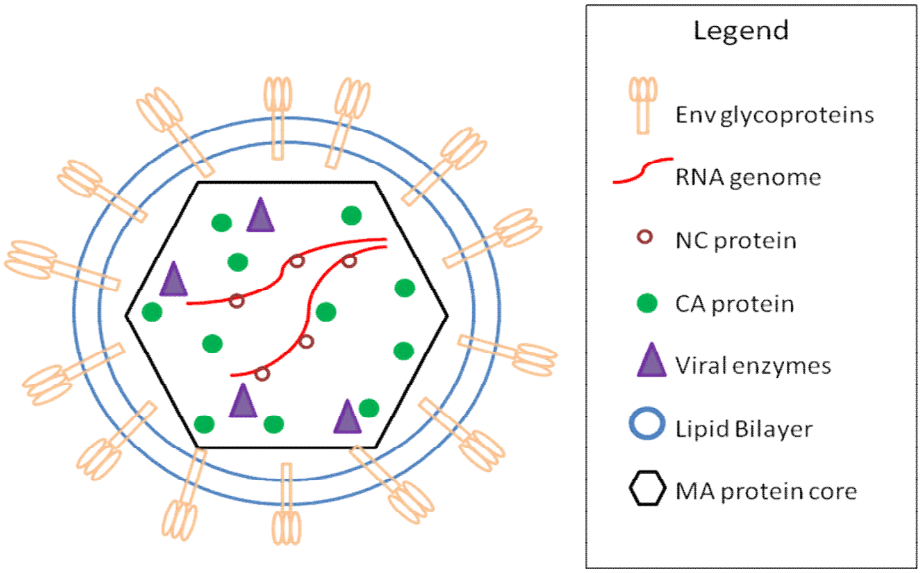


Figure 1.3: The FeLV virion.

1.2.3. Enzymatic proteins

The *pro-pol* gene is in-frame with the *gag* ORF and encodes the viral enzymes, which are packaged within immature virions. The precursor polyprotein (Pr180gag-pol) is translated from genomic mRNA and cleaved and processed into the individual proteins, being protease, reverse transcriptase and integrase.

The viral protease (Pro) displays many similarities to cellular aspartyl proteases (Jaskolski, Miller, Rao, Leis, & Wlodawer, 1990). Prior to budding, the protease is inactive and activity is triggered once the virion has budded. It catalyses the condensation of the viral core by cleaving structural precursor proteins, leading to maturation of the virion.

The Reverse Transcriptase (RT) enzyme mediates RNA-dependent-DNA synthesis, and transcribes the initial nascent DNA strand from the viral genomic RNA. This enzyme contains an additional RNase H function, which removes the RNA component of the DNA-RNA heteroduplex to allow cellular DNA polymerase to synthesise the complementary DNA strand (reviewed in Goff, *et al*. 1990 (Goff, 1990b)). The RNase H domain (an RNA-DNA hybrid specific ribonuclease) is located at the C terminus of the protein and is separate to the RT domain.

The integrase enzyme (IN) has multiple functions, including trimming of the dsDNA complex prior to integration and cleavage of the host DNA at the integration site. Integrase also possesses a ligation function to covalently fuse the viral and host DNA following integration (Bushman & Craigie, 1991).

1.2.4. Envelope glycoprotein

The overall structure of the *env* gene is highly conserved amongst the gammaretroviridae (Figure 1.4); as this protein mediates cellular entry there are numerous structural constraints which limit possible sequence variation. The production of functional Env glycoproteins is therefore a highly conserved process within retroviruses (Einfeld, 1996; Hunter & Swanstrom, 1990).The viral envelope itself is a host-originating phospholipid bilayer, from which the virally-encoded Env glycoproteins, in their native trimeric form, extend as "spikes". After translation from the subgenomic mRNA, the Env precursor protein, gp85, is cleaved by cellular proteases into the two constitutive proteins, being the Surface Unit (SU, or gp70) and the transmembrane (TM, or p15E) domains. The two domains remain associated through disulphide bridges and non-covalent bonds (Pinter, Lieman-Hurwitz, & Fleissner, 1978). Each domain of Env plays an essential role in the retroviral life cycle. SU mediates receptor binding and host cell entry, whilst the TM anchors the Env complex within the viral membrane.

During the processing of gp85, the N terminal signal sequence is recognised by the cellular transport machinery and directs the polyprotein into the endoplasmic reticulum (ER). The N terminal regions are translocated across the ER membrane; however the hydrophobic C terminal membrane anchor halts translocation at this point. Thus the N terminal region of gp85, the SU domain, is within the ER lumen whereas the C terminal TM domain remains within the cytoplasm. Within the lumen, the signal peptide is removed by host proteases, N-linked glycosylation occurs and disulphide bonds are formed. As a result the SU domain of mature retroviral virions is significantly more highly glycosylated than the TM domain. Correct glycosylation is required for future processing of the domains; additionally, although the sugar chains themselves may not be involved in receptor-Env binding, incorrectly glycosylated proteins reduce the infectivity of the virus (Knoper, Ferrarone, Yan, Lafont, & Kozak, 2009; Schultz, Rabin, &

Oroszlan, 1979). The oligomerisation of Env trimer complexes also occurs in the ER.

Env complexes are then transported to the Golgi apparatus where N-linked oligosaccharides are modified and O-linked oligosaccharides may be added to the SU domain. Cleavage of the SU and TM domains by cellular proteases also occurs in the Golgi. The Env proteins are then transported to the cellular membrane where they are incorporated into budding virions through interactions with viral core MA proteins.

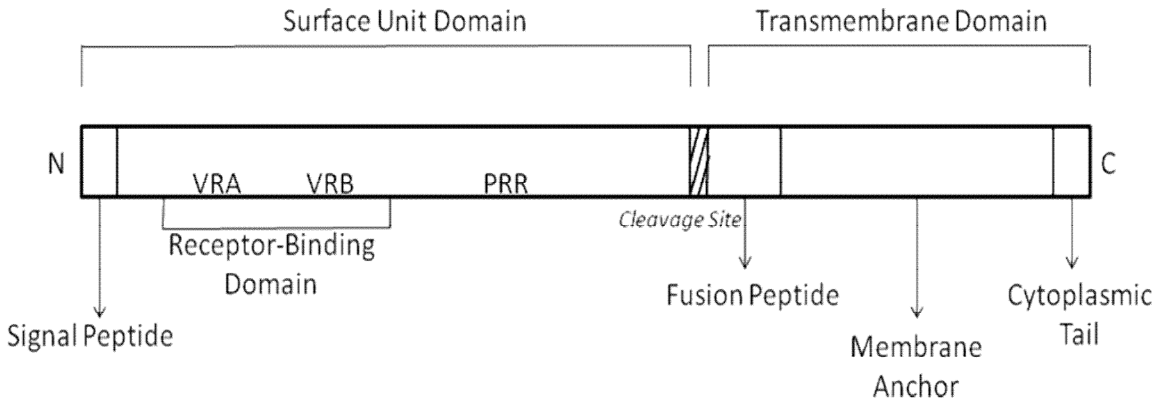


Figure 1.4: The FeLV Envelope protein.

1.2.4.1. Surface Unit domain

As the SU protein (gp70) is the major determinant of both cell tropism and disease outcome in FeLV, it has been studied intensively for many years (see Sections 1.8.1 and 1.9.1 for further details). However many aspects of this protein, including the genetic determinants of disease prognosis and interactions with host proteins, remain not fully understood. The SU domain of gammaretroviral envelope proteins is responsible for receptor recognition and hence entry into host cells. It is this protein which varies significantly between the FeLV subgroups, through alterations in its receptor usage. The main determinants of receptor binding are found in the N terminal region. There are two regions in this area (termed VRA and VRB) that are highly divergent between the gammaretroviridae, separated by a conserved region approximately 38 amino acids in length. Collectively these motifs constitute the receptor-binding domain (RBD) (Bae, Kingsman, & Kingsman, 1997; Battini, Danos, & Heard, 1998;

Battini, Heard, & Danos, 1992; Gray & Roth, 1993; Hoover & Mullins, 1991; Rigby et al., 1992).

Between the RBD and the C-terminal region of SU is a proline-rich region (PRR), which also varies significantly between the gammaretroviridae. The PRR is thought to act as a hinge between the N-terminal globular RBD and the C-terminal region (Barnett, Davey, & Cunningham, 2001; Fass et al., 1997). It is essential for viral infectivity as it allows conformational changes within the Env complexes (Lavillette, Ruggieri, Boson, Maurice, & Cosset, 2002) but does not appear to contribute to receptor recognition (Gray & Roth, 1993).

Another region of importance within the SU domain is the N terminal fusion motif (consisting of four sequential amino acids, SPHQ). This is distinct from the fusion peptide within the TM domain, which is protected by SU prior to the membrane fusion event. Mutations in the SU fusion motif disrupt the membrane fusion process although both incorporation of Env into virions and subsequent receptor binding may still occur (Bae, et al., 1997; Lavillette, Boson, Russell, & Cosset, 2001; Lavillette & Kabat, 2004). Mutations of the histidine are especially disruptive; this residue is involved in the formation and disruption of the disulphide bridges which link SU and TM. The isomerisation of these bridges is required for exposure of the fusion peptide and its subsequent insertion into the cellular membrane.

1.2.4.2. Transmembrane domain

In contrast to the SU domain, the TM domain (p15E) of the envelope glycoprotein is highly conserved between both the FeLV subgroups (Riedel, Hoover, Gasper, Nicolson, & Mullins, 1986) and retroviruses as a whole (Kobe, Center, Kemp, & Poumbourios, 1999; Patarca & Haseltine, 1984). Structurally, the TM domain forms a trimeric coiled complex (Fass, Harrison, & Kim, 1996). The TM domain consists of a short C-terminal cytoplasmic peptide, followed by a hydrophobic membrane spanning domain which anchors the protein within the viral membrane, and finally an N terminal ectodomain.

The cytoplasmic tail of TM is cleaved by the viral protease shortly before cellular infection, shortening it from p15E to p12E. The presence of this peptide (termed the R peptide) prevents membrane fusion until receptor binding has occurred. The R peptide was initially thought to be dispensable for viral infectivity (Perez, Davis, & Hunter, 1987), however it is now known that the cleavage of p15E to p12E is required to allow fusion and subsequent entry of the target cell (Bobkova, Stitz, Engelstadter, Cichutek, & Buchholz, 2002; Loving, Li, Wallin, Sjoberg, & Garoff, 2008; Song, Micoli, Bauerova, Pichova, & Hunter, 2005).

N-terminal to the R peptide is the so-called membrane anchor; this highly hydrophobic region prevents further translocation of the protein through the cellular membrane, ensuring the final Env trimer is a transmembrane complex. Between this region and the SU/TM cleavage site is the short TM ectodomain, which includes a hydrophobic peptide required for mediating host-viral membrane fusion essential for subsequent viral entry (Einfeld, 1996; Gallaher, 1987). This fusion peptide is buried within the trimer complex during virion maturation. Fusion is induced once the mature virion encounters a host cell membrane. SU-receptor binding and isomerisation of the disulphide bridges, aided by the SU fusion motif, may then occur. The TM ectodomain also contains a central highly conserved region associated with immunosuppression of the host, although a mechanism for this has not been established (Cianciolo, Bogerd, & Snyderman, 1988; Ogasawara et al., 1988). There is also evidence that particular residues in the FeLV ectodomain are essential for correct Env precursor processing within the ER and Golgi (Burns, Poss, Thomas, & Overbaugh, 1995). Thus both the SU and TM domains of Env are required for successful completion of the retroviral lifecycle.

1.3 The retroviral replication cycle

The retroviral replication cycle is a highly conserved process throughout the Retroviridae family, reflecting the conserved structure of these viral particles. When retroviral virions bind to their cognate receptor through non-convalent binding of the SU glycoprotein, a conformational change is triggered which exposes the hydrophobic fusion peptide within the TM region (Lavillette, et al., 2002). This catalyses membrane fusion and entry into the host cell.

Entry and Un-coating: Gammaretroviral cell entry is pH-independent, although pH levels within the virion may affect the kinetics of fusion peptide exposure. Following membrane fusion, the virion core is effectively uncoated of the lipid envelope and released into the cytoplasm where reverse transcription will occur. The virion core particle contains the dimeric RNA genome and the virally-encoded enzymes. Cellular tRNA molecules are also found within mature retroviral virions, as retroviruses utilise a specific tRNA during reverse transcription. FeLV utilises the tRNA(Pro) molecule (Laprevotte, Hampe, Sherr, & Galibert, 1984).

Reverse Transcription: Reverse transcription is a highly complex process which is still not fully understood, despite being heavily researched for many years (for an early review, see Gilboa, *et al*. 1979)(Gilboa, Mitra, Goff, & Baltimore, 1979). Importantly, the RT enzyme does not possess proof-reading or exonuclease activity. Mutations through misincorporation are therefore relatively common, leading to the high evolutionary rate observed in retroviral genomes (10^{-4} to 10^{-5} substitutions/site/cycle) (Overbaugh & Bangham, 2001).

The first step in FeLV reverse transcription is binding of the tRNA(Pro) to the primer-binding site (PBS[-]), an 18 bp sequence complementary to that of the tRNA, adjacent to the U5 region within the 5' LTR (Joshi, Van Brunschot, Robson, & Bernstein, 1990). Thus the tRNA acts as a primer to allow initiation of reverse transcription. The RT enzyme then extends the complementary region from the 3' end of the tRNA to the 5' end of the viral genome, by synthesising a singular DNA strand until it reaches the end of the R region. This is termed the "strong-stop" antisense DNA and is a short single-stranded segment. The RNase H function of RT allows degradation of the 5' R region of RNA, leaving this area of the strong-stop DNA available for complementary base-pairing.

The first strand transfer event now occurs, where the R region of the strong-stop DNA can bind to the 3' R region of the viral genome. It then acts as a secondary primer for RT, allowing reverse transcription of the remainder of the viral genome producing the full length nascent (antisense) DNA strand. This strand now contains the entire 3' LTR of the DNA due to the transfer of R and U5

domains in the strong-stop DNA. The RNase H motif of RT digests the RNA component of the heteroduplex, producing single stranded nascent DNA.

A RNase H-resistant area is located between the 3' U3 and the polypurine tract (PPR). This remaining RNA is now utilised as a third primer binding site (commonly called PBS[+]), initiating the positive sense DNA strand synthesis from the 3' terminus of the RNA to the end of the viral genome. This produces the U3, R and U5 regions of the "strong-stop" sense DNA strand. The second transfer event now occur, during which this short DNA segment translocates and base-pairs to the 3' end of the antisense DNA strand. This is then extended, forming a dsDNA complex which now contains blunt-ended LTRs at both termini. This entire process is thought to occur within the virion core. The dsDNA is now part of a high molecular weight pre-integration complex, which is transported into the nucleus.

Integration: Although retroviral particles contain two molecules of positive-sense genomic RNA, only a single dsDNA pre-integration complex and therefore a single provirus is produced from each virion (Hu & Temin, 1990). Integrase is packaged within the mature virion and acts upon the un-integrated complex, which is transported into the nucleus alongside viral structural and enzymatic proteins (Goff, 1990a). Although some retroviruses may actively enter the nucleus, gammaretroviruses passively enter during mitosis. Cleavage of the host DNA and the introduction of viral DNA occur in a concerted event; the enzyme makes a staggered cut within the host DNA, generating short overhangs with 5' phosphorylated ends which can non-covalently associate with the nicked viral DNA. Ligation (covalent fusion of the DNA molecules) is then mediated by a separate domain of the integrase enzyme. After integration, host enzymes correct the mismatched base pairs at the termini of the provirus, producing the 4 – 6bp of repeated sequence characteristic of retroviral integration sites (direct repeats). Although there is limited specificity in the targeting of retroviral DNA to specific host DNA sites, chromatin density may contribute, therefore increasing the chances of the provirus being inserted into a transcriptionally-active region. The provirus is then transcribed and translated as standard host genes.

Production of Viral Proteins and Nucleic Acid: Transcription of the provirus is initiated at the U3-R boundary of the 5' LTR. Polyadenylation of the transcript then occurs at the R-U5 boundary of the 3' LTR. Post-transcriptional processes, including methylation and capping of the viral RNA, are mediated by cellular enzymes (Stoltzfus, 1988) resulting in functional mRNA transcripts. Viral RNA may be either directly exported or spliced within the nucleus, producing the subgenomic RNA from which the majority of Env is translated. After export of the RNA into the cytoplasm, the expression of Env proteins occurs from membrane-bound polysomes, whereas the Gag and Pro-Pol protein precursors are translated by free cytoplasmic ribosomes from the full-length genomic RNA.

Assembly, Egress and Maturation: Within the gammaretroviridae, the assembly of structural proteins and genomic RNA into core viral particles occurs primarily at the cellular membrane. For Type C retroviruses such as FeLV, virion intermediates are therefore not detectable within the cytoplasm (Coffin, 1979). This is not a conserved process within the simple retroviruses; betaretroviruses assemble within the pericentriolar regions of the cytoplasm and are then trafficked to the membrane (Arnaud, Murcia, & Palmarini, 2007; Sfakianos & Hunter, 2003). Subgenomic mRNAs are excluded from the packaging process as RNA dimerisation and interactions with NC proteins depend upon a functional leader and packaging signal. The virion structural core, containing the RNA dimer, buds from the host membrane and thereby acquires both the lipid bilayer and Env glycoproteins. Cellular proteins are generally excluded from the emerging virion. Once released the virion undergoes maturation to become an infectious particle, hallmarked by cleavage of the Gag precursor proteins and the accompanying condensation of the viral core.

1.4 Immune responses to FeLV infection

FeLV infection activates multiple branches of the adaptive immune response, including antibody production and cytotoxic lymphocytes (CTLs) (Flynn, Hanlon, & Jarrett, 2000). Although most recovered cats produce an efficient antibody response, CTLs appear prior to VNAs (within a week of virus infection) and play an essential role in viral clearance (Flynn, Dunham, Watson, & Jarrett, 2002).

Cytokine responses are also essential for development of protective immunity (Hanlon et al., 2001). Initial FeLV viraemia induces both Type I and II interferon responses, as both IFN-ω and IFN-γ are up-regulated following infection (Garch et al., 2006).

However, chronic FeLV infection results in a decrease in both T lymphocyte responses and cytokines such as IFN-γ (Good, Ogasawara, Liu, Lorenz, & Day, 1990), contributing to the immunosuppression seen in many infected hosts. The mechanism of this is unknown, however recent investigations into feline neutrophils indicate that infection of these cells may contribute to FeLV pathogenesis. Neutrophils play a central role in the innate immune response by phagocytosing invading pathogens (Nathan, 2006). FeLV-induced immune-suppression is thought to be partially due to chronic overstimulation of the infected neutrophils, which are then unable to respond to novel pathogens (Wardini et al., 2009). This overstimulation is seen in both clinically ill and asymptomatic infected cats, although to a lesser extent in the latter group. These neutrophils also display reduced chemotactic responses (Kiehl, Fettman, Quackenbush, & Hoover, 1987) and impaired phagocytic activity (Hoffmann-Jagielska et al., 2005). Collectively these reduced responses contribute to the opportunistic infections often seen in FeLV-infected cats (Lewis, Duska, Stiff, Lafrado, & Olsen, 1986). Although it is not known how FeLV infection mediates these effects on neutrophils, it has been suggested that the Env TM domain may be responsible rather than viral replication itself (Lafrado, Lewis, Mathes, & Olsen, 1987). This region has been associated with the immunosuppressive effects of other retroviruses (Cianciolo, et al., 1988; Ogasawara, et al., 1988).

Although the degree to which T cell-mediated and humoral immunity each contribute to FeLV clearance and protection is still unknown, there has been significantly more research conducted upon the antibody responses of infected cats. This is mainly due to the assumption that the induction of VNAs was required for a protective vaccine. There is now increasing evidence that VNAs alone may not be sufficient for either protection against challenge or prevention of disease. Additionally, as research into other retroviruses continues to uncover novel host-virus interactions, it is becoming increasingly apparent that VNAs may

play a significant role in FeLV evolution within a host (see Chapter 4). Therefore much research is still required into the humoral responses to FeLV infection.

The first evidence that VNAs were involved in FeLV clearance was the observation that transfusion of blood between infected and naïve cats correlated with protective immunity (O. Jarrett, Russell, & Stewart, 1977). It was also documented that recovered cats continued to produce VNAs after viraemia had ceased, and these were notably absent from chronically-viraemic cats (Russell & Jarrett, 1978a). The degree of Env antibody response to FeLV is now known to be highly correlative with the disease outcome; by 3 weeks post-infection cats with low antibody titres can be accurately predicted to develop progressive infections whereas cats with higher titres will display regressive viraemia and antigenaemia from that point (Hofmann-Lehmann, et al., 2001).

In addition to Env-specific VNAs, cats mount an immune response against numerous *gag*-encoded proteins; however these do not provide protection or aid viral clearance (W. D. Hardy, 1993). The immunologically dominant region is the SU domain (Hoover & Mullins, 1991; Lutz, Higgins, Pedersen, & Theilen, 1979), however the receptor-binding regions and the neutralising epitopes are separate motifs within this protein (Ramsey, Spibey, & Jarrett, 1998). It is also probable that the immunogenicity of SU relies heavily upon its glycosylation, as is the case for other gammaretroviruses (Alexander & Elder, 1984). Recent investigations into potential recombinant vaccines have also identified neutralising antibodies against the TM domain (Langhammer, Fiebig, Kurth, & Denner, 2005; Langhammer, Hubner, Kurth, & Denner, 2006; Marciani et al., 1991).

Neutralising monoclonal antibodies (MAbs) specific to a conserved epitope within the central region of the SU domain are commercially available (Elder et al., 1987; Nunberg, Rodgers, Gilbert, & Snead, 1984). This epitope (C11D8) appears essential but not sufficient for a protective neutralising antibody response. Although the genetic drift of viral genomes away from this peptide correlates to an escape from clearance by the immune system (Sheets, Pandey, Klement, Grant, & Roy-Burman, 1992), external administration of these MAbs do not aid viral clearance (Weijer, UytdeHaag, Jarrett, Lutz, & Osterhaus, 1986). This indicates additional VNA binding sites within Env exist and contribute to the

antibody titre seen in infected hosts. Additionally, the C11D8 epitope is not found in endogenous FeLV elements (see Section 1.5) (McDougall et al., 1994; Sheets, Pandey, Jen, & Roy-Burman, 1993). However after the formation of FeLV-B genomes (see Section 1.8), a single substitution event occurs (C750T) which reintroduces the epitope into the exogenous viral genome (Pandey et al., 1995). It is thought that this mutation is driven by further recombination between FeLV-A and -B genomes occurring during viral replication, replacing the non-neutralising endogenous epitope with the exogenous sequence (Pandey, et al., 1995).

1.5 Outcomes of FeLV infection

FeLV infection is variably associated with acquired immunodeficiency syndrome (AIDS)-like immunosuppression, anaemia and various neoplastic haematopoietic disorders. The specific clinical manifestation reflects the subgroup present within the host (see Sections 1.7 to 1.10). Approximately 72% of domestic cats become infected after exposure (W. D. Hardy, 1993), however 60% of these are predicted to recover ("regressor" cats) whilst 40% progress to chronic infections and/or FeLV-related diseases (Hoover & Mullins, 1991). The likelihood of clearance correlates highly with the cats' age, with neonatal kittens displaying a high susceptibility to infection which decreases over time (Grant, Essex, Gardner, & Hardy, 1980; Hoover, Olsen, Hardy, Schaller, & Mathes, 1976). FeLV-infected cats generally live for 2.4 years following diagnosis, as compared to 6.3 years for healthy controls (J. Levy, 2009).

Following initial viraemia, cats can be categorised according to the eventual outcome; these are termed regressive, progressive, abortive and focal infections. There is evidence that the early viral load (in plasma, saliva, faeces and urine) correlates highly with the outcome of infection and hence the classification of the host. Regardless of the eventual outcome, all cats display a transient period of viraemia, hallmarked by detectable proviral and plasma viral RNA loads (Hofmann-Lehmann, et al., 2008; Lutz et al., 1980).

Regressive Infection: A majority of cats clear the infection through a combination of cell-mediated immunity and VNAs, and do not succumb to FeLV-

related diseases. As these cats only display transient viraemia and antigenaemia (Cattori et al., 2009), infection may only be detected through the presence of antibodies and/or proviral DNA (W. D. Hardy, Jr., et al., 1976; Hofmann-Lehmann, et al., 2001; Torres, et al., 2005). Infectious virus can occasionally be induced from bone marrow, indicating a latent infection remains in regressor cats (Madewell & Jarrett, 1983; Rojko, Hoover, Mathes, Krakowka, & Olsen, 1979; Rojko, et al., 1982). The duration of latency is highly variable (Pacitti & Jarrett, 1985). It is thought that the occasional reactivation of proviral DNA may contribute to the long term presence of VNAs in these so-called "recovered" cats (J. Levy, et al., 2008; Torres, O'Halloran, Larson, Schultz, & Hoover, 2008). Reactivation of a latent infection following years of viral inactivity has also been observed in a single experimental infection, however as this cat was additionally infected with FIV the reactivation may have been due to immunosuppression (A. K. Helfer-Hungerbuehler et al., 2010). The relevance of this particular incident is questionable considering there are no available data on the rate of natural infection reactivation after years of latency.

Whether latent FeLV proviruses contribute to disease is a contentious issue. FeLV latent infection has been associated with anaemia, panleukopenia and suppurative inflammation, as well as an increase in secondary non-viral infections (Suntz, Failing, Hecht, Schwartz, & Reinacher, 2010). However other research has not found a correlation between latent FeLV infection and general cytopenias in domestic cats (Stutzer et al., 2010). Additionally, FeLV latency has been documented as correlating with lymphosarcoma in domestic cats (A. T. Weiss, Klopfleisch, & Gruber, 2010). Given the disruptive and potentially oncogenic nature of the retroviral replication cycle, this is a plausible association; however other research has not been able to reproduce these findings (Stutzer, et al., 2010; Suntz, et al., 2010). It may be that the length of time required for the individual cat to clear the infection, and thus the degree of replication and reintegration the virus undergoes, affects the likelihood of disease occurring after viraemia ceases. Additionally, the role of latent viral infections in disease development may only become apparent now that vaccination is widespread, as this has caused the vast majority of infections to be non-progressive.

Progressive Infections: Chronic infections occur when the virus cannot be cleared or contained by the immune system and therefore becomes established within the bone marrow (Cattori et al., 2008; O. Jarrett, 1999; J. Levy, et al., 2008). Persistent viraemia and antigenaemia is observed, particularly within neutrophils and platelets which are unaffected by initial viraemia. FeLV-related disease may develop depending on both the infecting strain and the immunological response of the individual cat. The disease which manifests will depend upon which viral subgroups subsequently develop within the host. Compared to regressor cats, which do not maintain viral shedding, progressively-infected cats shed infectious virus as early as 3 weeks post-infection and maintain a high viral load (Cattori, et al., 2009).

Focal infections: Although rare, focal infections have been observed in both naturally-infected and experimentally infected cats (Roy-Burman, 1996). Focal infections are characterised by the host presenting with antigenaemia within specific isolated tissues, including spleen, lymph nodes, mammary glands or the small intestine although seroconversion and systemic viraemia do not occur (Hayes et al., 1989; Hoover, Schaller, Mathes, & Olsen, 1977; Pacitti, et al., 1986; Rojko, et al., 1982). It is not known to what extent these cats are at risk of developing disease. It is assumed that the detectable viral antigens are non-virion-associated and infection had been restricted to the affected organ/s (Miyazawa & Jarrett, 1997).

Abortive Infections: Abortive infections have been observed only in experimental infections to date. They are characterised by a confirmed initial infection followed by an absence of both secondary viraemia and detectable proviral DNA, indicating the cat successfully cleared all latently-infected cells (Pacitti & Jarrett, 1985; N. C. Pedersen, Johnson, & Theilen, 1984; Torres, et al., 2005). This is highly unlikely given the integrative nature of retroviral infections and the longevity of their target cells. However with the advancement of highly sensitive quantitative PCR assays the existence of abortive infections may require reconsideration, as it is possible they represent hosts with extremely low proviral loads undetectable by previous techniques.

1.6 Endogenous FeLV elements

Endogenous retroviruses (ERVs) are formed when the integration of viral dsDNA into the host genome occurs in either a germline cell or during early embryogenesis. The provirus is therefore present at this locus in every cell of the embryo, and the latent infection is transmitted vertically as a dominant Mendelian element. Degradation and an accompanying loss of function occurs as mutations accumulate within the provirus (Coffin, 1992), eventually resulting in non-functional endogenous retroviral elements. ERVs are generally conserved between individual members of a species and most are functionally inactive (Boeke & Stoye, 1997).

There are 8 to 15 FeLV-related endogenous elements (enFeLV) within the domestic cat genome (Benveniste & Todaro, 1975; Koshy, Gallo, & Wong-Staal, 1980; Okabe et al., 1976; Soe, Devi, Mullins, & Roy-Burman, 1983). They are confined to the genomes of the *Felis* genus, one of the 11 genera within the Felidae family. Therefore it appears that an ancestral exogenous FeLV initially became stably integrated into the genome of the last common ancestor of the *Felis* group, which now includes all small wildcats as well as domestic breeds (W. E. Johnson et al., 2006; Mattern & McLennan, 2000; O'Brien, 1986).

EnFeLV are polymorphic elements and therefore each provirus is not necessarily conserved between individuals (Koshy, et al., 1980; Roca, Nash, Menninger, Murphy, & O'Brien, 2005). Interestingly, privately-owned cats harbour a much higher enFeLV copy number than specific-pathogen free cats used for experimental purposes. Domestic cats also exhibit higher enFeLV loads than European wildcats (Tandon et al., 2007). This polymorphic nature indicates reinfection and germline integration has continued over the millennia and endogenisation of the cat genome is an on-going process. This is supported by the fact that most enFeLV elements have intact LTRs (Soe, et al., 1983; Soe, Shimizu, Landolph, & Roy-Burman, 1985), which would not be expected if they were conserved ancient ERVs.

Theoretically, enFeLV-derived virions would utilise the Pit1 receptor for cellular entry, as all enFeLV *env* genes studied to date contain Pit1-specific RBDs.

However these elements are generally non-functional and do not form infectious virions (Soe, et al., 1985); although expression of short transcripts has been documented (Busch et al., 1983; McDougall, et al., 1994; Niman, Akhavi, Gardner, Stephenson, & Roy-Burman, 1980) the degree of transcriptional activity they possess remains to be firmly established. It has been suggested that some defective elements contribute to the innate resistance to FeLV (McDougall, et al., 1994). This is mediated by the expression of a 35kDa Env fragment, corresponding to the N terminal region of SU, in both lymphoma and healthy lymphoid primary cell lines which prevents infection by FeLV-B, presumably by competitive receptor binding (McDougall, et al., 1994). This may explain the observation that purified FeLV-B cannot be transmitted between individual hosts (see Section 1.8) (O. Jarrett & Russell, 1978). However as cats are polymorphic for each individual enFeLV locus, it is unlikely that every cat would produce these protective Env fragments. It is possible that cats lacking the corresponding enFeLV are the individuals that succumb to FeLV-B-related disease. The protective peptide lacks the C11D8 VNA-inducing epitope (Neil, Fulton, Rigby, & Stewart, 1991) and would therefore be unlikely to induce a humoral immune response. A role for endogenous gammaretroviral elements in protection against exogenous infection has been previously observed in MLV infections in mice and is therefore a highly possible scenario (Bassin, Ruscetti, Ali, Haapala, & Rein, 1982; Ikeda, Ikeda, & Tsuchida, 1985; Jung, Lyu, Buckler-White, & Kozak, 2002; T. Wu, Yan, & Kozak, 2005).

In contrast to this potentially protective role against infection, there is also evidence that enFeLV proteins facilitate infection by exogenous FeLV-T, an otherwise entry-defective FeLV subgroup (see Section 1.10). It may be that individual enFeLV loci confer either protective or pathogenic qualities. However the exact mechanism by which the enFeLV peptide FeLIX contributes to cellular entry has not been elucidated.

It is apparent that further research is required to define the respective roles of individual enFeLV loci in FeLV disease and/or resistance. However recent research into enFeLV elements has focused upon the genomics and phylogenetics of these polymorphic loci. Full length enFeLV elements with putatively functional ORFs were recently identified, indicating they may be more active

than previously assumed (GenBank Accession numbers AY364318 and AY364319) (Roca, Pecon-Slattery, & O'Brien, 2004). That fact that some of these viral genomes possess identical 5 and 3' LTRs indicates they are relatively recent additions to the *Felis catus* genome, which became established between half a million to 2 million years ago (W. E. Johnson & Coffin, 1999). Supporting this hypothesis are the facts that these enFeLV genomes are only present in 9 - 15% of individual cats studied to date and have only been isolated from particular breeds (Roca, et al., 2005). They are also not found in closely-related *Felis* species, unlike truncated enFeLV loci (Roca, et al., 2004). Despite being "young" ERVs, these proviruses still display higher nucleotide homology to enFeLV than exogenous FeLV proviral genomes.

It is not known if these full-length enFeLV elements are being maintained through selective pressure or if they will degrade and become defective over time. The one potential benefit to the host (the protective role against FeLV-B infection) appears adequately provided by defective enFeLV elements (McDougall, et al., 1994). It would be of interest to investigate whether these full-length enFeLV genomes are isolated incidences of recent proviral germline integration or whether functional enFeLV genes are more widespread than previously assumed. The transcriptional activity of such loci, and their potential for incorporation into virions and subsequent horizontal transmission should also be addressed as this would have implications for the potential generation of pathogenic FeLV-B strains (see Section 1.8).

1.7 The FeLV subgroups and their host ranges

FeLV isolates can be classified into three main subgroups; FeLV-A, -B, -C (Russell & Jarrett, 1978b) which are distinguished both by their pathogenic potential and the receptors utilised for cellular entry. FeLV-T (Anderson, Lauring, Burns, & Overbaugh, 2000) represents a rare defective virus that confounds classical assays for viral subgroups; little is known about its frequency of occurrence in the field. Therefore the main genetic differences between the subgroups lie in the RBD of their respective SU proteins. Gammaretroviral cellular receptors are generally proteins displaying multiple transmembrane domains that function as transporters for small molecules (Overbaugh, Miller, & Eiden, 2001). The

receptor utilised by a simple retrovirus determines its classification into one of 11 known interference groups (Sommerfelt, 1999). Interference assays rely upon the fact that infection leads to downregulation or masking of the viral receptor upon the cell surface, preventing re-infection by a virus that would normally utilise the receptor. A cell can still be superinfected by a virus utilising a distinct receptor. Until recently, interference assays and *in vitro* host range analysis were the only available methods of classifying FeLV field isolates as the FeLV-A and –C receptors had not been identified. As the receptors for all FeLV subgroups have now been characterised and cloned, receptor usage assays utilising murine cell lines engineered to express the cognate receptors are commonly used. These allow more accurate analysis of the receptor choice of viral variants.

Although FeLV-A was generally regarded as ecotropic (i.e., able to only infect feline cells) (W. D. Hardy, 1993; O. Jarrett, Laird, & Hay, 1973; Sarma, Jain, & Hill, 1975) some isolates can infect canine and human cells *in vitro* (Moser, Burns, Boomer, & Overbaugh, 1998). However there are no recorded instances of humans or dogs being infected with FeLV (W. D. Hardy, Jr., et al., 1976; Schneider & Riggs, 1973). FeLV-B and FeLV-C are always isolated in conjunction with FeLV-A (O. Jarrett, Hardy, Golder, & Hay, 1978; Sarma & Log, 1973) and FeLV-A is the only naturally transmissible variant. FeLV-B and –C exhibit an expanded amphotropic host range, as they are both able to infect canine, human and mink cells *in vitro* (Anderson, et al., 2000; Boomer, Eiden, Burns, & Overbaugh, 1997; W. D. Hardy, Jr., et al., 1976; O. Jarrett, H.M. Laird, & D. Hay, 1969b; O. Jarrett, et al., 1973). FeLV-C is also able to infect guinea pig cells. The cell tropism of FeLV-T is limited to feline T cells, as this is the only known cell lineage to express FeLIX, the FeLV-T co-receptor (Lauring, Anderson, & Overbaugh, 2001) (See Section 1.10).

As FeLV-B and –C are generally more pathogenic than the transmissible FeLV-A, it is of significant interest to identify the blocks to transmission which are acting upon these subgroups. The observation that FeLV-B and –C cannot be transmitted without simultaneous FeLV-A infection was previously explained by the possibility of FeLV-A Env glycoproteins pseudotyping FeLV-B and/or FeLV-C cores (O. Jarrett, Golder, Toth, Onions, & Stewart, 1984). Pseudotyping has also been used to explain observations that simultaneous infection with FeLV-C and

enFeLV-FeLV-C chimaeric viruses significantly increases the rate of disease progression. In this case, the presence of enFeLV RBDs increases the tropism of the infectious virus, contributing to the broader host range observed in these experiments. However, only FeLV-C viruses established a chronic infection in this study, suggesting that the chimaeric Env preferentially packaged FeLV-C cores (Mathes et al., 1994).

However, recent studies have shown that co-expression of retroviral Env glycoproteins may induce the formation of heterotrimers which decrease the infectivity of the virus particles (Dewannieux & Collins, 2008). This was suggested to be a novel restriction mechanism of infectious retroviruses, and raises the question as to whether SU proteins form heterocomplexes in mixed subgroup FeLV infections. This may contribute to the lack of FeLV-B and –C transmission between hosts. Alternatively, it is possible that endogenous Env interact with those of infectious virus and induce heterotrimer formation; however this would require correct expression and glycosylation of the endogenous protein, something which is not definitively found in enFeLV elements to date.

1.8 FeLV-A

FeLV-A[Glasgow-1] is the prototypic strain of FeLV-A. However, much research has also been conducted on the weakly pathogenic FeLV-61E (Donahue et al., 1988). This was initially isolated as a helper virus for a highly pathogenic FeLV-FAIDS-inducing variant (see Section 1.10). FeLV-A is the only subgroup commonly transmitted between hosts and gives rise to FeLV-B and –C via intra-host evolution.

1.8.1. FeLV-A receptor: feTHTR1

Early research indicated FeLV-A utilised a 70kDa membrane protein (A. K. Ghosh, Bachmann, Hoover, & Mullins, 1992). It was later identified to be a thiamine transporter protein, referred to as THTR1, encoded by the SLC19A2 gene. The feline gene (feTHTR1) displays 93% amino acid identity to the human orthologue

(huTHTR1) (Mendoza, Anderson, & Overbaugh, 2006) which also has the capacity to function as a FeLV-A receptor. FeLV-A is the only retrovirus known to utilise this receptor and therefore creates a unique interference group.

Both the feline and human THTR1 genes are expressed in a broad range of tissues including small intestine, liver, kidney, skeletal muscle and peripheral blood lymphocytes (Diaz, Banikazemi, Oishi, Desnick, & Gelb, 1999; Dutta et al., 1999), supporting the broad ecotropic *in vitro* cell tropism of FeLV-A (Rojko, Hoover, Mathes, Olsen, et al., 1979). FeTHTR1 transcripts are also found in exceptionally high levels in oral mucosal tissues, corresponding to this being the initial site of FeLV-A entry and replication (K. A. Helfer-Hungerbuehler et al., 2011). Notably, the level of feTHTR1 transcripts in this tissue does not vary significantly in relation to a cats' age; therefore receptor expression levels cannot be responsible for the age-related resistance to FeLV-A infection that domestic cats display. It is also unlikely to be the lack of a functional receptor that protects most large felids from FeLV-A infection as feTHTR1 appears highly conserved across the Felidae family (K. A. Helfer-Hungerbuehler, et al., 2011).

The physiological role of THTR1 is to aid absorption of thiamine, or vitamin B1, in the intestinal tract and kidneys. It is not known whether FeLV-A binding and the subsequent infection impairs feTHTR1 function; however genetic disorders have been characterised indicating defective huTHTR1 proteins correlate with a decrease in intracellular thiamine and the onset of thiamine-responsive megaloblastic anaemia (Diaz, et al., 1999; Labay et al., 1999; Raz et al., 2000). The variety of non-thiamine-related disorders seen in infected cats does not support the hypothesis that FeLV-A binding to feTHTR1 causes a predictable impairment in function.

1.8.2. Pathogenesis of FeLV-A infection

FeLV-A isolates are often mistakenly regarded as minimally pathogenic (Donahue, et al., 1988; Roy-Burman, 1996) as severe FeLV-associated diseases more commonly develop in the presence of subgroups B or C. However highly pathogenic FeLV-A variants have been characterised, for example the FeLV-945 isolate (Chandhasin, Coan, & Levy, 2005; Chandhasin, Lobelle-Rich, & Levy,

2004) which causes non-T-cell multicentric lymphomas. This is due to a unique LTR and an unusually variable envelope sequence which together confer a replicative advantage and heightened pathogenicity to this isolate (Bolin, Chandhasin, Lobelle-Rich, Albritton, & Levy, 2011; Chandhasin, et al., 2005; Prabhu, Lobelle-Rich, & Levy, 1999). Infection with other FeLV-A isolates is commonly associated with thymic T-cell lymphoma, although a pathogenic mechanism has not been established (Neil, et al., 1991). Numerous other diseases have been associated with FeLV-A infection, including inflammatory and degenerative liver disease (Reinacher, 1989), chronic enteritis (Reinacher, 1987) and benign peripheral lymphadenopathy (Moore, Emerson, Cotter, & DeLellis, 1986). This confirms that most FeLV-A isolates have pathogenic potential, although it may not manifest as clearly as during FeLV-B or –C infection.

As FeLV-A does not encode a viral oncogene and infection is not routinely associated with a specific disease, the pathogenic mechanism/s have not been definitively established. As binding of FeLV-A Env to feTHTR1 does not appear to impair its function, it is possible FeLV-A pathogenesis is mostly mediated through insertional activation of cellular proto-oncogenes. Recent research indicates common retroviral integration sites exist within feline chromosomes A2 and B2 which may be targeted during FeLV-A infection of lymphoid cells (Fujino, Satoh, Ohno, & Tsujimoto, 2010). This has been suggested previously; the oncogene *c-myc* is activated in up to 30% of FeLV-induced tumours and leukaemias (L. S. Levy, Gardner, & Casey, 1984; Miura et al., 1987; Mullins, Brody, Binari, & Cotter, 1984; Neil et al., 1984), indicating it may be preferentially activated during integration. There is also evidence that the *flvi-2* locus is often affected by insertional mutagenesis during FeLV infection (L. S. Levy & Lobelle-Rich, 1992; L. S. Levy et al., 1993); this is additionally observed in multiple murine lymphomas and hence may be a conserved viral integration site within the gammaretroviridae (Uren, et al., 2005). Together *c-myc* and *flvi-2* activation contribute to approximately half of feline T-cell tumours (L. S. Levy, Lobelle-Rich, Overbaugh, et al., 1993). Other loci commonly involved in FeLV lymphoma development are *pim-1*, *bmi-1*, *flvi-1*, *fit-1* and *flit-1* (Fujino et al., 2009; Fujino, Ohno, & Tsujimoto, 2008; Levesque, Bonham, & Levy, 1990; L. S. Levy, Lobelle-Rich, & Overbaugh, 1993; Tsatsanis et al., 1994). It is possible these

proto-oncogenes contribute to human cancer progression, highlighting the role of FeLV as a model for other diseases and potential tool for oncogene discovery.

1.9 FeLV-B

FeLV-B isolates are found in approximately 40% of field cases, always alongside FeLV-A infection (Phipps, Hayes, Al-dubaib, Roy-Burman, & Mathes, 2000; Russell & Jarrett, 1976). The prototype strain is FeLV-B[Gardner-Arnstein] (Elder & Mullins, 1983; Nunberg, Williams, & Innis, 1984). FeLV-B genomes are formed via recombination of FeLV-A genetic material with endogenous proviral transcripts (Neil, et al., 1991; Overbaugh, Riedel, Hoover, & Mullins, 1988; Stewart et al., 1986). The phenomenon of retroviral variants arising through recombination is not restricted to FeLV; other examples include the pathogenic murine mink cell focus-forming viruses (L. H. Evans & Cloyd, 1984; Khan, 1984) and avian retroviruses (R. A. Weiss, Mason, & Vogt, 1973).

1.9.1. Evolution of FeLV-B envelope genes

The recombination event that leads to FeLV-B formation is hypothesised to occur during co-packaging of transcripts from both FeLV-A and enFeLV proviruses (Overbaugh, Riedel, et al., 1988; Tzavaras et al., 1990). Although the co-packaging of two distinct FeLV genomes within one viral core has not been directly observed, co-packaging of MLV retroviral RNAs resulting in a novel recombinant genome has been documented *in vitro* (Yin & Hu, 1997). Additionally, co-transfection of cell lines with multiple retroviral molecular clones gives rise to recombinant viruses, indicating heterodimeric RNA genomes must be produced (Pandey et al., 1991).

Although most of the enFeLV *gag* genes studied to date are highly mutated and thus assumed to be defective, both the packaging signal (Ψ) and leader sequences are intact (Berry, et al., 1988); it is therefore feasible that expression and packaging of RNA transcripts may occur from these loci. However the specific enFeLV loci that contribute to FeLV-B formation have not been identified; it is possible that the recently-characterised full length enFeLV *env*

genes (Roca, et al., 2004) contribute to the majority of FeLV-B genomes. Due to their intact LTRs and ORFs they are more likely to be transcriptionally active.

The endogenously-derived portions of FeLV-B *env* genes display >98% identity to enFeLV elements (Mullins, Hoover, Quackenbush, & Donahue, 1991), however the length of this enFeLV-related portion differs significantly between isolates and influences the replicative capacity of the final virus. This indicates that not all enFeLV loci have an equal capacity for replication-competent FeLV-B formation. The location of the recombination break points differs between isolates, although a central 250bp region of SU appears preferential (Sheets, et al., 1992). However, occasional 5' recombination points in FeLV-B genomes have been found in the *pol* gene indicating that this region must be transcriptionally active in at least some enFeLV loci (Overbaugh, Riedel, et al., 1988; Pandey, et al., 1991). This again indicates the full-length enFeLV loci are likely to be the contributing elements.

Although there is evidence from experimental infections that FeLV-A isolates differ in their capacity to recombine and produce FeLV-B (Phipps, Chen, Hayes, Roy-Burman, & Mathes, 2000), it is obvious that the transcriptional activity of specific enFeLV elements in the host is the main deciding factor determining whether FeLV-B arises during an infection. This is supported by the fact that the rate at which FeLV-B arises *in vitro* differs during infection of particular cells lines, an indication of the differing transcription levels of enFeLV loci in variable tissue types (Overbaugh, Riedel, et al., 1988).

Given the increase in pathogenicity and mortality associated with FeLV-B development in a host (see below), an accurate analysis of the transcriptional activity of various enFeLV loci and their subsequent contribution to FeLV-B formation would be highly informative. Recent advancements in both feline genomics and bioinformatics, as well as the ever-decreasing price of genetic sequencing, would now allow these questions to be readdressed.

1.9.2. FeLV-B receptor: fePit1

During formation of FeLV–B, the acquisition of a novel enFeLV-encoded RBD leads to a switch in receptor usage and subsequently an extended host range. FeLV-B can therefore infect human and canine cell lines *in vitro*, as well as the feline cell lines susceptible to FeLV-A (Hoover & Mullins, 1991). The cellular receptor for FeLV-B is the inorganic phosphate-sodium symporter, Pit1 (Rudra-Ganguly, Ghosh, & Roy-Burman, 1998). Both the human and feline proteins are functional viral receptors (Takeuchi et al., 1992) however the murine orthologue is not (Wilson, Farrell, & Eiden, 1994). This explains the restriction seen in rodent cell lines despite the broad expression of murine Pit1.

Some FeLV-B isolates can also utilise the homologous fePit2 cellular surface protein (Anderson, Lauring, Robertson, Dirks, & Overbaugh, 2001; Boomer, et al., 1997), which displays 60% amino acid identity to Pit1 and is the viral receptor for the amphotropic MLV (Miller, Edwards, & Miller, 1994; van Zeijl et al., 1994). The proportion of *env* which has originated from enFeLV loci determines whether a FeLV-B isolate can utilise Pit2 (Boomer, et al., 1997), as there are particular sequences within both the RBD and the SU C domain of Env that are required for successful utilisation of this protein (Boomer, et al., 1997; Sugai et al., 2001). The C domain of FeLV-B Env also contributes to the recognition of particular Pit1 orthologues (Faix, Feldman, Overbaugh, & Eiden, 2002), highlighting that the determinants of gammaretroviral receptor usage are not restricted to the prototypic RBD. It is thought that the C domain affects the post-binding steps of viral entry.

1.9.3. Pathogenesis of FeLV-B infection

Compared to other outbred species, both leukaemia and lymphoma occur amongst domestic cats at significantly high rates (Dorn, Taylor, & Hibbard, 1967). Specifically, FeLV-B infection is highly associated with lymphomas (Sheets, et al., 1993; Tsatsanis, et al., 1994; Tzavaras, et al., 1990), which occur in 10% of persistently FeLV-infected cats (Cotter, Hardy, & Essex, 1975; W. D. Hardy, Jr., et al., 1976). FeLV-B is also more commonly observed in

leukaemic cats than FeLV-A (O. Jarrett, et al., 1978; Tzavaras, et al., 1990). Despite these associations, a conserved oncogenic mechanism during FeLV-B infections has not been identified.

The most common FeLV-induced tumours are clonal T lymphoid tumours, which display fixed sites of viral integration across numerous cells (Casey, et al., 1981). They are generally classified according to their location; thymic (mediastinal), alimentary, multicentric (peripheral), and extranodal lymphomas have been identified and classified as "FeLV-B induced" (Lutz, et al., 2009). Although single tumours are most common, disseminated tumours involving multiple organs have been observed on occasion (Reinacher & Theilen, 1987). The long latency period associated with these diseases (between 2 and 3 years) implies FeLV-B infection is necessary but not always sufficient for lymphoma development, and its causative role(s) in these cancer progressions has not been determined. Notably, the presence of FeLV-B is specifically highly associated with thymic lymphoma, whereas non T-cell diseases (such as multicentric lymphoma) are associated with pure FeLV-A infections (Ahmad & Levy, 2010). This association indicates FeLV-B has an as-yet undefined pathogenic mechanism which causes such conditions.

It is generally thought that in addition to altering the clinical outcome, the development of FeLV-B accelerates disease progression, as a solely FeLV-A infection may remain asymptomatic indefinitely. However recent evidence indicates that co-inoculation of cats with FeLV-A and –B actually decreases both the rate of disease progression and development of chronic viraemia in a titre-dependent manner (Phipps, Hayes, et al., 2000). It may be that the genotype of the initial infecting FeLV-A contributes to the pathogenicity of the related FeLV-B.

It has been suggested that the main contributing factor to the increased pathogenicity of FeLV-B is the extended host range, conferred by the novel use of the fePit1 receptor. In addition to infection of naïve cells, this would also allow reinfection of chronically infected cells as the fePit1 protein would not be downregulated following the initial FeLV-A viraemia. However FeLV-B associated diseases do not always show a common cellular progenitor indicating this virus

does not preferentially target and disrupt a specific cell type. For example, neurological disorders have been associated with FeLV infection (Carmichael, Bienzle, & McDonnell, 2002) and recent studies found that both FeLV-B and enFeLV Env glycoproteins allow viral entry into cerebral endothelial cells (Chakrabarti, Hofman, Pandey, Mathes, & Roy-Burman, 1994). These are naturally resistant to FeLV-A and –C and so it appears FeLV-B would be the clinical cause of such symptoms. However this is not routinely observed; it is therefore highly difficult to predict disease outcome based purely upon the cellular tropism of the FeLV-B isolate. It is apparent, however, that FeLV-B formation is a hallmark for increased disease potential in the host. Research should be conducted into defining the molecular events which redirect disease outcome to lymphoma and/or leukaemia in such a large percentage of FeLV-B infections.

1.10 FeLV-C

FeLV-C is unique amongst the FeLV subgroups as it consistently induces pure red cell aplasia (PRCA) in infected cats, although it is not associated with any proliferative (neoplastic) disorders. It is extremely rare and is thought to affect only 1% of FeLV viraemic cats (Hoover, Kociba, Hardy, & Yohn, 1974; O. Jarrett, et al., 1984; O. Jarrett, et al., 1978; Mackey, Jarrett, Jarrett, & Laird, 1975). FeLV-C(Sarma), cloned and genetically sequenced in 1986 (Riedel, et al., 1986), is the prototype FeLV-C isolate. Experimental infections with this molecular clone induce the hallmark symptoms of FeLV-C infection, being fatal aplastic anaemia caused by a suppression of erythroid cellular development within 8 weeks of infection (Onions, Jarrett, Testa, Frassoni, & Toth, 1982; Riedel, et al., 1986). FeLV-C utilises the cellular protein FLVCR1 as a viral receptor (Tailor, Willett, & Kabat, 1999).

Similarly to FeLV-B, FeLV-C is only found alongside FeLV-A in naturally infected cats. Experimentally infected weanling kittens can clear FeLV-C infections unless it is administered alongside FeLV-A, and in these cases FeLV-C viraemia and the accompanying anaemia only appeared 20 weeks after FeLV-A (O. Jarrett, et al., 1984). However experimentally infected neonatal kittens can develop chronic viraemia and PRCA from FeLV-C alone, provided the virus is administered via

infected cell inoculation directly into the bone marrow (Dornsife, Gasper, Mullins, & Hoover, 1989). This correlates with two previous hypotheses; that FeLV in general displays age-restricted infectivity and FeLV-A is required to function as a helper virus to establish initial FeLV-B and FeLV-C infections (O. Jarrett, et al., 1984). It has been suggested that FeLV-A Env may pseudotype FeLV-C genomes, thus increasing the likelihood of the FeLV-C genome reaching cells wherein it may replicate and establish an infection (O. Jarrett, et al., 1984).

1.10.1. Evolution of FeLV-C envelope genes

FeLV-C was originally thought to arise through recombination with enFeLV, in a manner similar to that of FeLV-B (O. Jarrett & Russell, 1978; Overbaugh, Riedel, et al., 1988) as the SU of FeLV-C(Sarma) possesses two short regions with homology to enFeLV (Riedel, Hoover, Dornsife, & Mullins, 1988; Riedel, et al., 1986). FeLV-C also exhibits the extended host range of FeLV-B (thus it is not ecotropic) but has the additional capacity to infect guinea pig cells. However, most FeLV-C isolates do not contain enFeLV domains. It is now assumed that FeLV-C arises within a host after infection with FeLV-A through genetic drift of the viral genome, although this has never been shown experimentally. This leads to a switch in both the receptor usage and the pathogenic potential of the isolate. Thus FeLV-C isolates arise independently of each other, which is reflected by the lack of highly conserved regions within the Env of individual isolates (Rigby, et al., 1992), and the final genetic sequence partially reflects the original FeLV-A isolate. The specific mutations required within the Env protein to induce the FeLV-C phenotype and the pressures that may influence these mutations have not been identified.

Early research identified sequences within the RBD of specific isolates that correlated with the development of PRCA (Riedel, et al., 1988; Rigby, et al., 1992), and it was established that a mutation of 11 specific amino acids within this region of FeLV-A was sufficient to confer a FeLV-C cell tropism (Rigby, et al., 1992). However comparison of the RBDs from independently-isolated FeLV-C strains did not reveal any similarities, hence there does not appear to be a specific mutation essential for FeLV-C development. Mutations have been

identified that are conserved amongst numerous FeLV-C isolates; however when these are introduced into FeLV-A genomic backbones they do not alter the cell tropism (Brojatsch et al., 1992) and are not sufficient to induce PRCA. These mutations are also not conserved between all FeLV-C isolates characterised since this report.

As these isolates all induced PRCA in their hosts yet possessed differential mutations in the RBD, it appears that areas other than the traditional RBD of Env must contribute to both receptor recognition and disease progression. Mutations may be required across the entire Env protein to induce a switch from THTR1 to FLVCR and the subsequent PRCA. This hypothesis was supported by studies utilising chimaeric Env proteins, which found that replacing the 5' region of the FeLV-A RBD with that of FeLV-C did not induce hallmark PRCA symptoms (Rey, Prasad, & Tailor, 2008). Rather, the chimaeric viruses displayed an altered cell tropism and induced macrocytic anaemia. It was suggested that the chimaeras may have been able to utilise both THTR1 and FLVCR, albeit with lower efficiency (Rey, Prasad, et al., 2008).

Further investigations found the C-terminal region of the FeLV-C SU forms a second RBD, which binds FLVCR1 independently of the prototype N-terminal RBD (Rey, Prasad, et al., 2008). This "Cdom" is essential for successful viral binding and entry. Its binding to FLVCR1 is dependent upon the presence of the C2 disulphide-bonded loop within the C terminal of SU. However the sequence of the C2 loop is highly divergent between FeLV subgroup *env* genes, indicating it may indirectly play a role in receptor recognition. Although it appears that Cdom and the N-terminal RBD interact, soluble Cdom from FeLV-C is able to bind FLVCR1 in the absence of the N terminal RBD. The Cdom of FeLV-A may also recognise FLVCR1 independently of the traditional FeLV-C RBD (Rey, Prasad, et al., 2008). This observation supports the hypothesis that FeLV-C arises through the genetic drift of FeLV-A, and may explain why individual isolates may possess divergent sequences and yet consistently induce PRCA. It has been suggested that whilst the N terminal RBD confers receptor specificity, the Cdom provides a secondary receptor binding motif which binds to the viral receptor independently of the RBD, allowing viral entry to occur (Rey, Prasad, et al., 2008). A potential role for both the C2 loop and Cdom of SU in receptor

recognition is not restricted to FeLV, as similar hypotheses have been suggested for other gammaretroviruses (Barnett & Cunningham, 2001; M. Gemeniano, Mpanju, Salomon, Eiden, & Wilson, 2006; Lavillette, et al., 2001).

The hypothesis that the viral genome undergoes a series of mutations mediating the transition from FeLV-A to FeLV-C is supported by the recent discovery that an isolate previously characterised as being a mixture of FeLV-A, -B and –C was composed of a heterogeneous viral population including a tri-tropic Env protein able to use THTR1, FLVCR1 and FLVCR2 (Shalev et al., 2009). This Env contains a PRR and Cdom (including the C2 loop) similar to FeLV-A while the RBD region displayed mutations conserved with other FeLV-C genomes. This may contribute to the extended tropism seen in this isolate. It was suggested that this virus is in an intermediate stage of evolution towards FeLV-C. It is possible that eventually intermediate FeLV strains lose the ability to utilise feTHTR1 as mutations accumulate. Henceforth the viral variant would be limited in its transmission potential, explaining why FeLV-C is unable to transmit between cats.

1.10.2. FeLV-C receptor: feFLVCR1

As with the majority of retroviral receptors, FLVCR1 (feline leukaemia virus C receptor) is a membrane-spanning cellular transporter, with 12 transmembrane domains and 6 extracellular loops. The feFLVCR1 cDNA, sequenced prior to the discovery of the protein's function, was predicted to encode a protein approximately 560 amino acids in length, with a molecular weight of 60kDa (Tailor, et al., 1999). FLVCR1 was subsequently established as a haem exporter, a member of the major facilitator superfamily (Quigley et al., 2004; Tailor, et al., 1999).

The intracellular synthesis of haem proteins commences during maturation of BFU-E (Burst Forming Units – Erythroid) to CFU-E (Colony Forming Units – Erythroid). Under natural conditions a negative feedback system is in operation, as the haem proteins regulate the function of a transcriptional repressor, Bach1, which then regulates the transcription of globin genes (Rafie-Kolpin et al., 2000). Thus the presence of haem indirectly initiates the production of globins, which associate with the haem and are subsequently exported. It is hypothesised

that the wildtype function of FLVCR1 is as an “overflow valve” (Quigley, et al., 2004), operating to reduce levels of cytoplasmic haem in both erythroid precursors and erythrocytes. Inhibition of this function (such as binding of FeLV-C Env proteins) impairs erythroid maturation by increasing the cellular haem content, eventually leading to cellular apoptosis (Quigley, et al., 2004) and the non-regenerative anaemia seen in affected cats. Although expression of FLVCR1 is found in multiple haematopoietic lineages (Tailor, et al., 1999), impairment of this protein is only detrimental to erythrocytes and their progenitors (BFU-E and CFU-E cells) and does not affect granulocytes (Quigley et al., 2000).

The functional human orthologue, hFLVCR1, displays 88% nucleotide identity and 83% amino acid identity to the feline protein (Tailor, et al., 1999). A paralogue, hFLVCR2, has also been identified and displays 52% nucleotide identity to hFLVCR1. Although this protein functions as a haem transporter it cannot mediate infection by FeLV-C(Sarma) (Duffy et al., 2010). Although murine cells are resistant to all subgroups of FeLV, the murine FLVCR1 orthologue appears functional when over-expressed in host cells (Tailor, et al., 1999). There are multiple domains within FLVCR1 that contribute to its function as a viral receptor. Specifically, an aspartic acid residue (D487) within extracellular loop 6 confers receptor functionality upon FLVCR2 (J. K. Brown, Fung, & Tailor, 2006). Conversely this residue is not essential for FLVCR1, as replacement of D487 with the corresponding asparagine (N463) found in FLVCR2 does not deplete receptor functionality. The specific sequence of the first extracellular loop also appears necessary for the receptor function of FLVCR1, although individual essential residues within this loop have yet to be elucidated (J. K. Brown, et al., 2006).

Rearrangements within the hFLVCR1 gene have been associated with the presence of Diamond-Blackfan anaemia (Heyn, Kurczynski, & Schmickel, 1974), a fatal human congenital disorder characterised by an anaemia highly similar to feline PRCA. Other individuals with a similar disorder express alternatively spliced isoforms of FLVCR1 RNA transcripts (Rey et al., 2008). Thus impairment of the natural function of FLVCR1 may occur without viral interactions and may result in similar disease manifestations.

1.10.3. Pathogenesis of FeLV-C infection

During erythropoiesis, early erythroid progenitor cells (BFU-E) develop into late erythroid progenitor cells (CFU-E). CFU-E then develop into erythroid precursor cells, which later fully differentiate into erythrocytes. FeLV-C infection impairs the development of CFU-E cells from BFU-E both *in vivo* (Abkowitz, Holly, & Grant, 1987) and *in vitro* (Rojko et al., 1986), depleting the number of circulating CFU-E cells. The depletion of BFU-E is not as severe (Boyce, Hoover, Kociba, & Olsen, 1981; Onions, et al., 1982) although their growth kinetics are affected (Abkowitz, 1991; Abkowitz, Holly, & Adamson, 1987). The decrease in CFU-E and BFU-E coincides with the appearance of reticulocytopaenia and precedes the appearance of PRCA by approximately 2 to 3 weeks (Dornsife, et al., 1989). Clinical PRCA, hallmarked by a non-regenerative absence of circulating reticulocytes (Cotter, 1979; Mackey, et al., 1975) is evident by 6 weeks following experimental infection (Onions, et al., 1982); until the anaemia is quite far advanced non-specific clinical signs including fever, weight loss and fatigue may be observed. The SU domain of the Env of FeLV-C therefore acts as a dominant-negative protein, effectively inducing the PRCA phenotype by reducing and/or interfering with the cellular surface display of feFLVCR1 (Quigley, et al., 2000; Quigley, et al., 2004).

Colony Forming Units – Granulocyte-Macrophage (CFU-GM) cells, which originate and mature in the bone marrow, are infected but not functionally impaired during FeLV-C infection (Rojko, et al., 1986; Testa, Onions, Jarrett, Frassoni, & Eliason, 1983), however myelofibrosis (the replacement of bone marrow with scar tissue or collagen) has been observed in numerous FeLV-C infections (J. C. Olsen & Watson, 1980; Onions, et al., 1982). A pathogenic mechanism has not been determined and it is not known if this contributes to disease symptoms. It has been suggested that most cats simply die before these symptoms develop. FeLV-C replication is also associated with the aggregation and subsequent apoptosis of T lymphoma cells, a phenomenon only seen in infection with either FeLV-C or FeLV-C-based recombinants (Pandey, et al., 1991). This is thought to be due to the higher transcriptional activity of the FeLV-C LTR in these cell lines compared to fibroblastic cultures (Rojko et al., 1992). The implications of this phenomenon for the host have not been examined.

There have been attempts to treat the erythroid aplasia seen in FeLV-C infections with human interferon-α, however these treatments have produced conflicting results (Cummins, Tompkins, Olsen, Tompkins, & Lewis, 1988; Kociba, Garg, Khan, Reiter, & Chatfield, 1995). Treatment of naturally infected cats which displayed FeLV-C-associated disease with bovine interferon-ß also reported a decrease in symptoms and short-term recovery (Tompkins & Cummins, 1982). This is intriguing as FeLV-C infection disrupts the natural development of erythroid progenitor cells, making regeneration of erythrocytes highly unlikely whilst the virus is still actively replicating. Generally FeLV-C is regarded as fatal and with limited options for treatment.

1.11 FeLV-T

FeLV-T was isolated from a FeLV-A infected cat (Rohn, Linenberger, Hoover, & Overbaugh, 1994) and is a T-cell tropic subgroup of FeLV that induces lymphoid depletion and immunodeficiency in hosts. It appears to utilise the Pit1 receptor, despite having an apparently-THTR1-binding domain within Env. This is partially due to the lack of a functional fusion motif in the 5' region of SU, as the prototype FeLV-T virus possesses an asparagine (N) in place of the required histidine (H) in the fusion motif of SU (SPNQ, not SPHQ). To rescue this defect in cellular entry, FeLV-T requires expression of a cofactor in order to successfully infect T-lymphocytes (FeLIX). FeLV-T is the only gammaretrovirus identified to date that requires a cofactor for cellular entry (Lauring, et al., 2001; Overbaugh & Bangham, 2001) and thus does not display a traditional receptor-virus relationship. FeLIX (FeLV Infectivity X-accessory protein) is a truncated enFeLV envelope protein expressed in certain feline tissues, including T cells (Anderson, et al., 2000; Lauring, et al., 2001). As Pit1 is widely expressed in feline cells, FeLIX expression is the main factor limiting the tropism of FeLV-T. A mechanism of infection regarding how the combination of FeLV-T and FeLIX utilise the Pit1 receptor has not yet been elucidated.

FeLV-associated feline AIDs (FeLV-FAIDS) had been described prior to the discovery of FeLV-T, and the recent characterisation of FeLV-T and FeLIX mean that these observations may now be reinterpreted. For example, early research

identified an isolate that induced immunodeficiency, displayed a host range limited to T cells, and required the presence of a closely-related helper virus (Overbaugh, Donahue, Quackenbush, Hoover, & Mullins, 1988; Overbaugh et al., 1992). It is possible that this virus strain was an early isolation of FeLV-T.

1.11.1. FeLV-T envelope, FeLIX and Pit1 binding

FeLV-T arose through mutations in the SU domain of FeLV-A and the two subgroups display approximately 96% nucleotide identity (Donahue et al., 1991; Overbaugh, Donahue, et al., 1988). It is assumed that FeLV-T arose through genetic drift in a manner similar to that of FeLV-C (Gwynn, Hankenson, Lauring, Rohn, & Overbaugh, 2000; Rohn, Moser, Gwynn, Baldwin, & Overbaugh, 1998). The switch in receptor usage may result in FeLV-T being able to re-infect the initially infected cells, despite the down-regulation of the FeLV-A receptor (feTHTR1) which would usually prevent super-infection. This correlates with early evidence that FeLV-FAIDS isolates did not establish interference against homologous challenge (Moser, et al., 1998; Rohn, et al., 1998).

FeLV-T receptor specificity (that is, the ability to utilise Pit1 alongside a requirement for FeLIX expression) has been attributed to amino acids 6, 7 and 8 of the SU protein and the presence of a four amino acid insertion, with an additional adjacent mutation, in the C terminal of envelope glycoprotein (Cheng et al., 2006). This supports mounting evidence that both the C and N terminal regions of Env contribute to the receptor usage of FeLV variants, not merely the traditional RBD. This is further supported by the fact that some viruses displaying chimaeric envelope glycoproteins can utilise FeLV-C and FeLIX entry pathways (Cheng, et al., 2006).

FeLIX is 273 amino acids in length and displays 92.3% identity to the FeLV-B SU domain; in fact FeLV-B SU domains can functionally substitute for FeLIX (Lauring, Cheng, Eiden, & Overbaugh, 2002). It also possesses a putative signal peptide marking it for extracellular transport and is therefore a secreted peptide, as it lacks the hydrophobic transmembrane anchor. The secretion of FeLIX is confirmed by the fact that it functions *in trans*, as cell-free FeLIX

renders previously resistant Pit1-expressing cell lines susceptible to FeLV-T infection. However secretion is not essential for FeLIX function (Anderson, et al., 2000). Studies of the crystal structure of FeLIX indicate that it aids infection by catalysing the membrane-fusion event following receptor binding (Barnett, Wensel, Li, Fass, & Cunningham, 2003). This supports suggestions that FeLIX is an endogenous Env peptide that may rescue non-functional exogenous FeLV isolates. The concept of a soluble RBD transactivating the entry of heterologous retroviruses has been raised previously. In the case of the porcine endogenous retroviruses, the ability to infect otherwise restrictive cells is due to the presence of a soluble heterologous RBD and its cognate receptor (Lavillette & Kabat, 2004). It is possible that FeLIX allows entry of all non-functional FeLV isolates in this manner, regardless of their individual subgroup.

Notably, even though FeLV-B is able to utilise Pit2 for cellular entry, only Pit1 is able to mediate the binding of either FeLV-B SUs or FeLIX to allow FeLV-T progressive infection. Studies involving chimaeric Pit1/2 proteins have identified three regions within Pit1 that determine successful FeLIX binding, however these are not required for FeLV-B infection (Lauring, et al., 2002). Pseudotype assays with both FeLV-B and FeLV-T envelopes, conducted in the presence of soluble FeLIX indicated that FeLIX does not interfere with FeLV-B infection, confirming that they utilise different regions of Pit1 during viral binding (Shojima, Nakata, & Miyazawa, 2006). Therefore in addition to FeLIX, FeLV-T itself may be required to bind to Pit1 to ensure successful infection (Lauring, et al., 2002). To date, although FeLIX binding to Pit1 is known to be essential for FeLV-T infection, it is not known if FeLV-T itself is able to bind to any cellular receptor.

1.11.2. Pathogenesis of FeLV-T infection

FeLV-induced immunosuppression occurs in every infected cat to a degree, as all FeLV subgroups are able to infect haematopoietic cells. It may have numerous manifestations, including thymic atrophy, lymphopaenia, neutropaenia, and the loss of CD4+ and CD8+ lymphocytes (Ogilvie, Tompkins, & Tompkins, 1988; Orosz, Zinn, Olsen, & Mathes, 1985a, 1985b; Perryman, Hoover, & Yohn, 1972). FeLV-FAIDS is clinically characterised by persistent viraemia, weight loss and lymphoid hyperplasia followed by severe lymphoid depletion and a subsequent

high susceptibility to opportunistic secondary infections (Hoover, Mullins, Quackenbush, & Gasper, 1987). These clinical symptoms are assumed to be a result of cytopathic effect (CPE) within the infected lymphocytes, as FeLV-T infection of T cells induces syncytia *in vitro* (Rohn, et al., 1998). In addition to the lack of circulating T lymphocytes, immunosuppression may be partially due to the impaired ability of FeLV-T infected hosts to produce a primary antibody response against invading pathogens. This is associated with the presence of a 34 amino acid region within the C terminal of the SU domain of FeLV-T isolates. Although a mechanism has not been identified, it is thought that these isolates may display a heightened ability to infect and impair the function of certain immune cell subsets (Quackenbush et al., 1990).

Experimental inoculation of cats indicates a fatal immunodeficiency develops after an incubation period as short as 60 days, dependent on the age of the cat upon inoculation (Mullins, Chen, & Hoover, 1986). However survival rates depend highly upon the rate of immunosuppression; cats displaying a rapid depletion of circulating T-lymphocyte progenitor cells usually survive for approximately 3 months after the onset of clinical symptoms (Quackenbush, Mullins, & Hoover, 1989). However survival for up to a year has been observed in individuals displaying gradual immunosuppression, although this is associated with a higher rate of extranodal lymphoma (Hoover, et al., 1987; Mullins et al., 1989).

Unusually high levels of un-integrated viral DNA are often observed prior to the onset of clinical symptoms, mainly within the bone marrow, intestine and lymphoid tissues (Mullins, et al., 1986; Mullins, et al., 1991). Although it is difficult to detect *in vivo* due to the low level of synchronously infected cells, *in vitro* studies indicates this occurs a few days after infection (Hofmann-Lehmann, et al., 2001). It is not known if this contributes to or is a symptom of the clinical diseases seen in these infections.

It has been suggested that “discordant” cats, which present with antigenaemia but are negative in virus-isolation assay, may be infected with fusion-defective FeLV-T-like isolates which utilise the endogenous FeLIX expressed in T-lymphocytes. An assay has therefore been developed recently to detect defective FeLV-T virions in clinical samples. This relies upon an indicator cell

line which constitutively expresses FeLIX, thus allowing the otherwise-defective virus to replicate and induce a hallmark syncytia in the monolayer (Nakaya, Shojima, Hoshino, & Miyazawa, 2010). It remains to be seen whether this assay proves informative in the characterisation of potentially-defective viruses. It is true that isolates possessing defective fusion motifs have been identified previously (Chandhasin, et al., 2005), however as these were present within a whole viral population they may not require FeLIX expression to enter a cell. Although there are numerous aspects of FeLV-T infection and replication which remain un-investigated, the extreme rarity of this subgroup makes this research less of a priority compared to further characterisation of the pathogenic –B and –C subgroups.

1.12 Other feline retroviruses

Apart from FeLV, there are numerous other infectious retroviruses of felines which differ in their pathogenicity and disease associations. Two of these are complex retroviruses; feline immunodeficiency virus (FIV) is a highly pathogenic lentivirus, whilst feline foamy virus (FeFV) is an entirely apathogenic spumavirus. Within the simple retroviruses, feline sarcoma virus (FeSV) is a rare derivative of FeLV associated with spontaneous sarcoma development. Additionally, the domestic cat genome contains numerous non-FeLV related endogenous retroviral elements, including the infectious retrovirus, RD114.

1.12.1. Feline immunodeficiency virus

FIV was isolated in 1986 in the USA (N. C. Pedersen, Ho, Brown, & Yamamoto, 1987). As a lentivirus, it is a complex retrovirus displaying high similarity in both its genomic structure and pathogenic mechanisms to HIV. However HIV is commonly transmitted through sexual contact, whereas the primary mode of transmission of FIV is via salivary transmission. FIV has a worldwide distribution and the prevalence of infection varies between 1 and 15% of healthy cats, according to geographic region. Some estimates reach an infection rate of 44% amongst sick cats (Miyazawa & Mikami, 1993).

FIV infection is associated with a range of diseases, including gingivostomatitis, anaemia, neurological conditions and various degrees of immunosuppression (Ishida, Washizu, Toriyabe, & Motoyoshi, 1988; Yamamoto et al., 1989). Although FIV is often regarded as a fatal infection, disease progression may only occur after a long latency period. FIV is thought to escape immune clearance during this period by acquiring mutations in immunodominant hypervariable regions (Pancino et al., 1993). It is not known whether FeLV utilises a similar mechanism to avoid viral clearance.

The initial acute phase of FIV infection is characterised by high viral loads in circulating blood. This is followed by an extended asymptomatic phase during which cats may not be shedding infectious virus particles. The third phase of infection is characterised by a generalised lymphadenopathy, followed by severe AIDS-related disorders and a final terminal AIDS condition. Opportunistic infections, including upper respiratory tract infections and gastrointestinal problems, commonly occur during this latter period, at which point the immune system is chronically depleted (Ishida & Tomoda, 1990; Miyazawa, 2002).

Vaccines that induce protective immunity are commercially available, however as vaccinated cats are serologically indistinguishable from infected cats their use is not routinely recommended (J. Levy, et al., 2008). Cats produce antibodies against SU, TM, and multiple *gag*-encoded proteins after approximately four weeks of infection (Hosie & Jarrett, 1990). However the duration of this humoral response is questionable (Kohmoto et al., 1998). Protection is also provided by CTL activity which contributes to viral clearance (Flynn et al., 1995).

Similar to FeLV, the FIV genome contains the *gag*, *pol*, and *env* essential genes (Maki et al., 1992; Olmsted, Hirsch, Purcell, & Johnson, 1989). However unlike gammaretroviruses the Gag and Pol proteins are not produced from a singular polyprotein; although a single RNA transcript is produced, Pol is only translated following a ribosomal frameshift (Morikawa & Bishop, 1992). Additionally, both the TM and SU domains of Env are heavily glycosylated (Maki, et al., 1992; Olmsted, et al., 1989; Poss, Dow, & Hoover, 1992), in comparison to FeLV where glycosylation is restricted to the SU domain. FIV also contains three accessory genes, *vif*, *rev* and *ORF-A*. Rev is a regulatory protein involved in the transport

of immature viral mRNAs from the nucleus into the cytoplasm preceding translation (Kiyomasu et al., 1991; Phillips et al., 1992; Tomonaga et al., 1993). The role of ORF-A has yet to be fully elucidated, however it appears to be involved in post-transcriptional processing of RNA and is essential for viral infectivity (M. C. Gemeniano, Sawai, Leutenegger, & Sparger, 2003). The Vif (viral infectivity factor) protein is conserved amongst lentiviruses and is critical for virus expression from otherwise-resistant cells.

1.12.2. Feline foamy virus

Feline foamy virus (FeFV), also known as feline syncytium-forming virus, is a complex retrovirus of the Spumavirus genus. It was isolated in 1969 (Fabricant, Rich, & Gillespie, 1969; Riggs, Oshirls, Taylor, & Lennette, 1969) and epidemiological studies indicate a worldwide distribution amongst both domestic and wild felids (Daniels, et al., 1999; Winkler, Lochelt, & Flower, 1999). Prevalence of infection is estimated to be between 14 to 28% of domestic cats (Miyazawa, 2002) however it has been suggested that this may rise to 70% amongst older cats (Flower, Wilcox, Cook, & Ellis, 1985; Winkler, Flugel, Lochelt, & Flower, 1998; Winkler, et al., 1999). This is reflective of the fact that infections are of a lifelong duration with chronic viraemia (German, Harbour, Helps, & Gruffydd-Jones, 2008; Meiering & Linial, 2001). The virus displays a broad cell tropism both *in vitro* and *in vivo* (German, et al., 2008) (Gaskin & Gillespie, 1973), although the receptor has yet to be identified. Additionally, the methods of transmission have not been elucidated (Winkler, et al., 1999), although it is suspected to be through mutual grooming and/or fighting (i.e., an oronasal primary route of transmission, similar to that of FeLV and FIV).

Spumaviruses are generally regarded as apathogenic (Meiering & Linial, 2001). However CPE, including syncytia formation and vacuolisation leading to apoptosis, has been observed in some infected cell lines (Meiering & Linial, 2001) and there has been recent controversy regarding whether or not they are truly apathogenic (German, et al., 2008). FeFV infection has been associated with chronic progressive arthritis, although this may merely be due to the high prevalence of the virus (N. C. Pedersen, Pool, & O'Brien, 1980). Supporting this hypothesis is the fact that experimentally infected cats did not display any

clinical symptoms through six months of infection, although histopathology was noted in the lungs and kidneys upon autopsy (German, et al., 2008). Multiple previous studies of experimentally infected cats also indicated FeFV is purely apathogenic (Schwantes, Ortlepp, & Lochelt, 2002) (Alke, Schwantes, Zemba, Flugel, & Lochelt, 2000; Gaskin & Gillespie, 1973). Despite the asymptomatic infection, cats mount immune responses to FeFV infection and antibodies can be detected approximately two weeks post-infection (German, et al., 2008). This serology can be used to distinguish between the two genetically-distinct subtypes of FeFV (Flower, et al., 1985) which display 57% nucleotide identity within the SU domain of their Env proteins (Winkler, et al., 1998). However this does not correlate to differences in their host range, as would be expected for FeLV.

As spumaviruses are complex retroviruses, their genomes contain additional accessory genes as well as the standard *gag, pol* and *env* ORFs. FeFV possesses two accessory genes, both encoding non-structural proteins; *tas* (previously termed *bel-1*) and *bet*. The *bet* ORF is not essential for viral replication and is thought to aid in viral cellular defence by binding to feline restriction factors (Chareza et. al., 2012). However the Tas protein is essential for viral replication and plays a role in transactivation of the LTR promoter elements (Keller et al., 1991). Despite the conserved presence of accessory genes, there are numerous features that distinguish spumaviruses from their complex lentiviral relatives:

(1) Although the majority of intracellular (immature) virions contain viral RNA, mature foamy virus particles contain DNA (Roy et al., 2003). The RNA is reverse transcribed within the particle before viral adsorption and cell entry (Yu, Baldwin, Gwynn, Yendapalli, & Linial, 1996).
(2) Spumavirus genomes contain an internal promoter within the *env* gene which drives transcription of both *tas* and *bet* (Lochelt, Flugel, & Aboud, 1994).
(3) FeFV *pol* genes are expressed from spliced sub-genomic transcripts (Bodem, Lochelt, Delius, & Flugel, 1998), whereas in other retroviruses a large polyprotein is translated and cleaved into the respective viral enzymes.
(4) The FeFV virions appear immature, even after budding (a hallmark of spumaviruses). This is partially due to inefficient cleavage of the Gag polyproteins and to the Env protein containing a different leader peptide to

other retroviruses. This induces virion release from the ER, rather than the plasma membrane (Goepfert, Wang, & Mulligan, 1995).
(5) FeFV is also unique as the Gag proteins do not localise to the nucleus at any point in the viral replication cycle (Bodem, et al., 1998), unlike the simian foamy viruses (Schliephake & Rethwilm, 1994). The Gag proteins of other retroviruses (including FeLV) translocate to the nucleus in order to interact with nucleic viral RNA prior to nuclear export (Garbitt-Hirst, Kenney, & Parent, 2009).

Due to its apathogenicity and broad cell tropism, FeFV has not been studied in detail in recent years, and the majority of recent research has centred around its use as a viral vector for gene therapy applications (Bastone & Lochelt, 2004; Schwantes, Truyen, Weikel, Weiss, & Lochelt, 2003). Interest in the spumaviruses has also been revived recently by the discovery of an endogenous spumavirus within the genome of the two-toed sloth (*Choloepus hoffmanni*) (Katzourakis, Gifford, Tristem, Gilbert, & Pybus, 2009) indicating these viruses have been present as infectious agents of mammals for at least 100 million years. This was the first endogenous foamy virus to be characterised and indeed one of the first endogenous complex retroviruses (the first being the discovery of an endogenous lentivirus of rabbits in 2007 (Katzourakis, Tristem, Pybus, & Gifford, 2007)).

1.12.3. Feline sarcoma virus

Feline sarcoma virus (FeSV) is a rare retrovirus which induces approximately 2% of fibrosarcomas in cats. Although this virus has not been the focus of recent studies, early research indicated it was especially prevalent in young cats presenting with fibrosarcomas (Snyder, 1971). FeSV isolates are all genetically unique, as the virus arises from recombination events between exogenous FeLV-A and host cellular DNA (Frankel, Gilbert, Porzig, Scolnick, & Aaronson, 1979; Guilhot, Hampe, D'Auriol, & Galibert, 1987). The recombination events ensure the FeSV genome does not contain intact *env* sequences; thus all FeSV viruses are replication defective (Henderson, Lieber, & Todaro, 1974) and require the continued presence of replicating FeLV to spread. Infected cells therefore express chimaeric virus-host proteins that induce malignancy. Although the

specific oncogenic mechanism differs between isolates, the activation and transduction of a cellular proto-oncogene is essential. For example, the *fes* gene is activated in the Snyder-Theilen FeSV strain (Sherr, Fedele, Oskarsson, Maizel, & Woude, 1980), inducing transformation of the infected cells.

Although systemic FeLV viraemia may be observed in the affected cat, the FeSV virus is contained within the tumour itself (de Noronha, Grant, Lutz, Keyes, & Rowston, 1983). Therefore FeSV is not transmissible under natural conditions, although cats have been experimentally infected with a mixture of both FeSV and FeLV viruses (de Noronha, et al., 1983). Transfection of naïve cells with the recombinant proviral genome, in the absence of FeLV, is also sufficient to induce initial transformation of the host cell (Barbacid, 1981) although the virus is unable to spread.

FeSV has been reported worldwide and there is no indication that specific FeLV-A strains are more likely to result in FeSV formation. However there is evidence of preferential transduction and activation of cellular tyrosine-specific protein kinases (Barbacid, Beemon, & Devare, 1980; Hampe, Gobet, Sherr, & Galibert, 1984; Naharro et al., 1983; Van de Ven, Khan, Reynolds, Mason, & Stephenson, 1980; Ziemiecki et al., 1984). These genes tend to be highly conserved across species and transduction of orthologues by other oncogenic retroviruses has been documented (reviewed in Hampe, *et al* 1984) therefore they may represent common retroviral integration sites. FeSV-induced tumours are also distinct from the vaccination site-associated sarcomas initially described in 1991 (Hendrick & Goldschmidt, 1991). These are associated with an increased inflammatory response following vaccination with adjuvanted inactivated FeLV and rabies vaccines (Hendrick, Goldschmidt, Shofer, Wang, & Somlyo, 1992; Macy, 1995).

FeSV was previously associated with the presence of FOCMA (feline oncornavirus-associated cell-membrane antigen) (Essex, Grant, Cotter, Sliski, & Hardy, 1979), which was originally termed a “non-virion tumour specific surface antigen” (Sliski, Essex, Meyer, & Todaro, 1977). FOCMA is detected at the cellular surface of infected lymphoid cells within the tumour, but not within non-transformed cells regardless of their FeLV status. It was thought to be a cellular protein, induced by either transformation events or the expression of FeSV recombinant

proteins (Sliski, et al., 1977) as it was found to be immunologically distinct from viral antigens (Stephenson, Essex, Hino, Hardy, & Aaronson, 1977). It was also suggested that FOCMA may be incorporated into FeSV pseudotypes (Sliski & Essex, 1979), therefore cats with sufficient anti-FOCMA antibodies will clear FeSV infection and exhibit tumour regression, while FeLV continues to circulate (Essex, 1977; Essex, Klein, Snyder, & Harrold, 1971).

However FOCMA is now known to be a combination of several viral components and not a tumour-specific antigen as previously thought (Lutz, et al., 2009). The absence of previous evidence for this may be partially explained by the fact that although anti-FOCMA MAbs displayed high reactivity to FeLV-C antigens (Vedbrat et al., 1983), the antibodies only bound to immature viral particles. FOCMA-specific antibodies were also found in the sera of FeLV "regressor" cats, which had successfully cleared the active infection but may express occasional immature viral proteins (Rojko, et al., 1982).

1.12.4. Endogenous retroviruses: RD114 and FcEV

The genome of the domestic cat, *Felis catus*, contains ERVs from 3 families, being the primate retrovirus MAC-1, RD114- and FeLV-related elements (enFeLV) (O'Brien, 1986). Recently a further ERV, *Felis catus* endogenous virus (FcEV) was identified as a Type C ERV present as multiple defective copies throughout the domestic cat genome (van der Kuyl, Dekker, & Goudsmit, 1999).

Similarly to enFeLV, the RD114 viruses are only found within the *Felis* genus and have been identified in the genomes of numerous wild small felids (Benveniste & Todaro, 1975; Reeves & O'Brien, 1984). The *Felis* genus split from the Felidae main lineage approximately 6.2 million years ago (W. E. Johnson, et al., 2006), indicating exogenous RD114 integrated into the *Felis* ancestral germline at this time. RD114 is a recombinant retrovirus possessing the *gag-pol* and LTR regions of FcEV (van der Kuyl, et al., 1999) alongside the *env* gene of baboon endogenous retrovirus, an inducible endogenous betaretrovirus found in numerous primate species. Comparison of the baboon endogenous retrovirus and FcEV genomes identified two highly homologous regions which would facilitate recombination (van der Kuyl, et al., 1999). FcEV and RD114 also both contain an

intergenic spacer between the *pol* and *env* genes (van der Kuyl, et al., 1999), which is unusual for gammaretroviruses (Thayer et al., 1987). During recombination these regions ensured reading frames were not disrupted, allowing for the production of a functional retrovirus.

RD114 belongs to the Type D interference group (Sommerfelt & Weiss, 1990) and the RD114 receptor is a neutral amino acid transporter (Rasko, Battini, Gottschalk, Mazo, & Miller, 1999; Schnitzer, Weiss, Juricek, & Ruddle, 1980). This gene is highly conserved between species and widely expressed across various tissues (Green, Lee, & Rasko, 2004). The *in vitro* host range of RD114 is therefore broad and yet does not include murine cells, similarly to FeLV (Kakimi et al., 1990; Rasko, et al., 1999). Until recently RD114 was regarded as an endogenous xenotropic virus as the majority of virus isolates could not exogenously infect feline cells (Livingston & Todaro, 1973). Although released at low levels in numerous feline cell lines (Baumann, Gunzburg, & Salmons, 1998; East, Knesek, Allen, & Dmochowski, 1973; Fischinger, Peebles, Nomura, & Haapala, 1973; O. Jarrett & Ganiere, 1996; Okabe, Gilden, & Hatanaka, 1973) and inducible in others (Livingston & Todaro, 1973; Todaro, Benveniste, Lieber, & Livingston, 1973) it was not thought to be expressed *in vivo*. However there is recent evidence that RD114 may be able to infect various feline cells (Sakaguchi, Okada, Shojima, Baba, & Miyazawa, 2008), although mechanisms to restrict this may exist. Resistance to infection by two RD114-related viruses with distinct envelope proteins has been observed (Haapala, Robey, Oroszlan, & Tsai, 1985), indicating some feline cells restrict viral infection via an envelope glycoprotein-independent method. This is in addition to a reported novel restriction mechanism, which indicated that both the producer cell line and the glycosylation state of the host cell receptor determine the infectious titre of the virus (Dunn, Yuan, & Blair, 1993), similar to results found in ecotropic MLV studies (Knoper, et al., 2009). There is also evidence that host-mediated silencing may reduce potential proviral expression (Spodick, Ghosh, Parimoo, & Roy-Burman, 1988). The extent of this silencing may be reduced in tissue culture, leading to the elevated retroviral expression *in vitro* compared to that found in actual tissue.

The copy number of FcEV and RD114-related sequences within the domestic cat genome is approximately 20 (Reeves, Nash, & O'Brien, 1985). However, only a single copy of RD114 is responsible for viral production (Reeves, et al., 1985). The fact that the *env* genes of many FcEV proviruses are heavily deleted whilst RD114 has maintained functionality indicates there is a selective pressure upon RD114, supported by the fact that all RD114-related proviruses identified to date contain intact LTRs (Reeves, et al., 1985). There may be pressure to maintain functional 5' LTRs to drive transcription of the *gag-pol* genes, which would contribute to the intracellular transcripts found in many studies (Niman, et al., 1980).

Increased RD114 expression has been observed in numerous malignant tissues, predominantly lymphomas. However many of the cancer-affected cats exhibiting elevated RD114 expression were also exogenously infected with FeLV. It is possible that expression of RD114 is up-regulated following FeLV infection, although whether this is a contributing factor to cancer development or a subsequent effect has not been determined. However FeLV-negative lymphomas also exhibit elevated RD114 expression in some cases (Niman, Stephenson, Gardner, & Roy-Burman, 1977), indicating the putative FeLV-R114 interaction is not essential for RD114 production in malignant tissues. There are also reports of CPE (mainly syncytia formation) occurring in numerous infected cell lines (Germain, Roullin, Qiao, de Campos Lima, & Caruso, 2005; Klement & McAllister, 1972; Rand & Long, 1973). Despite these observations, no diseases are definitively associated with RD114 expression or infection and there is no evidence that domestic cats produce RD114-specific antibodies (Mandel, Stephenson, Hardy, & Essex, 1979). Research upon this endogenous virus is therefore not a priority when compared to the numerous aspects of FeLV which require attention.

1.13 Scope and aims of this thesis

After an extensive review of the literature, it is apparent that despite the volume of research conducted since the discovery of FeLV (W. F. Jarrett, et al., 1964) there remains many areas in which further study is required. These include clinical aspects, such as the development of safer and more efficient vaccines

and treatments, identification of the protective correlates of the feline immune response and the respective roles of both VNAs and CTL responses in FeLV protection. Considering the threat FeLV represents to endangered wild felids, such as the Iberian lynx and Florida panthers (M. A. Brown, et al., 2008; Luaces et al., 2008), this should be a priority for FeLV research despite the decreasing prevalence of the virus in domestic cats.

In addition to clinical research, there are many questions regarding FeLV genetics and retroviral biology that require renewed attention, such as investigating the factors which restrict inter-host transmission of FeLV-B and -C. Considering the recent advancements in molecular biology and ever-expanding knowledge of host-retroviral interactions (mainly due to HIV research), many of these areas may now be revisited.

Firstly, as FeLV-B and –C are highly pathogenic, it is of significant interest to ascertain which factors influence the development of these subgroups within the host. Is it purely based upon the infectious strain or do host-derived factors play a role in the development of FeLV variants? If FeLV-B evolution simply requires the presence of enFeLV transcripts, what factors prevent transmission of either wholly enFeLV virions and/or purely FeLV-B viruses to naïve hosts? The recent discovery of polymorphic putatively-functional enFeLV genomes (Roca, et al., 2004) has provided novel tools which will prove useful for this research.

Regarding FeLV-C evolution, what factors influence the development of these viral variants, and why does this occur in only a minority of natural infections? Is development of FeLV-C purely due to the genotype of the initially infectious FeLV-A; or does the host immune system affect FeLV-C development, in a process similar to that seen in other retroviruses to escape the humoral response? The recent discovery of dual-tropic Env variants in a FeLV-C primary isolate (Shalev, et al., 2009) suggests that FeLV-A evolves into FeLV-C through the stepwise acquisition of mutations. If this is the case, can this process be mimicked *in vitro* and the Env determinants of FLVCR1 usage mapped?

It is apparent that these questions, which may have been difficult to address previously, now have the potential to be solved using modern molecular techniques. The overall aims of this thesis were therefore:

- To identify Env determinants which may predispose FeLV-A strains to preferentially develop into FeLV-C variants;
- To investigate potential roles of the host humoral response in FeLV-C evolution;
- To determine the potential for inter-host FeLV-B transmission without the additional presence of a FeLV-A “helper” virus;
- To characterise the functionality of enFeLV elements and the possibility of enFeLV horizontal transmission between hosts.

The results of these investigations will contribute to the knowledge of feline genetics and immunology, FeLV clinical prognosis and retroviral biology and evolution.

2. Materials and Methods

2.1 Cell culture techniques

2.1.1. Maintenance of cell lines

Mus dunni tail fibroblast (MDTF) cells (ATCC Catalogue CRL-2017) and guinea pig foetal (104C1) cells (C. H. Evans, Cooney, & DiPaolo, 1975) were maintained in low-glucose DMEM (Life Technologies, Paisley, U.K.), supplemented with 10% foetal bovine serum, 100U/mL penicillin and 100µg/mL streptomycin. MDTF cells expressing the human Pit1 protein were kindly donated by Prof C. Tailor, University of Toronto.

Human embryo kidney (HEK293T) cells (Graham, Smiley, Russell, & Nairn, 1977) and QN10 (S+L- feline embryonic fibroblastic AH927) cells were maintained in high-glucose DMEM (Life Technologies), supplemented with 10% foetal bovine serum, 100U/mL penicillin and 100µg/mL streptomycin.

Feline embryo A (FEA) cells (O. Jarrett, et al., 1973) were maintained in high-glucose DMEM (Life Technologies), supplemented with 10% foetal bovine serum, 100U/mL penicillin, 100µg/mL streptomycin, 2mM L-Glutamine and 0.1 mg/mL sodium pyruvate. FEA cells chronically infected with multiple strains and isolates of FeLV were obtained from the University of Glasgow Companion Animal Diagnostics facility.

All cells were regularly subcultured when approaching confluency. During subculturing, cells were washed with phosphate-buffered saline (PBS) and treated with 0.5-2mL of 0.05% Trypsin-EDTA (Life Technologies).

2.1.2. Transient transfection of adherent cells

Adherent cells were transfected with Superfect Reagent (Qiagen, Crawley, U.K.) according to the manufacturer's protocol. Cells were seeded the day before transfection into 10cm plates, to approximately 60% confluency. The day of transfection, 60µL of Superfect reagent was mixed with 12µg plasmid DNA and made up to 300µL with incomplete, serum and antibiotic-free DMEM. Following 15 minutes incubation at room temperature, the DNA-Superfect complexes were then added directly to the cells and allowed to incubate for 3 hours at 37°C. DNA complexes were then removed and complete DMEM added to plates. After three days, transfection products were harvested, passed through a 0.45µm filter and stored at -80°C prior to use.

2.1.3. X-Gal staining of lacZ-expressing cells

Pseudotypes encapsidating transcripts of the pMFG plasmid lead to expression of the lacZ reporter gene after infection of the target cell. X-gal staining was performed to detect this expression. Briefly, confluent monolayers of cells were washed in PBS before being fixed with 0.5% glutaraldehyde (in PBS) for a minimum of 30 minutes. Fixative solution was removed and cells re-washed before the addition of staining solution (PBS containing 0.02% X-Gal, 3mM ferro-cyanide, 3mM ferri-cyanide, 1.3mM $MgCl_2$). Plates were stored at 4°C overnight before the lacZ-expressing cells (indicated by a blue appearance) were counted manually.

2.1.4. Stable transduction of cell lines

To engineer MDTF cell lines that stably expressed specific retroviral receptors, adherent cells were infected with VSV-G-enveloped pseudotypes. The pseudotypes encapsidated transcripts of the pFB-NEO plasmid containing the cDNA of the desired receptor. Constructs were kindly provided by Prof. C. Tailor, University of Toronto. Prior to transduction, $1x10^5$ cells were seeded in a T25 flask (Thermo Scientific, Loughborough, U.K.) and allowed to adhere

overnight. The media was then removed and 2mL of pseudotypes, including 4µg/mL hexadimethrine bromide (polybrene, Life Technologies), was added on to the cells. After eight hours incubation, this medium was removed and replaced with whole DMEM. Selection of transduced cells was induced two to three days later by the addition of 800µg/mL G418 (Life Technologies). Cells were subcultured twice weekly upon confluency for three weeks, at which point G418 concentration was reduced to 400µg/mL to maintain expression of the target genes.

2.1.5. Interference assay

Interference assays are a traditional technique in retrovirology used to classify receptor usage of novel viruses. FEA cells chronically infected with FeLV-A, -B and –C ("indicator" cells) were kindly provided by the Companion Animal Diagnostics Unit, University of Glasgow. To classify novel FeLV strains, naïve FEA cells were infected with a sample of the primary isolate and cultured for 10 days. At this point, supernatant from the cells was titrated upon the various indicator cells lines. 3 days post-infection, cells were scored for cytopathic effect. Superinfection was indicated by the presence of multiple rounded, apoptosing cells, as opposed to the fibroblastic monolayer of FEA cells usually displayed. A lack of superinfection indicated the novel sample contains that particular subgroup of FeLV as the downregulated receptor is not available for cellular entry.

2.1.6. Reverse interference assay

Traditional interference assays require the viruses to have identical producer cell lines (in the case of FeLV, FEA cells are used). For this reason, reverse interference assays were utilised to compare receptor usage of viruses from a range of producer cells, including HEK293Ts. Reverse interference assays also allow detection of which viral receptors are masked or downregulated within a cell line infected with a novel isolate. Briefly, virus was harvested from chronically infected HEK293T and FEA cell lines and passed through a 0.45 nm filter, before being titrated on to QN10 (S+L-) cells at 20% confluency (the

primary indicator cells). QN10 cells are feline fibroblastic cells stably expressing a defective sarcoma virus genome, which may be co-packaged and transmitted during infection with an exogenous functional retrovirus. Transformation of this cell line is therefore indicative of the sarcoma viral genome being mobilised by a functional FeLV; these mixed-population virions possess the envelope glycoprotein of the novel sample. After transformation was observed, supernatant was harvested, filtered and titrated upon FEA cells chronically infected with FeLV of known or unknown classification (the secondary indicator cells). The presence of transformation, indicative of superinfection, was scored 3 days after infection.

2.1.7. QN10 focus-forming assays

QN10 (S+L-) cells were seeded into 6 well plates at 20% confluency and allowed to adhere overnight prior to infection. Titrations of infectious virus was used to infect the cells for two hours at 37°C. 72 hours post-infection, the numbers of foci were manually counted and the viral titre (in FFU/µL) was calculated.

2.2 Protein-based assays

2.2.1. Reverse transcriptase detection assay

A commercially-available ELISA-based kit was used to detect and/or quantify RT activity within viral supernatants (C-type RT Activity Kit, Cavidi Technology, Uppsala, Sweden). This utilises a 96-well ELISA plate coated with an RNA template, which is reverse-transcribed by RT present within the sample. During reverse transcription BrdUTP is incorporated into the nascent DNA strand. In accordance with manufacturer's instructions, samples were prepared with the provided Dilution Buffers before the ELISA plate was incubated at 33°C for three hours. After washing, the RT-Product Tracer component was added and the plate incubated at 33°C for 90 minutes, during which time an alkaline phosphatase-labelled anti-BrdU antibody binds to the RNA-DNA heteroduplexes. Following a

secondary wash, alkaline phosphatase substrate is added to the sample. The processing of this substrate produces a colorimetric reaction which can be quantified using a spectrophotometer (Ascent Multiskan, U.K.).

2.2.2. SDS-PAGE and immunoblots

Immunoblots were performed to detect expression of specific proteins in both cell lysates and concentrated virus preparations (prepared by ultracentrifugation of cell-free virus supernatant, at 30 000 rpm for one hour in a Beckman Ultracentrifuge with a SW40 rotor). Samples were diluted to the desired volume using BPB Protein Loading Buffer and heated to 90°C for five minutes prior to electrophoresis. Electrophoresis was conducted in either 4-12% gradient precast polyacrylamide gels (Life Technologies) at 90V for two hours in MES buffer, or in SDS-polyacrylamide gels of variable percentages in Tris-Glycine buffer. Proteins were transferred to nitrocellulose membranes using the iBlot transfer system (Life Technologies).

Membranes were blocked overnight using 2% skimmed milk powder and 0.1% (v/v) Tween-20 in PBS. Primary antibodies were used at suitable dilutions in blocking buffer, for one hour at room temperature. The anti-capsid (p27) MAb (clone VPG19.1) was used unpurified as a hybridoma culture supernatant at a dilution of 1:500. Purified anti-SU (gp70) MAb was used at $1:10^5$. Anti-RD114 SU primary antibody (a kind gift from Hans Lutz) was used at $1:10^4$. The anti-HA MAb was used unpurified as a hybridoma culture supernatant at a dilution of 1:500.

After incubation with the primary antibody, membranes were washed three times in PBS with 0.1% Tween-20 prior to incubation with secondary antibody. Biotinylated secondary antibodies from various species were utilised (Vector Laboratories, Peterborough, U.K.) at a 1:1000 dilution in blocking buffer for one hour at room temperature. The “ABC-AmP” kit (Vector Laboratories) was then utilised to allow highly sensitive detection of proteins. Following treatment with the secondary antibody, an alkaline phosphatase-labelled avidin complex was bound to the membrane. Proteins were then visualised using a chromogenic alkaline phosphatase substrate.

Cell membranes to be assayed in immunoblots were prepared from cultured cells according to previous descriptions (J. K. Brown, et al., 2006). Briefly, $1x10^6$ MDTF cells were Dounce-homogenised in 1mL PBS, after which the nuclei were removed by centrifugation at 1000g for 15 minutes. Membrane fractions were then pelleted by ultracentrifugation at 30,000rpm for one hour at 4°C before being resuspended in 20µL PBS or bromophenol blue protein loading buffer.

2.2.3. Immunofluorescence

Immunofluorescence was utilised to detect expression of specific proteins in cell cultures. For the detection of haemagluttinin (HA)-tagged retroviral receptors, $3x10^4$ receptor-expressing MDTF cells were seeded into each well of 8-well multi-test glass slides (Flow Laboratories, Surrey, U.K.) and allowed to adhere overnight. Cells were fixed in 3.7% paraformaldehyde for 10 minutes. Rabbit anti-HA IgG (Sigma-Aldrich, Gillingham, U.K.) was used at a 1:35 dilution (in PBS with 0.1% azide, 1% bovine serum albumin) at room temperature for 45 minutes. Cells were washed before incubation with a 1:500 dilution of FITC-labelled goat anti-rabbit IgG (Sigma-Aldrich) for 30 minutes prior to visualisation using a UV microscope (Leica Microsystems, U.K.).

For the detection of internal enFeLV Env proteins within FEA cells, a permeabilisation step was included after fixation by paraformaldehyde. This involved incubating cells with cold methanol at -20°C for 10 minutes, before washing and incubating with the primary antibody (murine anti-gp70 MAb).

2.2.4. Flow cytometry

MDTF cells were dispersed using 1mM EDTA before 50µL of supernatant containing the protein of interest was incubated with 10^5 cells, for 30 minutes on ice. Cells were washed with PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBS-BSA-Az). 0.5µg of PE-labelled antibody specific for human IgG (eBiosciences, Hatfield, U.K.) was then added to each reaction and incubated for a further 30 minutes on ice. Cells were washed and re-

suspended in PBS-BSA-Az before being analysed on a Beckman Coulter Cytomics FC500 flow cytometer.

2.3 Molecular cloning techniques

2.3.1. Cellular nucleic acid extraction

Genomic DNA was extracted from $1x10^6$ cells using a commercially available kit (QIAamp Blood Mini kit, Qiagen) according to manufacturer's instructions. This protocol utilises DNA binding to a silica gel membrane before repeated washing in 70% ethanol-based buffers. Purified gDNA was eluted with water and its concentration and purity analysed with a Nanodrop spectrophotometer (Thermo Scientific).

Whole RNA was extracted from feline tissue samples using a commercial kit (RNEasy, Qiagen). Tissue samples were taken from uninfected negative control cats during in a previous study, snap-frozen in liquid nitrogen and stored at -80°C. Briefly, 300mg of tissue was disrupted with a mortar and pestle in liquid nitrogen without thawing. Lysates were passed through a homogenising column (QIAshredder, Qiagen) before the RNA was purified using the RNEasy columns and protocol. Final RNA concentration and purity was analysed with a Nanodrop spectrophotometer.

2.3.2. Viral RNA extraction

Viral supernatant from confluent cultures was harvested and passed through a 0.45µm filter, prior to centrifugation in a Beckman ultracentrifuge (SW40 rotor) at 30 000rpm for one hour at 4°C. Viral pellets were resuspended overnight at 4°C in PBS. Viral RNA was extracted using the QIAamp UltraSens Viral kit (Qiagen) according to manufacturer's instructions. As the silica-based membranes utilised in this protocol bind all nucleic acid, following elution with water RNA was DNase-treated to remove potential contaminating cellular gDNA (Amplification-grade DNase, Life Technologies). In order to allow

subsequent cDNA synthesis, DNase was inactivated by EDTA-treatment and heating the sample to 65°C for 15 minutes.

2.3.3. cDNA synthesis

First-strand cDNA synthesis was conducted with a commercial MLV RT enzyme (Life Technologies) according to manufacturer's instructions. In order to preferentially reverse-transcribe polyadenylated mRNA (including viral genomes) oligo-dT primers were used. Dithiothreitol was included to reduce secondary structures within the template RNA. Ribonuclease inhibitors (RNaseOUT, Life Technologies) were included in all cDNA synthesis preparations.

2.3.4. Plasmid constructs

The pMDG plasmid (Naldini et al., 1996), expressing vesicular-stomatitis virus G protein (VSV-G), was used to produce pseudotypes that would enter cells through endocytosis.

The pMFG plasmid (Ohashi et al., 1992), encoding ß-galactosidase (lacZ) with a MLV-packaging signal, was used to detect pseudotype entry and retroviral integration through expression of the lacZ reporter gene. LacZ expression was measured by 5-bromo-4-chloro-3-indolyl-ß-d-galactoside (X-Gal) staining.

The pCMVi plasmid (Towers et al., 2000), encoding MLV gag-pol, was used to produce MLV(FeLV) pseudotypes. The FGA construct contains a functional molecular clone of the FeLV-A(Glasgow-1) viral genome, in the pUC18 vector.

Retroviral expression vectors, based upon the pFB-NEO plasmid (Agilent Technologies, Wokingham, U.K.) containing the complete cDNA of various retroviral receptors with 5' haemagluttinin (HA) tags were kindly obtained from Prof C. Tailor (J. K. Brown, et al., 2006; Rey, Prasad, et al., 2008). The pVR1012 plasmid (Vical Inc., San Diego, U.S.A.) is an expression vector

modified from the pUC plasmid (Hartikka et al., 1996) containing a cytomegalovirus early promoter to drive target gene expression.

The pTORSTEN mammalian expression vector drives expression of soluble proteins with an incorporated C-terminal human IgG1-Fc tag upon transfection of susceptible cell lines (Spiller et al., 2006).

2.3.5. General cloning techniques

pVR1012 (described above), pcDNA3.1 (Life Technologies) and pBR322 (Bolivar et al., 1977) vectors were utilised for cloning various genes and sections of FeLV genomes. Although most PCR protocols incorporated restriction enzyme sites to facilitate cloning into pVR1012, TA cloning was conducted with the pcDNA vector (TOPO TA Cloning, Life Technologies).

2.3.5.1. Polymerase chain reaction

Oligonucleotide primers for PCR were obtained from MWG Biotech (Ebersberg, Germany). All PCRs were conducted using a pre-prepared, commercially available mastermix (High Fidelity PCR Master, Roche, U.K.) and thermal cycling was conducted by a GeneAmp PCR System 9700 (Applied Biosystems, Warrington, U.K.). A list of primers and conditions for specific PCRs is included in Appendix 8.2.

2.3.5.2. Purification of PCR products

PCR products were electrophoresed on 0.8-1.5% agarose gels containing ethidium bromide, generally at 100V for one hour in TBE buffer. Amplicons were visualised under UV light and gel-excised using sterile scalpels. PCR products were then purified from the agarose using the QIAquick Gel Extraction kit (Qiagen) according to manufacturer's instructions. This protocol effectively precipitates DNA with isopropanol before binding it to a silica-based membrane and repeated ethanol washing, prior to elution with water.

2.3.5.3. Restriction enzyme digestion and ligation

When cloning inserts into specific vectors (for example, pVR1012) restriction digests were conducted upon both the vector and PCR product to create ssDNA overhangs to aid ligation. All restriction digests were conducted according to manufacturer's instructions, regarding the suitable buffer(s) and the presence of bovine serum albumin in the reaction. Digestions were conducted at 37°C. Adequate digestion was visualised by an alteration of the migration patterns of the DNA upon gel electrophoresis (see above). DNA was purified from agarose as previously described. Overnight ligation of DNA fragments was conducted with T4 ligase (Life Technologies) at 16°C.

2.3.5.4. Transformation

DH5α Max Efficiency competent cells [genotype F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k-, m_k+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1] (Life Technologies) were transformed with ligation products according to the manufacturer's protocol. Briefly, 2μL of ligation products were added to 20μL competent cells and incubated on ice for at least 30 minutes before being heat-shocked at 42°C for 45 seconds. After further two minute incubation on ice, 900μL SOC broth (Life Technologies) was added and cells were incubated at 37°C for an hour whilst being shaken continuously. Cultures were spread on to LB agar plates containing the desired antibiotic (kanamycin at 50μg/mL or ampicillin 100μg/mL) and incubated overnight at 37°C. Positive transformants (containing the desired insert within the vector) were identified by restriction digest of DNA isolated from overnight cultures of individual bacterial colonies.

2.3.6. Preparation of plasmid DNA

A commercial kit was used to isolate small volumes of plasmid DNA, according to manufacturer's instructions (QIAquick Minikit, Qiagen). Glycerol stocks of

bacterial cultures were prepared by mixing 500µL of actively-growing culture with 500µL sterile 60% glycerol, before being stored at -80°C. Large-scale plasmid purification from up to 500mL of actively-growing culture was conducted using commercial kits (HiPure Maxiprep Kit, Qiagen).

2.3.7. Genetic sequencing

All DNA was sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Briefly, a PCR was conducted using specific primers and the recommended conditions (1 cycle at 96°C, 1 minute; followed by 25 cycles of (1) 96°C, 10 seconds; (2) 50°C, 5 seconds; (3) 60°C, 3 minutes). Sequencing reaction products were precipitated using the ethanol/sodium acetate method. 100µL of DNA was added to 1µL 3M sodium acetate and 200µL 100% ethanol. After mixing the solution was stored at -80°C for at least 30 minutes. DNA was precipitated by a 10 minute centrifugation at 13 000 rpm using a benchtop microcentrifuge, then washed once with 200µL 70% ethanol. DNA pellets were air-dried thoroughly before being re-suspended with 20µL highly-deionised formamide (Applied Biosystems). Sequencing reaction products were analysed using an ABI Prism 3130xl Genetic Analyser (Applied Biosystems).

2.3.8. Site-directed mutagenesis of plasmids

The QuickChange Lighting Mutagenesis kit (Agilent Technologies) was utilised to enable accurate and efficient mutation of specific nucleotides within the FeLV-A(Glasgow-1) molecular clone and the pVR1012 construct encoding the FeLV-A(Glasgow-1) Env gene. The protocol was conducted according to manufacturer's instructions, however PCR products were precipitated with ethanol/sodium acetate following Dpn1 treatment. They were then re-suspended in a small volume of water prior to transformation into competent cells.

2.4 *In silico* and bioinformatics techniques

2.4.1. RNA structural models

RNA secondary structures were modelled using the programs RNAStructure Version 5.3 (Reuter & Mathews, 2010), the mFold webserver (available online, http://mfold.rna.albany.edu/?q=mfold) (Zuker, 2003) and the Alifold web-server (available online, http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi) (Gruber, Lorenz, Bernhart, Neubock, & Hofacker, 2008) using default parameters (pH 7.0, temperature of 37°C).

RNAStructure Version 5.3 predicts structures within nucleic acid sequences based upon the minimal possible free energy of the structure(s). Although an optimal structure is produced, this program also allows visualisation of alternative structures with less optimal free energies. This program requires the input of two distinct RNA sequences and allows modelling of both homo- and hetero-dimers.

The mFold webserver predicts nucleotide secondary structures based upon the thermodynamic stability, including free energy, of the resulting model(s). Multiple predictions are produced for each input sequence; these often contain conserved structural motifs which are predicted to be highly stable.

The Alifold webserver requires input of a multiple sequence alignment, within which the individual sequences can be assumed to possess similar secondary motifs (for example, as a ClustalW alignment or FASTA file). The output file is a single structural prediction based upon the consensus sequence. The relative colour coding of the individual nucleotides reflects their conservation within the original alignment and/or their ability to base-pair (see Chapter 6 for further details). This structural model takes into account the minimal free energy as well as to the phylogenetic relationships between the input sequences.

Both the mFold and Alifold webservers model the secondary structures within monomeric RNA. To model dimeric RNA, the two RNA sequences are arranged consecutively with a string of >9 uridine bases separating them. This synthetic "linker" forms a hairpin structure from which the monomeric sequences extend, allowing interactions between them to be modelled.

2.4.2. Multiple sequence alignments

Genetic data was analysed with DNADynamo (Blue Tractor Software Ltd, U.K.). Both protein and nucleotide multiple sequence alignments were created with Seaview Version 4.3.2 (Galtier, Gouy, & Gautier, 1996; Gouy, Guindon, & Gascuel, 2010). Seaview utilises the ClustalW2 program for maximum alignment and parsimony (Larkin et al., 2007).

2.4.3. Transcription factor screens

MatInspector was used to screen LTRs for potential TF binding sites. This web-based software is available from Genomatix Software (www.genomatix.de). This program analyses a genetic sequence (the input sequence, provided by the user) for the presence of annotated transcription factor binding sites and other promoter and enhancer elements (Cartharius et al., 2005; Quandt, Frech, Karas, Wingender, & Werner, 1995). A quality rating is assigned to each detected site; this is based upon the degree of similarity between the input sequence and a matrix describing the TF family.

2.4.4. Graphs and statistics

Graphs were constructed using SigmaPlot 8.0. Error bars represent the standard error of the mean (SEM). Statistical tests (paired and unpaired Student's T tests) were performed using SigmaPlot 8.0.

3. Specific FeLV-A strains may be predisposed towards conversion to FeLV-C

3.1 Introduction

There are three main subgroups of FeLV; FeLV-A, -B and –C, from which only FeLV-A appears to be transmitted efficiently between hosts (W. D. Hardy, Jr., et al., 1976; W. D. Hardy, Jr., Old, Hess, Essex, & Cotter, 1973). Despite FeLV-C strains being fully replication competent *in vitro* (Riedel, et al., 1986), replication *in vivo* is thought to require the continued presence of FeLV-A. Thus the A subgroup is commonly referred to as a "helper" virus required for transmission and dissemination of novel subgroups within the host. FeLV-A is often mistakenly termed the "low-pathogenicity" variant (Donahue, et al., 1988) as approximately 60% of exposed cats mount a competent immune response and successfully clear infection following a transient viraemia (Hoover & Mullins, 1991; N. C. Pedersen et al., 1977). However, FeLV-A is pathogenic and in cats in which a chronic infection is established a range of clinical signs may develop, including immunosuppression, lymphoma and anaemia (Chandhasin, et al., 2005; Mackey, et al., 1975; Reinacher, 1989).

The disease association and clinical prognosis of an infection is influenced by both the genotype of the FeLV-A isolate and the presence of other subgroups which arise *in vivo* (O. Jarrett, 1992). FeLV-C arises within the infected host through the gradual acquisition of mutations within the viral genome (Rigby, et al., 1992) and is thus frequently identified alongside a concurrent FeLV-A infection (O. Jarrett, et al., 1978; O. Jarrett & Russell, 1978; Sarma & Log, 1973). FeLV-C is found in approximately 1-2% of chronically infected cats and its emergence is associated with the development of PRCA (O. Jarrett, et al., 1984; O. Jarrett, et al., 1978; Mackey, et al., 1975). This non-regenerative anaemia is fatal within approximately 2-3 months of FeLV-C arising in the cat (Onions, et al., 1982; Riedel, et al., 1986).

The development of FeLV-C infection from an initial infection with a FeLV-A isolate is accompanied by an alteration in the receptor usage of the virus, from

the thiamine transporter feTHTR1 utilised by FeLV-A (Mendoza, et al., 2006) to the haem transporter, feFLVCR1 utilised by FeLV-C (Tailor, et al., 1999). The binding of FeLV-C to feFLVCR1 impairs the normal cellular function of the protein, preventing haem transport in erythroid cells and resulting in a depletion of erythrocyte precursors (Quigley, et al., 2000; Quigley, et al., 2004). Although the widespread cellular distribution of both feTHTR1 and feFLVCR1 means that FeLV-A and –C are able to infect multiple lineages of haematopoietic cells (lymphoid, erythroid and myeloid), the pathogenic potential of subgroup C viruses appears to be conferred by the ability to interfere with the function of feFLVCR1 on erythroid progenitor cells, rather than the widespread infection of diverse cell types (Dean, Groshek, Mullins, & Hoover, 1992).

The development of FeLV–C is due to alterations within the SU domain of the Env glycoprotein (Brojatsch, et al., 1992; Riedel, et al., 1988; Rigby, et al., 1992). These mutations affect the RBD, the region that determines the cognate receptor used for cellular entry. Accordingly, mutations within this region of Env alter the cellular tropism of the virus and FeLV-C possesses an expanded *in vitro* host range (Boomer, et al., 1997; W. D. Hardy, Jr., et al., 1976; O. Jarrett, et al., 1969b; O. Jarrett, et al., 1973). Previous studies have demonstrated that a 241 amino acid region within the Env of prototype FeLV-C(Sarma) conferred the ability to induce PRCA in experimental infections (Riedel, et al., 1988). Subsequently, the primary determinant of this phenotype was mapped more precisely to a string of 92 amino acids within the RBD of isolates of FeLV-C cloned biologically (Brojatsch, et al., 1992; Rigby, et al., 1992). It was noted that there was limited conservation between the sequence of individual isolates of FeLV-C (Brojatsch, et al., 1992; Rigby, et al., 1992), supporting the assertion that there is minimal inter-host transmission of FeLV-C and that each isolate arises *de novo* within a unique host. Protein signatures or structures that are conserved between all FeLV-C isolates have yet to be identified, and the critical residues that are essential and sufficient to confer FLVCR binding upon Env have not been elucidated.

It has been assumed that the acquisition of the Env mutations that define the C subgroup would lead to the emergence of two distinct viral populations with non-overlapping receptor tropisms within the host, resulting in the FeLV-A/FeLV-

C co-infections observed in clinical cases. However, recent studies have suggested that the evolution of FeLV-C *in vivo* may be a gradual process, with viruses displaying intermediate phenotypes and receptor usages co-existing within the infected host (Shalev, et al., 2009). Indeed, virus isolates have since been identified that utilise both THTR1 and multiple FLVCR1 paralogues (Shalev, et al., 2009). Presumably, these FeLV-A/C dual-tropic viruses would eventually give rise to a FeLV-C isolate which would utilise feFLVCR1 solely.

Previous research conducted in our laboratory had used 7 primary FeLV field isolates which had been confirmed previously to contain both subgroups A and C by interference assays (Table 3.1) (Adema, 2003). Comparisons between the *env* genes of these subgroup A strains with those of prototypic FeLV-A would allow identification of mutations which may contribute to an initial interaction with FLVCR1. Their presence would therefore enhance the possibility of FeLV-C development occurring following genetic drift during subsequent cycles of replication.

Table 3.1: Primary FeLV isolates.

FEA cells were infected with 7 primary FeLV isolates previously classified as being either A/C or A/B/C co-infections through interference assays (Adema, 2003). Three isolates had been partially characterised previously.

Isolate	Subgroup Classification	Previous References
FA27	A/C	Onions, 1982; Brojatsch, 1992; Rigby 1992
FS246	A/C	Onions, 1982; Brojatsch, 1992; Rigby 1992
FZ215	A/B/C	Brojatsch, 1992; Rigby 1992
FA621	A/C	N/A
FX343	A/C	N/A
L3128F	A/C	N/A
L3950F	A/C	N/A

FEA cells were chronically infected with the FeLV-A/C primary isolates and proviral *env* genes were cloned from genomic DNA. The PCR conditions, “FeLV-Env”, are detailed in Appendix 8.2. Consistent with their designations by interference, multiple unique *env* sequences were obtained from each isolate,

confirming the heterogeneity of the viral populations within these isolates. The majority of variation between Envs was located within the region encoding the RBD (Figure 3.1). These results indicated that primary isolates of FeLV-A/C mixed infections contain semi-conserved polymorphisms. By comparing the amino acid sequences of the novel variants with the prototypic FeLV-A(Glasgow-1) (Stewart, et al., 1986) and FeLV-C(Sarma) (Riedel, et al., 1986) strains, each Env was tentatively identified as either the subgroup A or subgroup C component of the isolate. Criteria for classification as a likely FeLV-C Env were the presence of substantial amino acid substitutions and/or length polymorphisms in the "Vr1" region (Rigby, et al., 1992) of the RBD. Viral variants that appeared genetically to be of the FeLV–B subgroup (present in isolates FY981 and FZ215) and which showed characteristic evidence of recombination with endogenous FeLV sequences were not included in subsequent analyses.

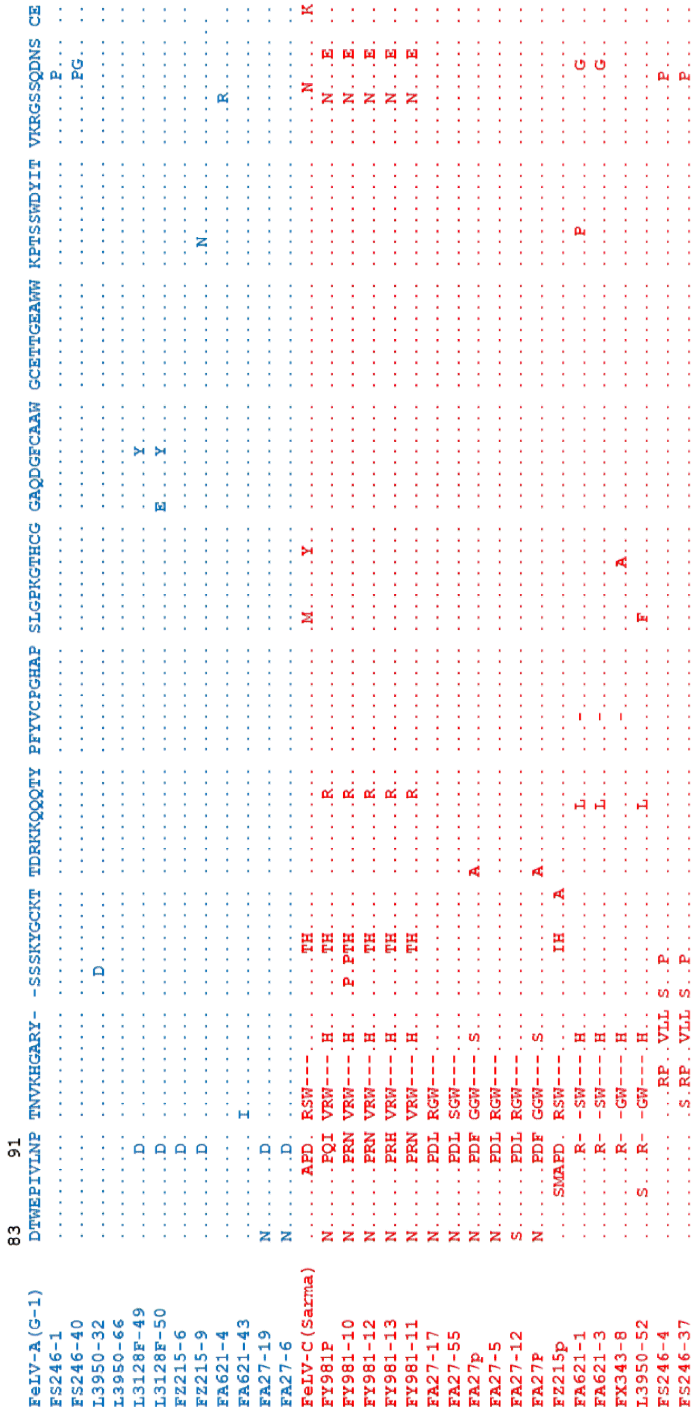


Figure 3.1: RBDs from the *envs* of anaemogenic strains of FeLV.

FEA cells were infected with primary isolates of FeLV containing both subgroups A and C from cats with anaemia. *Env* genes were amplified from purified genomic DNA and the nucleic acid sequences of multiple clones determined. Sequences in blue were putatively assigned to subgroup A; sequences in red were assigned to the FeLV-C component/s of the isolate. Dots indicate conservation with the prototype FeLV-A(Glasgow-1) sequence. Dashes are included to maintain alignment conservation. Residues 83 and 91 in the N terminal region are annotated. Figure adapted from (Adema, 2003).

The sequences of the FeLV-A components were highly conserved between isolates, despite the lengthy interim between their isolation. It is widely assumed that all retroviruses acquire significant levels of variation over time due to the error prone enzymatic activity of reverse transcriptase, the actions of cellular antiviral factors and the selective pressure from the host immune response (Overbaugh & Bangham, 2001). However, while this is indeed the case for HIV, and to a lesser extent FIV, most isolates of FeLV-A date are remarkably similar (Donahue, et al., 1988). In comparison, FeLV-C is thought to be poorly transmissible and each isolate arises independently within the infected cat. This is reflected by the high degree of divergence observed in the RBD region of Env between isolates. Despite this heterogeneity, no mutations were identified that were conserved across all the FeLV-C *env* sequences.

Within the A/C mixtures Envs were identified that were ostensibly subgroup A by sequence alignment and yet bore point mutations from the reference strain FeLV-A(Glasgow-1) (Figure 3.1). It can be assumed that the FeLV-A Envs were the parental viruses of the FeLV-C isolates within their respective hosts; therefore these mutations may have affected the receptor utilisation of the FeLV-A Env, increasing the likelihood of FeLV-C developing.

Within the FA27 isolate, variants were identified with an aspartate to asparagine substitution D83N, a residue that varied in two of the novel FeLV-Cs. This non-conservative mutation (D83N) was documented previously in the FY981 virus, a variant that is able to utilise FLVCR1, FLVCR2 and THTR1 (Shalev, et al., 2009). Similarly, as asparagine to aspartate substitutions (N91D) was present in three of the novel FeLV-As (L3128F, FZ215 and FA27), ablating a potential site for N-linked glycosylation and aligning with a region of FeLV-C Envs that is critical to the determination of the subgroup C phenotype (Riedel, et al., 1986; Rigby, et al., 1992). Asparagine-91 of FeLV-A(Glasgow-1) is replaced with a Serine (S91) in both FeLV-A(3281) and FeLV-A(61E) (Donahue, et al., 1988). In contrast, FeLV-A(Rickard) which has been shown to give rise to FeLV-C *in vivo* (Chen, et al., 1998; Phipps, Chen, et al., 2000) contains an aspartate residue (D91). D91 is also found in FeLV-C(Sarma) (Riedel, et al., 1986) and FeLV-A(945) (Levesque, et al., 1990). As the D83N and N91D substitutions were localised to the primary

determinant of FeLV-C receptor usage, these residues may contribute to the evolution of FeLV-C from FeLV-A.

The aims of this study were therefore to investigate whether the D83N and N91D mutations confer an expanded receptor tropism to the FeLV-A Env (resulting in a dual-tropic A/C virus). Alternatively, such mutations within FeLV-A *env* may influence its ability to evolve further into dual-tropic variants. This would allow an insight into the sequence of molecular events that lead to the development of a highly pathogenic retroviral variant.

3.2 Results

3.2.1. D83N and N91D-bearing mutants of FeLV-A (Glasgow-1) are replication-competent

The wildtype genotype of FeLV-A(Glasgow-1) Env is D83:N91 (DN). Using site-directed mutagenesis, three mutants of the FeLV-A(Glasgow-1) molecular clone were developed, designated FeLV-A(Glasgow-1) D83:D91 (DD), N83:D91 (ND) and N83:N91 (NN). HEK293T cells were then transfected with the functional molecular clones and a chronic FeLV infection of each mutant virus was established. Active infection was confirmed after two weeks in culture by immunoblotting for both viral capsid protein p27 and Env protein gp70 (Figure 3.2).

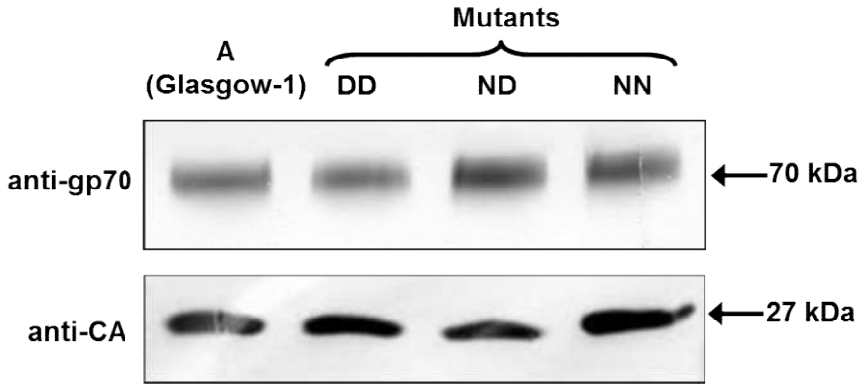


Figure 3.2: Mutants of the FeLV-A(Glasgow-1) molecular clone produce replication-competent virus.

Three mutants of the FeLV-A(Glasgow-1) molecular clone, possessing mutations at amino acids 83 and/or 91, were transfected into HEK293T cells. While FeLV-

A(Glasgow-1) has the genotype D83:N91, the mutants DD, ND and NN were D83:D91, N83:D91 and N83:N91 respectively. Viral supernatant was harvested 72 hours post-transfection, filtered and ultracentrifuged, separated by SDS-PAGE, immunoblotted and probed with either anti-gp70 (SU) or anti-p27 (CA).

3.2.2. The N91D mutation increases viral entry through THTR1

During the preparation of virus stocks of the four variants, it was noted that the DD mutant appeared to grow more efficiently in HEK293T cells. This was unlikely to be a reflection of transfection efficiency, as it was observed in multiple independent experiments utilising a range of transfection volumes from independently-derived DNA stocks. In order to measure the approximate titre of the four virus preparations, serial dilutions of the HEK293T-derived viruses were prepared, plated onto QN10 (S+L-) cells and the number of foci per µL quantified. This S+L- assay is used widely to confirm the presence of an infectious feline gammaretrovirus (Russell & Jarrett, 1976) and indicated that despite transfection of matched inputs of the four molecular clones into HEK293T cells, the four viruses displayed differences in infectious titre. DD achieved a significantly higher titre than the reference FeLV-A(Glasgow-1) while ND and NN achieved lower titres (Figure 3.3). These observations suggested that the combination of residues D83 and D91 conferred an enhanced replicative capacity upon FeLV-A.

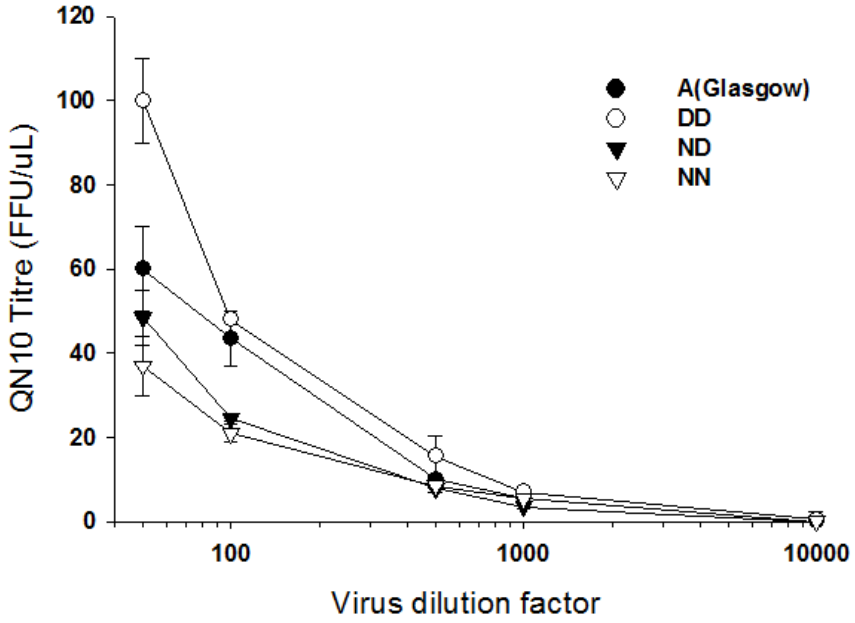


Figure 3.3: The DD mutant of FeLV-A(Glasgow-1) displays a higher viral titre. FeLV-A(Glasgow-1) (A) or the DD, ND and NN mutants were transfected into HEK293T cells and the supernatant recovered. Serial dilutions of each supernatant were prepared and plated onto QN10 (S+L-) cells. 72 hours post-infection, foci were scored manually; values represent the mean +/- SEM of two independent experiments. The increase in titre between A(Glasgow) and the DD mutation is statistically significant (Students *t* test, p value= 0.0474).

As QN10 assays were designed to detect infectious virus rather than quantify viral entry, the Glasgow-1 (A), DD, ND and NN *envs* were subcloned into a mammalian cellular expression vector in order to produce MLV(FeLV) *lacZ* pseudotypes (murine leukaemia virus virions bearing the FeLV Envs and carrying a *lacZ* marker gene), thus facilitating quantification of viral titre based solely on viral entry. Following infection of HEK293T cells with matched inputs (equal RT value) of the MLV(FeLV) *lacZ* pseudotypes, DD Env-bearing pseudotypes yielded a higher titre than those bearing the parent FeLV-A(Glasgow-1) Env (Figure 3.4). In contrast, ND Env-bearing pseudotypes achieved a lower titre than Glasgow-1 Env-bearing pseudotypes. As these pseudotypes undergo a single cycle of infection, the data suggest that the DD mutation enhanced replication at the stage of viral entry.

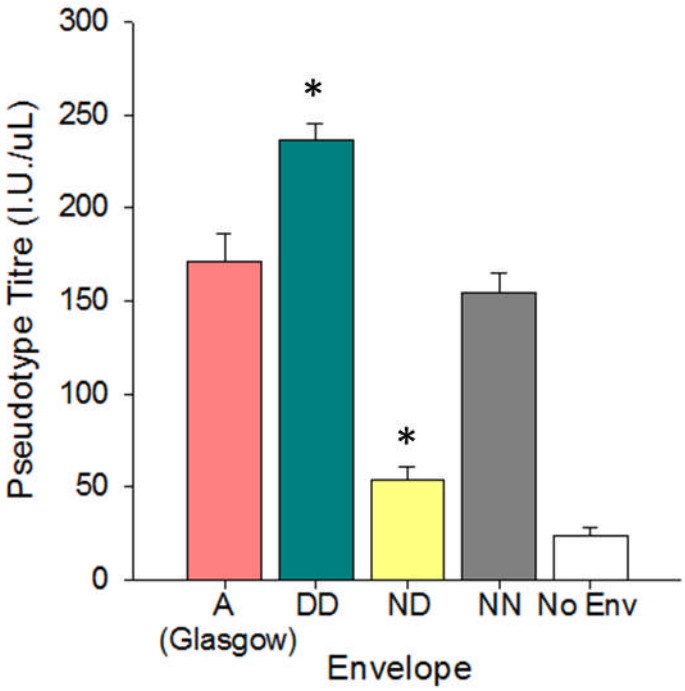


Figure 3.4: The DD Env supports more efficient infection of HEK293T cells. Matched inputs (RT activity) of MLV(FeLV) lacZ pseudotypes bearing the Glasgow-1 (A), DD, ND or NN Envs, were plated onto HEK293T cells. 72 hours post-infection, the cells were stained for expression of lacZ and counted manually. Values are presented as infectious units per µl (I.U./ µl), mean +/- SE of three independent experiments. The increase in titre for the DD mutant and the decrease in titre of the ND mutant are statistically significant in comparison with A (Glasgow-1) (unpaired T test, p=0.031 and 0.001, respectively).

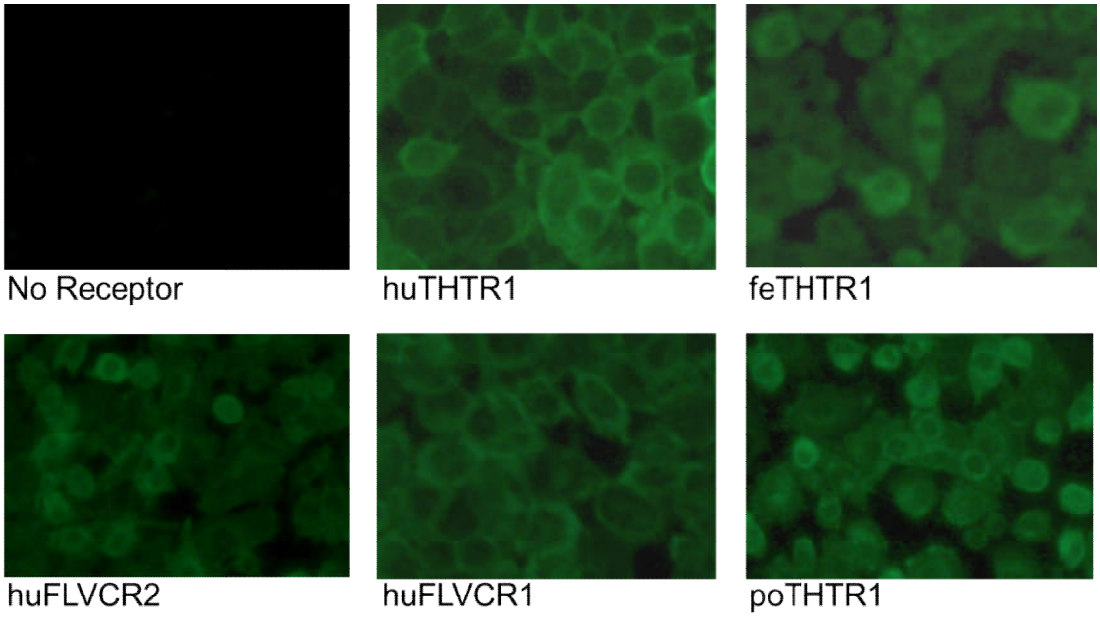
HEK293T cells are susceptible to all subgroups of FeLV (O. Jarrett, H. M. Laird, & D. Hay, 1969a; Sarma, Huebner, Basker, Vernon, & Gilden, 1970) and presumably express all the cognate receptors, although this has never been confirmed directly. Hence although the DD Env appeared to confer enhanced viral entry, this assay did not allow identification of the receptor(s) being utilised by the virus. Therefore a range of cell lines were developed to allow accurate analysis of viral receptor tropism. MDTF and 104C1 cells expressing a range of FeLV-A and -C receptor homologues were engineered by retroviral insertion of the receptor cDNA (Table 3.2). The expression of the receptor proteins was confirmed using immunofluorescence against a C-terminal HA tag (Figure 3.5). Wildtype MDTF cells are resistant to all FeLV-C subgroups, and 104C1 cells are only susceptible to FeLV-C (Sarma, et al., 1975). Therefore

manipulation of these cells to express specific receptors would allow accurate assessment of virus-receptor binding and cellular entry.

Table 3.2: Receptors expressed in MDTF and 104C1 cells.
Cells were transduced to express a range of FeLV-A and-C receptors from *Felis catus*, *Sus scrofa* and *Homo sapiens*.

Receptor	Wildtype Function	Species of Origin	FeLV Subgroup	Known Receptor Functionality
feTHTR1	Thiamine transporter	*Felis catus*	FeLV-A	Functional
huTHTR1	Thiamine transporter	*Homo sapiens*	FeLV-A	Functional
poTHTR1	Thiamine transporter	*Sus scrofa*	FeLV-A	Functional
huFLVCR1	Haem transporter	*Homo sapiens*	FeLV-C	Functional
huFLVCR2	Putative haem transporter	*Homo sapiens*	FeLV-C	Limited functionality (selected isolates)

(A)



(B)

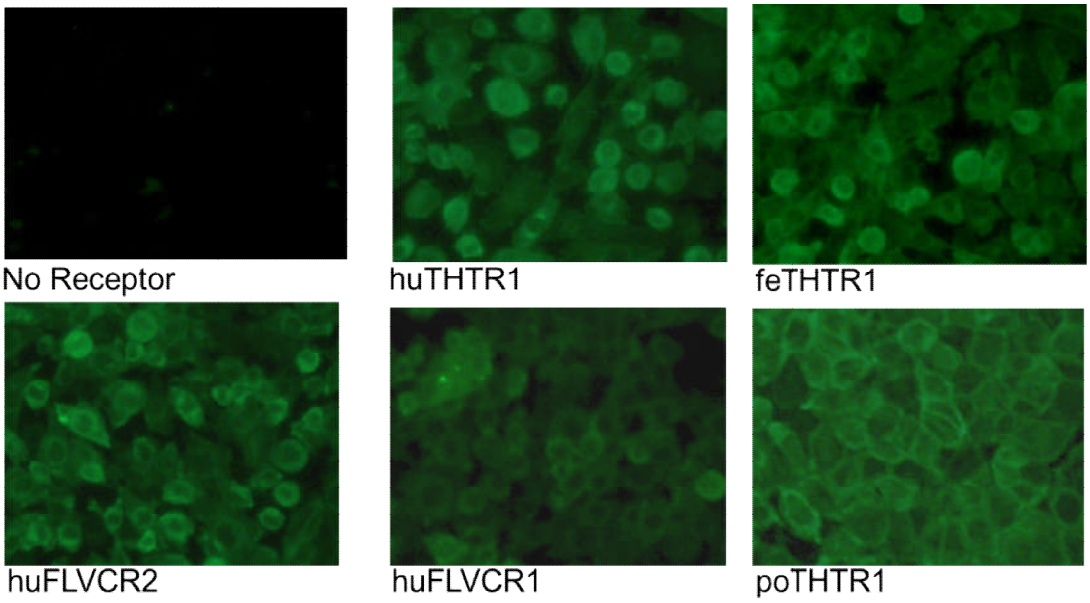


Figure 3.5: Stably transduced cells express a range of retroviral receptors. MDTF (A) and 104C1 (B) cells were transduced with VSV-G-enveloped pseudotypes, encoding retroviral receptor cDNA transcripts cloned into the pFB-Neo construct (kindly provided by C. Tailor, University of Toronto). After selection with G418, immunofluorescence was conducted upon paraformaldehyde-fixed cell monolayers to detect surface expression of the HA-tagged receptor proteins.

The lacZ pseudotype assay was repeated using the MDTF cells, expressing a range of FeLV receptors. Cells were infected with matched inputs (RT activity) of MLV(FeLV) *lacZ* pseudotypes and the efficiency of infection quantified. The results indicated that pseudotypes bearing the DD Env displayed a significant increase in the usage of all three THTR1 orthologues in comparison with the wildtype FeLV-A(Glasgow-1) Env (Figure 3.6). In contrast, C(Sarma) Env-bearing pseudotypes displayed a marked preference for hFLVCR1-expressing MDTF cells.

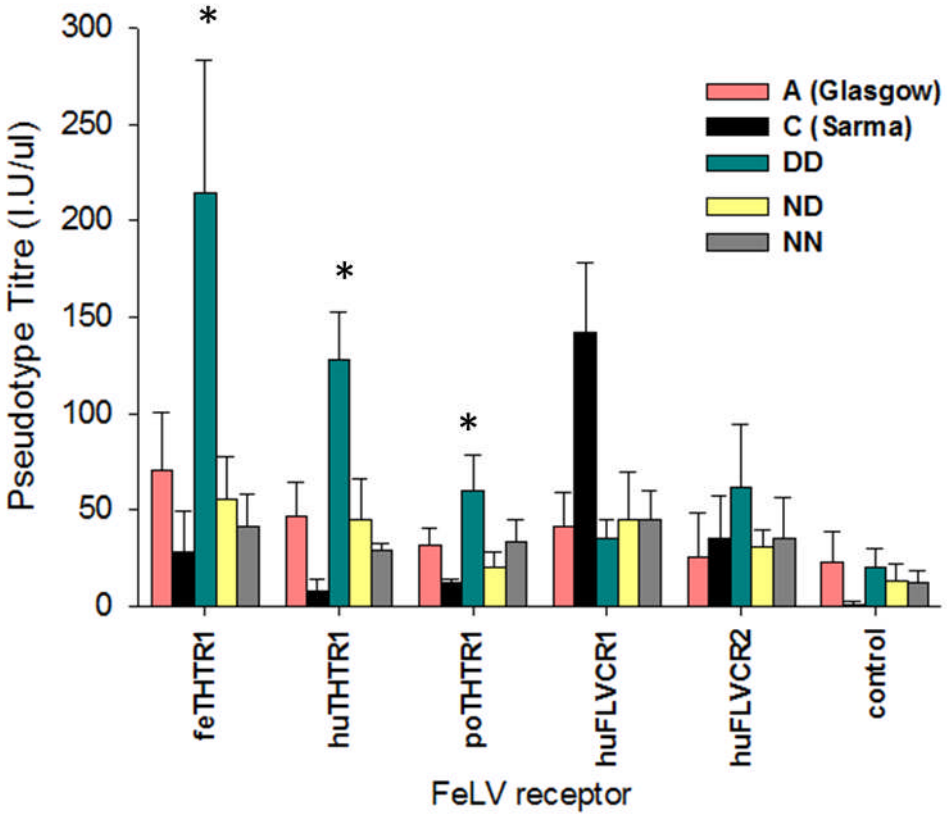


Figure 3.6: The DD Env confers enhanced utilisation of THTR1 homologues. MLV(FeLV) lacZ pseudotypes bearing the A (Glasgow-1), C (Sarma), DD, ND or NN Envs were plated onto MDTF cells expressing the feline (fe), human (hu) or porcine (po) THTR1 homologues, human FLVCR1 or human FLVCR2. 72 hours post-infection, cells were stained for expression of lacZ and counted manually. Values represent the mean +/- SEM of three independent experiments. The increase in titre associated with the DD mutation upon feline, human or porcine THTR1 is statistically significant in comparison with A (Glasgow-1) (unpaired T test, p=0.0063).

3.2.3. Enhanced binding of FeLV D83:D91 SU to viral receptors

In vitro studies with MLV(FeLV) pseudotypes had suggested that the combination of D83 and D91 in Env conferred enhanced viral entry. This led to the question as to whether this effect was mediated by increasing binding to the viral receptor(s), THTR1 (FeLV-A receptor), FLVCR1 (FeLV-C receptor) and Pit1 (FeLV-B receptor). The SU domains of the mutant FeLV Env proteins were cloned into the pTORSTEN mammalian expression vector (PCR "FeLV SU", details in Appendix 8.2) and recombinant SU proteins were expressed transiently in HEK293T cells as C-terminal fusions with human IgG-Fc. Immunoblot analysis with anti-gp70 MAb and anti-human IgG Fc confirmed the antigenicity and yield of the SU-Fc proteins respectively (Figure 3.7).

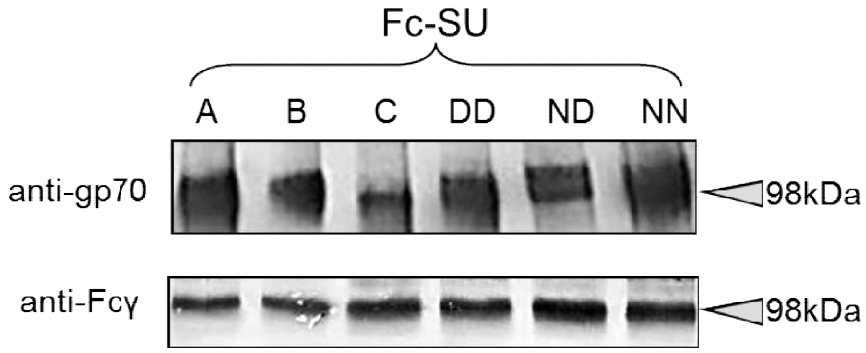


Figure 3.7: Expression of soluble Fc-tagged FeLV SUs.
HEK293T cells were transfected with the pTORSTEN vector into which the SU domains of the Glasgow-1 (A), Gardner-Arnstein (B) and Sarma (C), or the Glasgow-1 mutants DD, ND and NN had been cloned. Matched volumes of supernatant were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for both gp70 (upper) and the human IgG Fc tag (lower).

SU-Fc binding to receptor-expressing MDTF (murine) or 104C1 (guinea pig) cells was then assessed by flow cytometry using matched inputs of SU-Fc proteins (informed by immunoblotting of the recombinant SU-Fc proteins with anti-IgG) from FeLV-A(Glasgow-1) (A), FeLV-B (Gardner-Arnstein) (B) and FeLV-C (Sarma) (C) or the FeLV-A(Glasgow-1) mutant D83:D91 (DD), N83:D91 (ND) an N83:N91 (NN). The A, DD, ND and NN SU-Fc proteins bound to MDTF-expressed feline and

human THTR1 with comparable efficiency and with a reduced efficiency to porcine THTR1 (Figure 3.8). In contrast, B SU-Fc bound to huPit1 expressing MDTF cells while C SU-Fc bound most efficiently to human FLVCR1-expressing cells, confirming the specificity of the interactions. Of the four FeLV-A SU-Fcs, the DD mutant appeared to display higher binding than the A, NN and ND SU-Fcs to each of the THTR1s suggesting that the DD combination may enhance the Env-receptor interaction, implicating a molecular basis for the enhanced replication and viral entry.

These findings were mirrored using independently generated cell lines derived from 104C1 cells (Figure 3.8). The binding studies with the 104C1 cells also indicated that the NN and ND SU-Fc proteins bound less efficiently to 104C1-expressed THTR1s than the parental A Su-Fc, suggesting that while mutations such as DD may enhanced Su-Fc binding the converse may be true of NN and ND mutations. Significant weak binding of all SU-Fcs was noted to control MDTF and 104C1 cells although it was notable that the A and B SU-Fcs had higher background binding than the C Su-Fc protein, with the DD SU-Fc displaying the highest binding to control cells. As THTR1 are expressed widely and MDTFs express a murine THTR1, this background binding is most likely due to either endogenously expressed murine THTR1 or a related protein. It is notable that while the DD-SU Fc bound with a higher efficiency than the other A SU-Fcs to the control MDTF cells, it bound with a similar efficiency to huFLVCR2-expressing cells. As the A, NN, ND and DD SU-Fcs bound to huFLVCR2-expressing cells with similar efficiencies (Figure 3.8, red lines) these data strongly suggest that the enhanced binding of the DD SU-Fc to diverse receptors is a specific property of the DD SU receptor binding domain and not a reflection of variations in the amount of viable SU-Fc in the preparation. Given that the development of FeLV-associated PRCA is marked by a shift in receptor usage from THTR1 to FLVCR, the enhanced binding afforded by the DD mutation may be highly significant in the spread of such variants into compartments expressing the FLVCR1 receptor; this likely represents the first step towards the biological selection of subgroup C viruses.

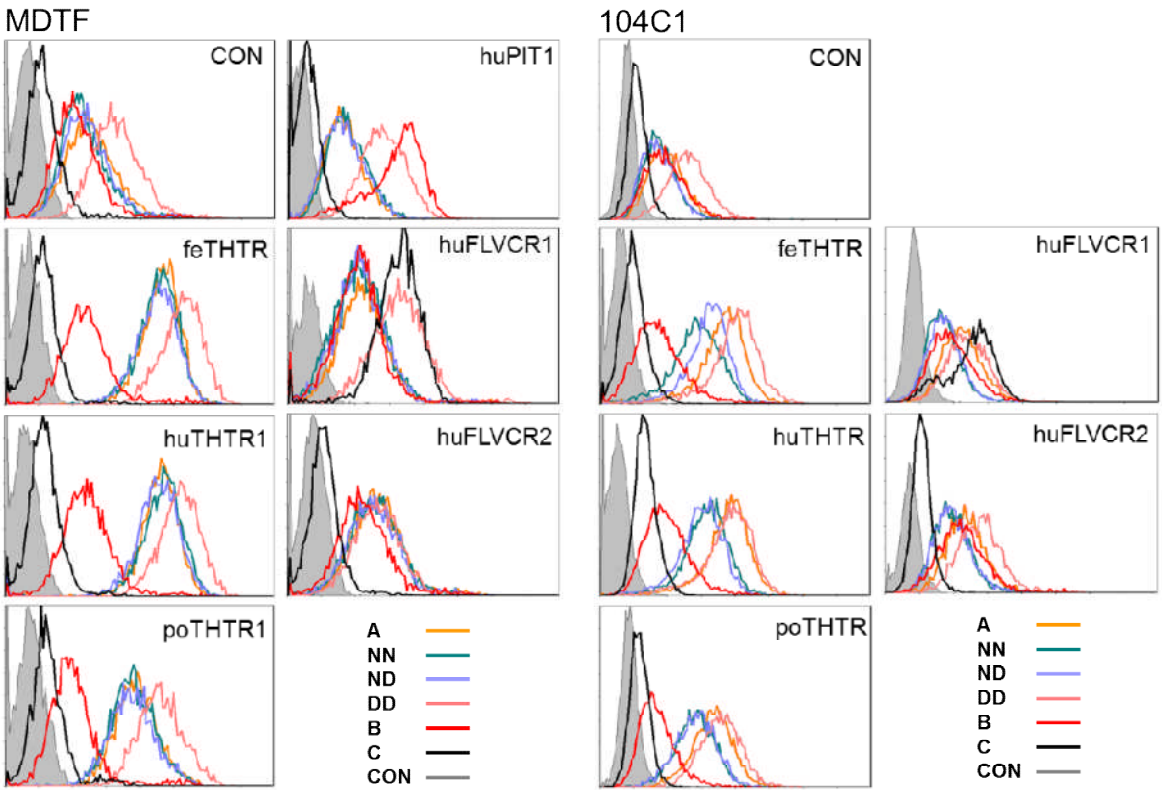


Figure 3.8: The DD mutations confer enhanced binding to multiple receptors. Matched volumes of supernatant containing the Fc-SU fusion proteins from Glasgow-1 (A), Gardner-Arnstein (B), Sarma (C), or the mutants DD, ND and NN, were added to either MDTF or 104C1 cells expressing feline, human or porcine THTR1, human FLVCR1 & 2, human Pit1 (MDTF only), or vector only (CON). Fc-SU binding was detected by flow cytometry with PE-conjugated anti-human IgG-Fc. Each histogram represents 10,000 events collected in LIST mode and are representative of two independent analyses. Ordinate displays number of events while abscissa displays fluorescence intensity.

3.3 Discussion

The amino acid sequence of the receptor binding domain (RBD) of the A subgroup of FeLV varies little between isolates, constraining the virus to usage of the thiamine transporter THTR1 for infection. The switch in subgroup from A to C in cats with pure red cell aplasia is marked by amino acid alterations in the RBD that shift receptor usage from THTR1 to the haem transporter FLVCR1. While all anaemogenic strains of FeLV bear such substitutions, little is known about the genesis of the A to C switch. To date, each isolate of FeLV-C studied

has displayed a unique RBD sequence, suggesting that recombination with endogenous *env* sequences is an unlikely source of the mutated RBD. In contrast, the emergence of subgroup B viruses is associated with recombination between exogenous and endogenous FeLV *env* sequences (Pandey, et al., 1991; Sheets, et al., 1992; Stewart, et al., 1986). A more likely mechanism for the derivation of subgroup C viruses would be the acquisition of mutations *in vivo* in response to a selective pressure from the host, either through pressure to escape the adaptive immune response or through receptor availability in the tissue in which the virus replicates. Such a mechanism predicts the presence of variants with an intermediate tropism; subgroup A viruses with point mutations in the RBD that confer an enhanced or expanded receptor usage. Previous studies identified a subgroup C virus, FY981 that had retained the ability to utilise the subgroup A receptor THTR1 for infection (Shalev, et al., 2009), confirming that there are indeed "dual-tropic" or "poly-tropic" viruses amongst primary isolates of anaemogenic strains of virus. FY981 is actually a poly-tropic virus as it is able to utilise a third receptor (FLVCR2) in addition to THTR1 and FLVCR1 (Shalev, et al., 2009).

3.3.1. The D83:D91 motif enhances viral replication

Here, it was demonstrated that subtle variations in the RBD of subgroup A viruses may have significant effects on the way the viruses interact with their receptors, potentially predisposing the viruses to *in vivo* mutagenesis. Accordingly, the presence of the combination of D83 and D91 in the background of FeLV-A(Glasgow-1) was sufficient to enhance receptor binding, viral entry and viral replication. Residue D91 is particularly intriguing as it is present in the well-characterised Rickard strain of FeLV. In two separate studies examining recombination in FeLV infection, it was noted that inoculation of cats with a molecular clone (pFRA) of the Rickard strain of FeLV resulted in 1 of 3 (Phipps, Chen, et al., 2000) and 1 of 5 (Chen, et al., 1998) cats developing an FeLV-C associated anaemia. As FeLV-C is thought to arise in <1% of infected cats, the high incidence of FeLV-C emergence following inoculation with FRA (33% and 20% respectively) may suggest an enhanced propensity for the development of FeLV-C. Mechanistically, a scenario can be envisaged whereby some subgroup A viruses may be inherently more pathogenic than others due to an enhanced

ability to infect and spread in the infected host, a feature determined largely by the affinity of the Env for the viral receptor. Indeed, such a virus (FeLV-945) has been described and shown to have a higher binding affinity for its receptor (Bolin, et al., 2011). Mapping the determinants of the enhanced binding of the FeLV-945 SU to feline cells suggested that the major determinant of the enhanced binding of the 945 SU resided in variable region B (VRB) of gp70. FeLV-945 is a D83:D91 virus, similar to the DD mutant examined in this study; however, inserting the VRA of 945 in the background of FeLV-61E (an SU that binds with a lower affinity to its receptor) did not confer an enhanced binding upon the 61E SU, suggesting that multiple determinants in gp70 may contribute to the receptor binding affinity. However, it should also be noted that the 61E SU varies from the Glasgow-1 SU at a number of other residues across gp70; the context in which D83:D91 is expressed may be critical to its effect on binding affinity. Moreover, in this study, SU fusion proteins (dimeric) with a C-terminal IgG Fc-tag were expressed and binding was assessed on both mouse and guinea-pig cells expressing individual receptors, whereas the 945-SU proteins (Bolin, et al., 2011) were expressed as C-terminal HA tagged proteins (monomers) and binding assessed on the feline lymphosarcoma cell line 3201, a cell line that produces a soluble 35kDa endogenous FeLV Env protein capable of viral interference (McDougall, et al., 1994). Such experimental differences may modulate both the affinity and the specificity of the Env-receptor interaction in the two systems. For example, it has been shown that the context in which the receptor THTR1 was expressed altered the efficiency of receptor usage by FeLV (Shalev, et al., 2009) while soluble endogenous FeLV Env produced from 3201 cells conferred infectivity on the otherwise defective FeLV-T Env (Anderson, et al., 2000). Irrespective of the differences in the experimental systems, the enhanced binding of the Glasgow-1 DD mutant SU-Fc to THTR1 was consistent with the enhanced entry and replication of the virus, while the high affinity binding of the 945-SU was consistent with enhanced binding of intact virus particles from FeLV-945 to the same cells (Bolin, et al., 2011).

3.3.2. FeLV-A RBDs display promiscuous receptor binding

The binding assays with Fc-tagged FeLV SUs suggest that all FeLV-A RBDs are able to bind to both THTR1 and FLVCR1 homologues, an observation not described previously. It is possible that both the affinity of the FeLV SU for the receptor and its ability to induce fusion once bound combine to determine the eventual route of viral entry. These data may predict that during FeLV-C evolution, additional mutations accumulate during long-term viral replication and that these mutations decrease SU affinity for THTR1 whilst increasing the relative affinity for the FLVCR1 homologues. Such viral evolution would eventually result in an Env capable of mediating fusion and entry via FLVCR1, producing the FeLV-C phenotype and associated PRCA symptoms. This theory is supported by the observation that FeLV-C (Sarma) displayed a severely limited ability to bind to THTR1 despite possessing the D83:D91 motif. However this altered binding affinity may be mediated by a range of mutations across the SU, not comprising a single binding motif, explaining why individual FeLV-C Env proteins are functionally but not genetically conserved. It is possible that the overall final Env conformation, rather than specific individual residues, permits FLVCR1-mediated membrane fusion and cellular entry. The deletions which were observed in multiple FeLV-C *env* clones from the primary isolates may be essential for decreasing the affinity of the THTR1-SU interaction and allowing FLVCR1-mediated entry. This deletion may represent the final mutation in the progression from FeLV-A to -C.

3.4 Conclusions

There are particular caveats which must be taken into account during interpretation of these results. Specifically, the higher-affinity of DD-Env proteins to receptors assumes this mutation does not alter the proportion of Env incorporated within a virion. Similarly, RT enzyme activity was used to ensure an equivalent amount of virus was utilised in the pseudotype assays; it was assumed mutations would not influence RT protein packaging within virions. The degree of receptor expression upon the surface of stably-transfected MDTFs was

observed to be of a similar value; however small discrepancies in their expression levels may have influenced results.

These studies indicate primary isolates of FeLV-A and C consist of heterogeneous viral populations and the subgroup A components of these isolates display a range of subtle mutations in Env. In the context of FeLV-A(Glasgow-1), the combination of D83 and D91 in gp70 allowed increased binding to both THTR1 and FLVCR1 and enhanced both viral entry and replication. Such properties may predispose viruses to evolution from subgroup A to subgroup C by enhancing spread of the virus into cellular compartments where ability to use FLVCR1 is selected preferentially. These data provide a first step towards elucidating why FeLV-C emerges infrequently in infected animals and provide further evidence that despite a high degree of genetic homogeneity, not all FeLV-As are equal.

4. Investigation of potential factors which may drive FeLV-A to -C evolution

4.1 Introduction

In the previous chapter, mutations were identified which may predispose particular strains of FeLV-A isolates to evolve into subgroup C variants by altering the receptor-binding properties of Env. Subsequent studies, described in this chapter, aimed to model the process of FeLV-A to –C evolution *in vitro*. If, as predicted, the acquisition of substitutions such as D83N and N91D render the virus more likely to evolve from subgroup A to subgroup C, this evolutionary process may be observable if a similar viral milieu to that observed *in vivo* could be recreated *in vitro*. Unfortunately, the site of viral replication and evolution *in vivo* remains unclear and therefore it can only be surmised that it is a site in which both A and C receptors are expressed. Moreover, additional selective pressures may be placed upon the evolution of the viral Env protein *in vivo* from the adaptive (acquired) immune response of the host.

It was predicted that the FeLV-A genotype, host humoral immune response and the relative availability of cognate receptors upon target cells would be the main determinants of FeLV-C development. These factors are commonly regarded as the two main selective pressures shaping the evolution of retroviral variants (Overbaugh & Bangham, 2001). *In vitro* models mimicking these pressures would allow accurate mapping of the viral mutations and receptor usage alterations occurring in real time. As the presence of the D83:D91 motif within Env enhanced SU binding to FLVCR1, indicating that these viral variants may be predisposed towards FeLV-C conversion *in vivo,* the replication-competent mutants of FeLV-A(Glasgow-1), D83:D91, N83:D91 and N83:N91 (DD, ND and NN respectively) were included in the *in vitro* viral replication models. This would allow comparison of the relative capacity of each Env to evolve further towards a FLVCR-utilising variant.

The *in vitro* models also aimed to determine whether the presence of gp70-specific antibodies during long-term FeLV-A replication would result in the

accumulation of mutations previously associated with FeLV-C, and whether this would be more likely to occur in the D83:D91 mutant compared to wildtype FeLV-A(Glasgow-1). For many retroviruses, the host humoral immune response exerts a pressure upon circulating virions which selects for those able to escape virus neutralising antibodies (VNAs) (Manrique et al., 2007; Nakowitsch et al., 2005; Rwambo, Issel, Hussain, & Montelaro, 1990). In the case of HIV, such mutations additionally correlate with the development of a specific disease and the acquisition of an extended cell tropism in a scenario parallel to that of FeLV-C (Berger, Murphy, & Farber, 1999). It is possible that FeLV virions possessing the DD Env are able to circumvent these antibodies more efficiently than minimally-pathogenic FeLV-A strains, either by their heightened cell entry rates or through alteration of neutralising epitopes. Alternatively, during replication in the presence of gp70-specific antibodies FeLV-A may acquire specific mutations to avoid neutralisation, which would then interact synergistically with residues 83 and 91. These mutations may induce the conformational changes presumably required within the SU domain to enhance the preliminary FLVCR1-SU binding, eventually allowing FLVCR1-mediated entry. The development of dual-tropic viruses and eventual FeLV-C phenotype may therefore be a result of this antibody-mediated escape.

In contrast, the development of viral variants with altered tropism may occur if the novel receptor is the only such available protein (Overbaugh & Bangham, 2001). Subscribing to this theory, the presence of VNAs would have minimal effect upon receptor usage without this additional pressure upon the virus. Therefore *in vitro* assays in which virus was cultured within cells expressing solely FLVCR homologues, both with and without prior growth on permissive FEA cells, were also conducted. This would theoretically allow the expansion of low-titre FeLV-C viral variants. This technique is similar to traditional methods of purifying FeLV-B or –C from mixed subgroup primary isolates upon selective cell lines (Adema, 2003). It must be highlighted that this is a less physiologically relevant method of mapping the accumulation of Env mutations, as FLVCR1 is not the receptor initially encountered by the virus *in vivo*. However these additional studies would allow comparison of the extent to which both receptor availability and the host humoral response influence viral evolution.

The overall aims of these studies were therefore to characterise the relative roles that VNAs, receptor availability and the initial FeLV-A genotype play in the development of FeLV-C and dual-tropic viral variants, using *in vitro* models of viral evolution.

4.2 Results

4.2.1. D83N and N91D Env mutations do not alter neutralisation susceptibility

In order to investigate whether the humoral immune response played a role in the evolution of FeLV-C, sera from FeLV-infected cats were pooled and screened for reactivity with gp70. During recovery from infection, cats mount a neutralising response that targets gp70. Accordingly, by pooling sera from recovered cats, a polyclonal serum was generated which reacted with gp70 on immunoblot (Figure 4.1A) and which neutralised infection with FeLV (Figure 4.1B). When the relative sensitivities of FeLV-A(Glasgow-1) and the DD, ND and NN mutants to neutralisation by either the pooled cat serum or a monoclonal antibody targeting gp70 were compared, no significant differences were detected, suggesting that the substitutions in amino acids 83 and 91 did not confer resistance to either of these neutralising antibodies. As it is likely that, in individual cats, the response to gp70 may be epitope-specific and that the specificity of the response will vary between cats, the possibility that N83D or N91D-containing variants may have either a heightened or reduced sensitivity to neutralisation *in vivo* cannot be discounted.

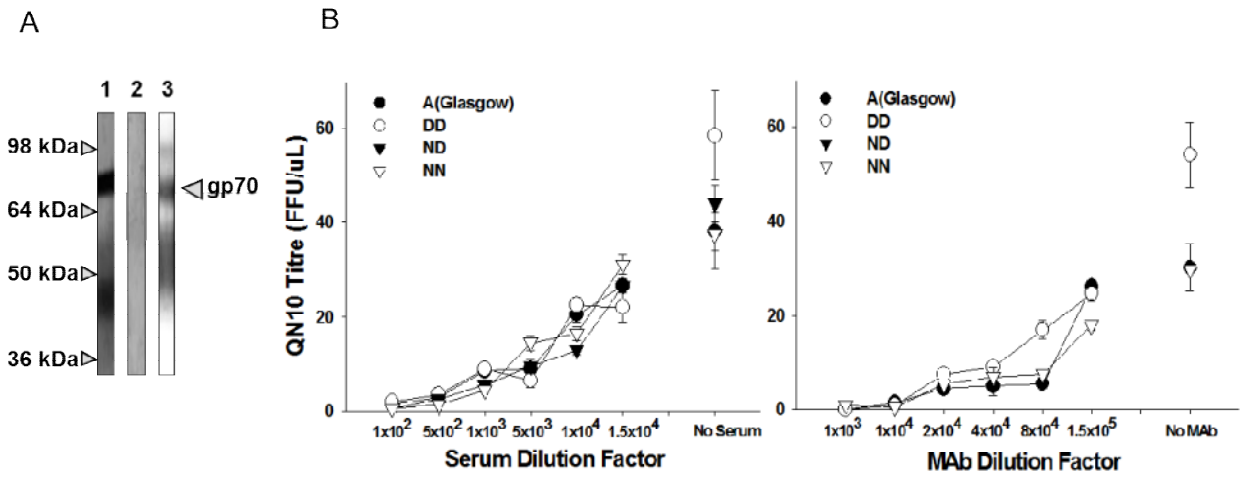


Figure 4.1: Neutralisation of FeLV by either pooled serum from FeLV-recovered cats or anti-gp70 monoclonal antibody.

(A) Purified FeLV-A (Glasgow-1) was separated by SDS-PAGE and transferred to nitrocellulose membrane. Strips of membrane were probed with pooled FeLV-positive sera (lane 1), pooled FeLV-negative sera (lane 2) or murine anti-gp70 MAb (lane 3). (B) 100 I.U. of FeLV-A (Glasgow-1) (A) or the DD, ND and NN mutants were incubated for two hours with serially-diluted pooled FeLV-positive serum or anti-gp70 MAb, before being titrated on QN10 cells. 72 hours post-infection, foci were enumerated manually. Values represent the mean +/- SEM of two independent experiments.

4.2.2. Long-term replication of FeLV in vitro under immune pressure does not produce FeLV-C variants

Next, it was asked whether culturing each of the viruses in the presence of sub-optimal neutralising concentrations of antibody would promote the emergence of variants with distinct receptor usages. FEA cells were infected with either FeLV-A(Glasgow-1) or the DD, ND and NN mutants (MOI = 0.01) and a productive infection was established. Anti-gp70 MAb (1:20^4 vol/vol) or pooled FeLV-positive cat serum (1:500 vol/vol) were added to the culture medium after the first subculture (72 hours post-infection). Ten days post-infection, the culture supernatants were harvested, concentrated 100-fold by ultracentrifugation and screened by immunoblot for the presence of viral p27 CA protein. While p27 was detected in the concentrated culture supernatant from cells infected with virus

in the absence of antibody at both 10 and 28 days post-infection, both the polyclonal cat serum and, more markedly, the MAb, suppressed virus production at 10 days post-infection (Figure 4.2). As p27 production was detected in all cultures by 28 days post-infection, the data confirmed a sub-optimal neutralising antibody concentration was being used and that the culture system was biased towards the emergency of neutralisation escape mutants.

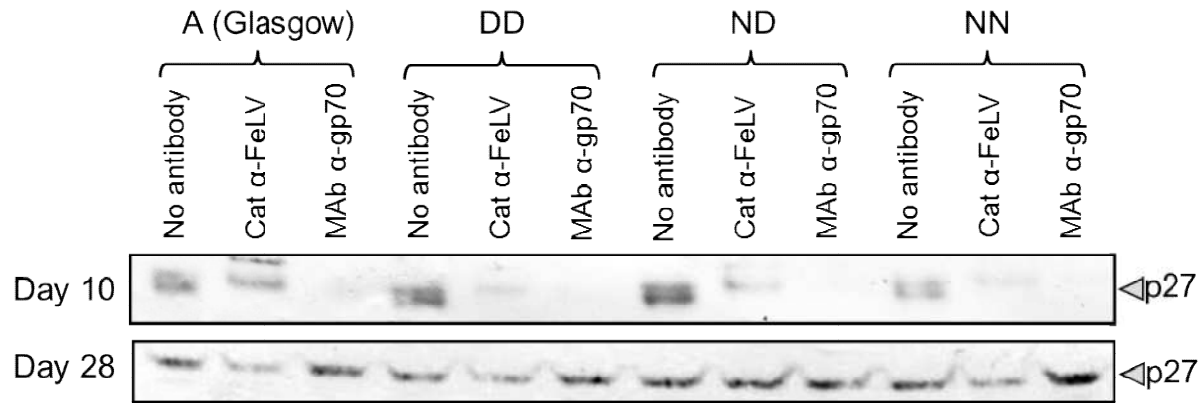


Figure 4.2: Replication of FeLV in the presence of sub-neutralising concentrations of anti-FeLV antibodies.

FEA cells were infected with FeLV-A(Glasgow-1) (A) or the DD, ND and NN mutants in the presence of sub-optimal neutralising concentrations of either pooled serum from FeLV-positive cats (1:500) or anti-gp70 MAb ($1:20^4$ vol/vol). Supernatant was harvested from infected cultures at 10 and 28 days post-infection and concentrated by ultracentrifugation; viral pellets were then separated by SDS-PAGE, transferred to nitrocellulose and stained for capsid protein (p27).

The cultures were maintained for fifty days in the presence of antibody with routine sub-culture, after which the culture supernatants were harvested from all cultures, concentrated by ultracentrifugation and viral RNA was isolated. *Env* genes were amplified, cloned and their nucleic acid sequences determined ("FeLV Env" PCR, detailed in Appendix 8.2). A total of 62 *env* sequences were determined from the 12 cultures (~5 sequences per culture) and were compared with the inoculum used for infection.

A total of 59 mutations, 42 of which were non-synonymous, were identified (Figure 4.3). Appendix 8.3 contains a multiple sequence alignment displaying the nucleotide mutations. With rare exceptions, each mutation was identified in a single individual clone. When comparing the mutations which arose to those described previously or those observed in the original primary isolates (Figure 3.1), no evidence was seen for the selective expansion of variants with FeLV-C-like sequences. The level of variation varied widely between viruses and while nine non-synonymous substitutions were detected in variants amplified from the culture infected with the ND mutant, in the presence of anti-gp70 MAb (D30G, T49A, R263K, D305G, T336A, L476O, R482G, M591T, L608P), no substitutions were detected in the culture infected with the NN mutant in the presence of anti-FeLV polyclonal antibody. Mutations were dispersed across both SU (gp70) and TM (p15E) and were not focussed within variable regions A and B (VRA and VRB) or the proline rich region (PRR). The data indicate that under the culture conditions utilised for this study, the four variants had equal propensities to acquire mutations *in vitro* and that the combinations of either ND, DD, DN or NN did not alter significantly the likelihood of the Env acquiring non-synonymous mutations.

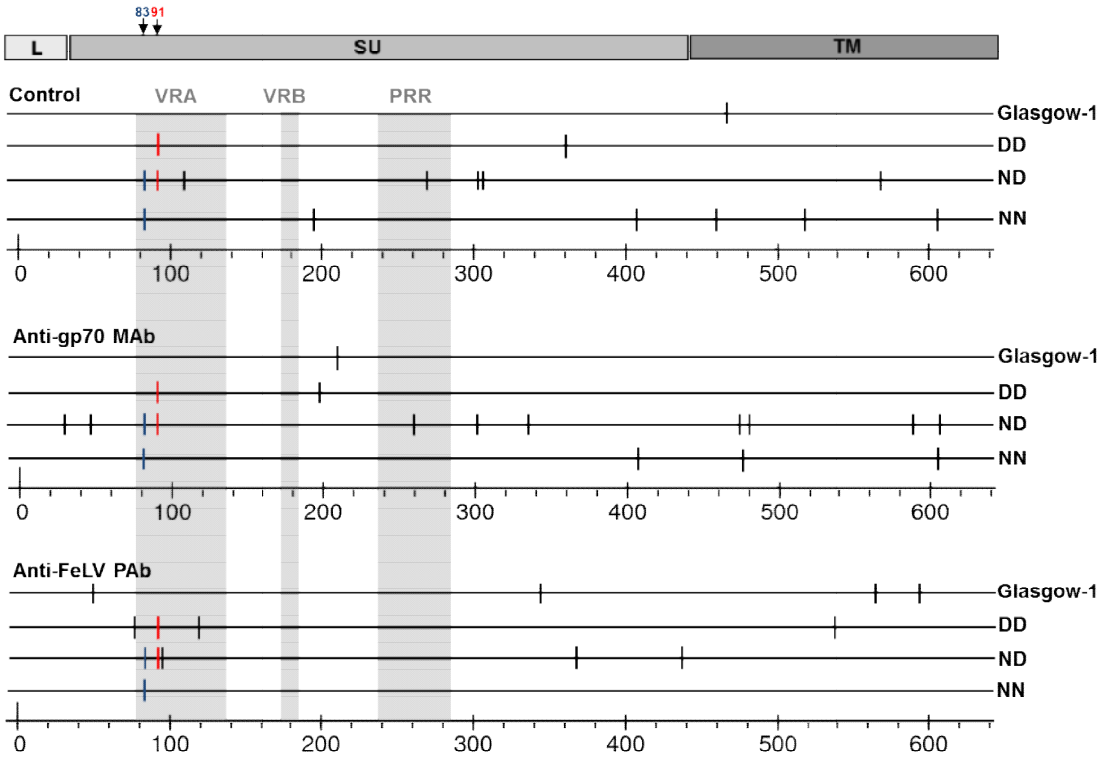


Figure 4.3: Acquisition of non-synonymous mutations in the Envs of FeLV-A mutants following long-term culture.

FEA cultures were infected with FeLV-A (Glasgow-1), or the DD, ND and NN mutants in the presence of pooled feline sera from FeLV-positive cats, anti-gp70 MAbs, or with no antibody (control). 50 days post-infection, env sequences were amplified from purified viral RNA and their nucleic acid sequence determined. Nucleic acid sequences are detailed within Appendix 8.3.

As an accumulation of mutations within the RBD was not observed within any culture it is unlikely a switch in receptor usage had occurred. Additionally, mutations had not occurred in either the N terminal fusion motif (SPHQ), the cysteine residues involved in formation of intra-SU disulphide bridges, the SU/TM cleavage site (FRR/EP) or the fusion peptide immediately downstream of this site, indicating all clones were presumably functional. However it remains possible that particular substitutions affected receptor-binding and/or cellular entry. Specific Env proteins were therefore selected and their receptor usage analysed in subsequent experiments.

4.2.3. Evolution of non-functional FeLV Env proteins during long-term viral replication

Env proteins containing mutations between residues 220 to 400 were selected. This Env section encompasses the proline rich region (PRR) and the C-terminal "C domain" (Cdom) of gp70; both these motifs affect receptor usage (Rey, Prasad, et al., 2008). It has been suggested that whilst the N terminal RBD confers receptor specificity, the Cdom provides a secondary receptor binding motif which binds to the viral receptor independently to that of the RBD (Rey, Prasad, et al., 2008). This is based upon observations that the FeLV-A Cdom binds weakly to FLVCR1; binding being dependent upon the SU C2 loop, a disulphide bridge formed between C342 and C396. Specific mutations in this loop therefore may also affect FLVCR1 usage. It was therefore hypothesised that substitutions observed in either the C2 loop, Cdom or PRR may enhance SU-FLVCR1 binding and potentially contribute to either the FeLV-C phenotype or receptor dual-tropism.

In total, 12 mutations were located in this region (summarised in Table 4.1). Two clones contained two mutations each; however, four *env* clones also contained downstream frameshift mutations, caused by either the loss or gain of a cytosine residue. As these occurred within a string of cytosines encoding residues 337 to 400 of Env, these mutations may have been due to "slipping" of the DNA polymerase during the cloning process. As these *env* clones would produce nonsense proteins, they were not investigated further.

Table 4.1: Mutations arising within the PRR and Cdom during long-term viral replication.

Parental Genotype	Culture Conditions	Mutation/s	Functionality
D83:D91	MAB	R263K	Functional
N83:D91	AB-free	V274A, frameshift	N/A
D83:N91 (wildtype)	MAB	T297A, frameshift	N/A
N83:D91	MAB	D305G	Non-functional
N83:D91	AB-free	T309A, T364A	Functional
N83:D91	AB-free	D311G	Functional
N83:D91	MAB	L316R, T336A, frameshift	N/A
D83:N91 (wildtype)	Feline Sera	S344P, frameshift	N/A
D83:D91	AB-free	P366L	Non-functional
N83:D91	Feline Sera	N374D	Non-functional

MLV(FeLV) *lacZ* particles, pseudotyped with the six remaining FeLV Env mutants, were produced by transient transfection of HEK293T cells. Anti-gp70 and anti-CA immunoblots were conducted upon concentrated pseudotypes to ascertain the level of Env incorporation (Figure 4.5).

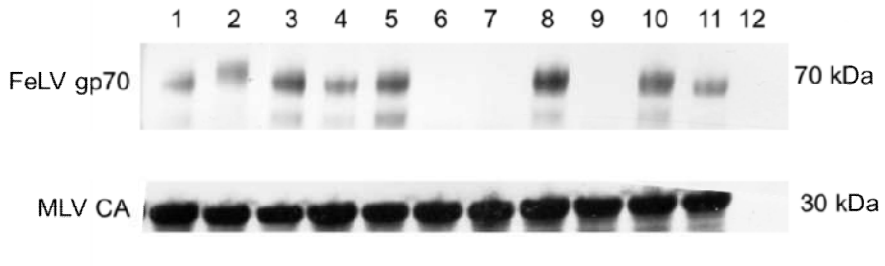


Figure 4.4: Mutant FeLV Env proteins display inconsistent levels of incorporation into MLV(FeLV) pseudotypes.
Pseudotypes were concentrated by ultracentrifugation, separated by SDS-PAGE and probed for the presence of both FeLV Env (upper) and MLV CA (lower) proteins. Lanes: (1) Glasgow-1 A; (2) Sarma C; (3) DD; (4) ND; (5) NN; (6) ND-D305G; (7) DD-P366L; (8) DD-R263K; (9) ND-N374D; (10) ND-D311G; (11) ND-T309A-T364A; (12) HEK293T supernatant.

Although equivalent levels of CA protein were detected in all pseudotypes, Env protein was not observed in the preparations from the ND[D305G], DD[P366L] and ND[N374D] *env* clones (lanes 6, 7 and 9 of Figure 4.4). This indicates that either these glycoproteins were not able to pseudotype MLV virion cores, despite possessing all the required motifs for a functional gammaretroviral envelope, or they were no longer recognised by the gp70 MAb. Re-sequencing of these non-functional genes confirmed that the open reading frames were intact and that only the indicated mutations were present.

4.2.4. Specific FeLV Env mutations within the C-domain of SU increase cellular entry via THTR1

Assuming that the ND[D305G], DD[P366L] and ND[N374D] *env* clones were non-functional, they were not included in subsequent receptor-usage assays. The MLV(FeLV) *lacZ* pseudotypes possessing the remaining novel proteins (DD[R263K], ND91[T309A, T364A] and ND[D11G]) were corrected to an equivalent RT activity

and titrated upon the range of receptor-expressing MDTF cells described in Chapter 3. The results indicated that ND[T309A, T364A] and ND[D11G] displayed a heightened ability to utilise THTR1 homologues (Figure 4.5). The increase in THTR1 usage by DD[R263K] was attributed to the presence of the D83:D91 motif. However none of the mutants acquired a significant ability to utilise the FeLV-C receptor homologues. It must also be highlighted that two of the three clones of interest arose from FEA cultures that were antibody-free. It appears therefore that genetic drift, rather than antibody-mediated escape, was responsible for these mutations and their effects upon cellular entry and viral titre.

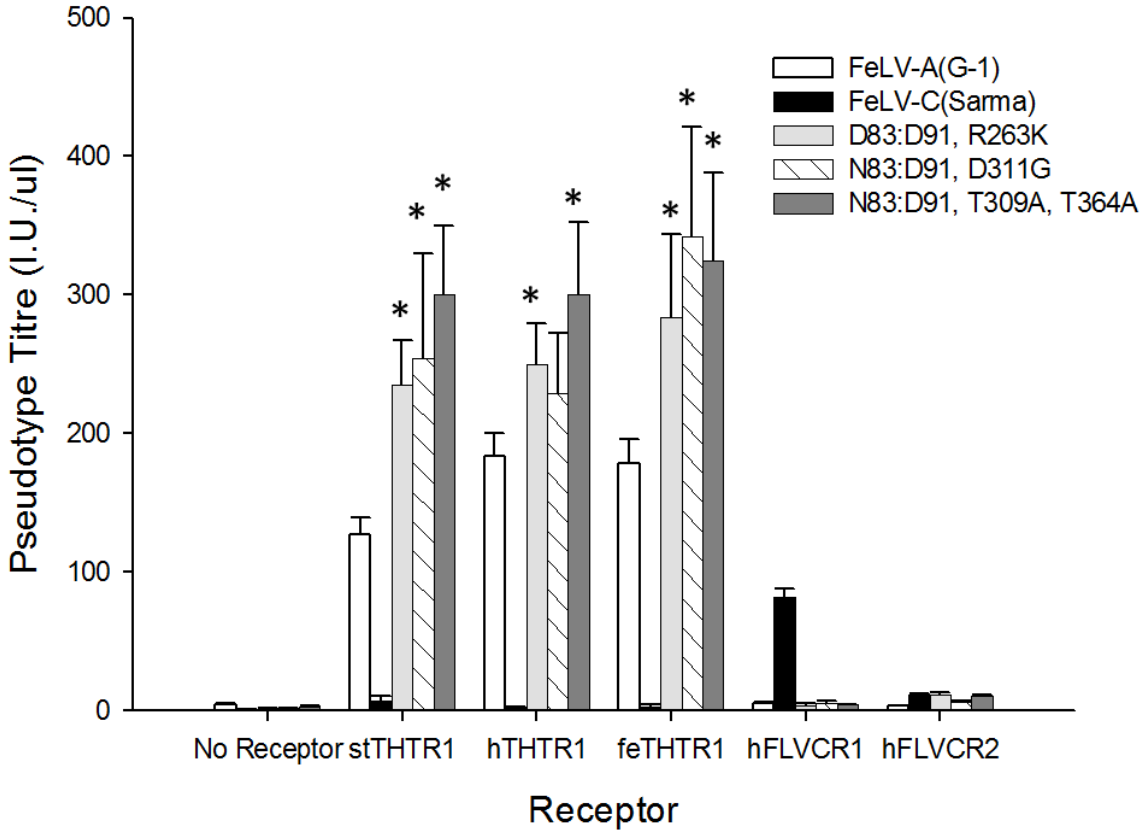


Figure 4.5: Specific Env mutants which arise through genetic drift increase cellular entry via THTR1 homologues.

MLV(FeLV) *lacZ* pseudotypes were titrated upon MDTF cells expressing porcine (st), feline (fe) and human (h) homologues of the THTR1 protein, as well as hFLVCR1 and hFLVCR2. 72 hours post-infection, cells were stained for lacZ expression. Values represent the mean +/- SEM of three independent experiments. The increase in titre seen in DD[R263K], ND[D311G] and ND[T309A, T364A] upon all three THTR1 orthologues is statistically significant (Students unpaired *T* test, p=0.0145).

The long-term viral replication assay described above indicated that VNAs did not drive the evolution of FeLV-C variants, regardless of the initial FeLV-A genotype; it appeared that mutations arose through genetic drift rather than antibody-mediated escape. Although some of these mutations abrogated functionality, others enhanced viral entry through the THTR receptor.

4.2.5. FeLV-A culture within MDTF-huFLVCR1/2 cells does not produce FeLV-C variants

The next aim was to investigate whether receptor availability contributed to the evolution of FeLV-C or of dual-tropic viral variants. As FEA cells are susceptible to FeLV-A,-B and -C, continued use of this cell line in the previous assay did not exert a purifying effect upon the viral cultures. Therefore the long-term replication assay was reproduced using MDTF cells expressing either huFLVCR1 or huFLVCR2 proteins.

Initially, MDTF-huFLVCR1 cells were infected at a MOI of 0.01 with the four FeLV-A(Glasgow-1) based viruses. After the first subculture (72 hours post-infection), antibodies were included in the culture medium at sub-neutralising concentration to aid development of neutralisation-escape mutants. The cells were subcultured upon confluency for 50 days. Supernatants were regularly harvested, concentrated by ultracentrifugation and screened by immunoblot for the detection of the FeLV CA and Env proteins. However, replicating virus was not detected in any of the cultures at any point, regardless of the presence of antibodies.

The third long-term replication assay was conducted upon MDTF-huFLVCR2 expressing cells. Antibodies (either pooled sera or the Env MAb) were not included in this assay as stocks of these preparations were limited. As described previously, the four FeLV-A(Glasgow-1)-based mutant viruses were used to infect MDTF-huFLVCR2 cells and cells were passaged for 50 days. Concentrated cellular supernatant was tested regularly for the presence of viral particles by immunoblots against both CA and gp70. However, after 50 days in culture no virus replication was detected in any of these cultures.

4.2.6. Expansion of FeLV-A subpopulations upon MDTF-huFLVCR1/2 cells

Neither homologue of FLVCR is the initial receptor encountered by FeLV-A virions, indicating that initial growth upon MDTF-huFLVCR1/2 cells is a physiologically inaccurate model. A lack of active virus production during these latter models was therefore partly expected. It must also be highlighted that a pure culture of virus, produced from an infectious molecular clone, was used in these assays which does not mirror the natural scenario. A natural isolate of FeLV-A would contain a cloud of viral variants within the sample, some of which may give rise to FeLV-C viruses after purification by growth on MDTF-huFLVCR1 cells. However, using a primary field isolate in an *in vitro* model would prevent distinction between those mutations which arose during the purifying expansion process and those which were initially present in the FeLV-A inoculum.

To more closely mimic the natural scenario whilst still utilising whole viral molecular clones, both MDTF-huFLVCR1 and –huFLVCR2 cells were infected with virus from the final passage of the FEA cells infected in the first long-term assay. Although the final *env* analysis had not detected viral mutations potentially leading to expanded receptor usage, the nature of the methods (PCR followed by *env* cloning) was not exhaustive and did not provide a fully conclusive result. It is possible that dual-tropic viruses were present in some cultures and had not been detected during the cloning analysis. Further culture of the viruses on both MDTF-huFLVCR1 and -huFLVCR2 cells would allow amplification and purification of variants able to utilise these receptors.

MDTF-huFLVCR1 and –huFLVCR2 expressing cells were therefore infected with concentrated viral preparations from the originally infected FEA cells, harvested at 50 days post-infection. Viral supernatant had been stored at -80°C. Antibodies (either pooled feline sera or the Env MAb) were not included in the media. Cultures were passaged upon confluency for 50 days. Supernatant was harvested regularly and concentrated by ultra-centrifugation before being screened by immunoblotting for the detection of both CA and Env proteins. Virus production was not detected in any of the cultures at any point throughout this assay. This

was confirmed by a RT detection assay conducted upon cellular supernatant at the conclusion of the experiment.

These studies indicated that if low levels of FeLV-C/dual tropic variants were present at the conclusion of the FEA long-term replication assay, further culture upon MDTF-huFLVCR1/2 cells did not allow selection and expansion of these variants. It remains possible that FeLV-C progenitor viruses were present within the FEA viral cultures at extremely low levels. To quantify the sensitivity of this FeLV-C expansion method, a control experiment was performed to confirm the length of passage required for an FeLV-A/C mixture to replicate to detectable levels upon MDTF-huFLVCR1 cells. A preparation of replication-competent FeLV-A (Glasgow-1) was prepared, and "spiked" with serial dilutions of FeLV-C (FY981C). These viral preparations were then used to infect MDTF-huFLVCR1 cells. FeLV-A(Glasgow-1) alone was included as a control for viral genetic drift. Cellular supernatant samples were taken daily and cells were subcultured upon confluency. By 50 days post-infection, replicating FeLV-C could be detected from an initial dilution of $1:10^{-3}$ (vol/vol) by RT detection assay (Figure 4.7). This indicates that FeLV-C viruses initially contributed less than 0.1% of the viral population during the long-term viral replication assays.

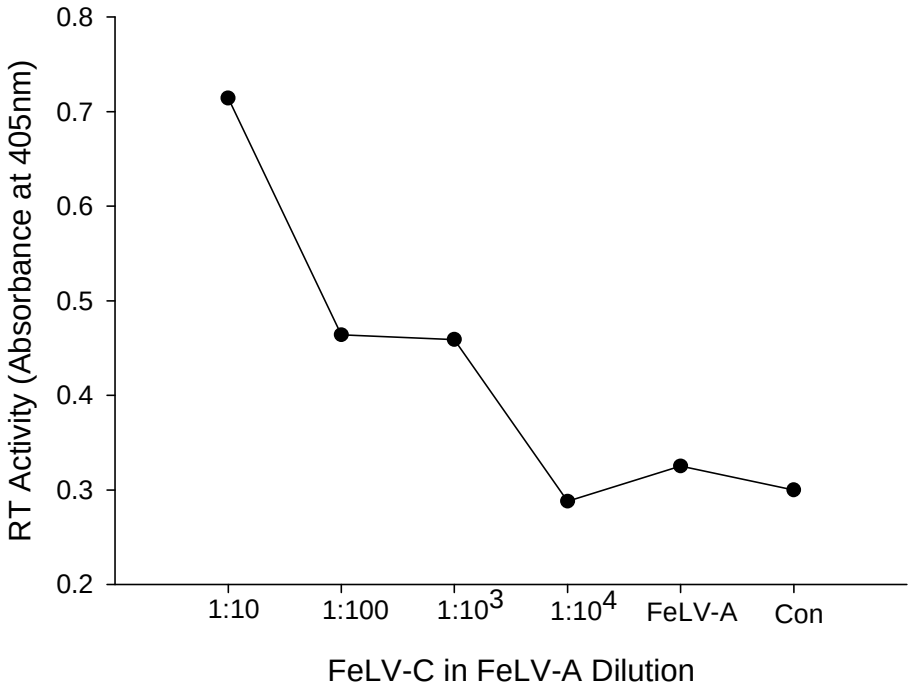


Figure 4.6: A 1:10^3 dilution (vol/vol) of FeLV-C within a FeLV-A population is detectable by RT detection assay after 50 days in culture upon MDTF-huFLVCR1 cells.

10-fold dilutions of FeLV-C within a stock of FeLV-A were cultured in MDTF-huFLVCR1 cells. RT activity, measured by absorbance at 405nm, was measured in cell-free supernatant fifty days post-infection. FeLV-A (only) viruses and a control sample of cell- and virus-free supernatant (Con) were included as controls.

To summarise, the development of FeLV-C or dual-tropic viral variants from any FeLV-A strain was not observed in any long-term viral replication models, regardless of the presence of VNAs or cognate receptor availability. As these two variables are thought to be the main influences upon viral subgroup evolution, these results were unexpected. It may be that pressures other than antibody-mediated escape and receptor availability are the determinants of FeLV-C evolution, regardless of the genotype of the infectious FeLV-A strain.

4.3 Discussion

Having established that subtle variations in the VRA of FeLV-A could have a significant impact upon the biological properties of FeLV-A(Glasgow-1) (Chapter 3), the subsequent aim was to mimic the selective pressures exerted upon FeLV *in vivo*. By culturing virus in the presence of sub-optimal concentrations of either monoclonal anti-gp70 antibody or pooled serum from FeLV-infected cats, the acquisition of non-synonymous mutations over time was observed, although a shift from subgroup A (THTR1-using) to subgroup C (FLVCR1-using) viruses was not demonstrated. Subgroup C viruses emerge in an estimated 1% of anaemic cats, suggesting that a relatively rare set of circumstances combines to drive their evolution. It is possible that the epitope specificity of the antibody response elicited following infection may prove critical in determining the composition of variants that evolve, and so serum from cats from which FeLV-C had been isolated rather than a diverse pool of FeLV-infected cat sera would be required to mimic this response in vitro. While the working hypothesis was that the humoral immune response influences the likelihood of FeLV-C emerging in infected cats, other factors may have a significant impact upon viral evolution; for example VRA may constitute a T cell epitope in some cats and pressure to escape a cellular immune response may play a role in driving variation in VRA.

4.3.1. The role of VNAs in viral evolution

It is possible the antibodies in this experiment were used at too low a concentration to drive selection of escape mutants. The antibody concentrations were calculated so as to neutralise the majority of virus present but allow a continuous low level of replication. However, the clones from the viral cultures containing each antibody preparation (either pooled feline sera or gp70-MAb) do not contain mutations in common areas. Mutations did not localise to specific areas; rather, they are randomly and evenly distributed throughout the *env* gene. This indicates the antibody concentration in either preparation was not sufficient to induce purifying selection; had this occurred the mutations would have clustered within the targeted epitopes. It would then have been of additional interest to investigate if the mutations which arose conferred resistance to homologous and/or heterologous neutralisation. However the

limited availability of the antibody preparations made this impossible in this study. These results would have provided further evidence as to whether antibody-mediated escape was occurring, regardless of the association of the mutations with the FeLV-C subgroup.

It must be highlighted that the selective pressure provided by VNAs does not alter the mutation rate of a virus, but rather exerts a purifying effect on the circulating mutants and alters the spectrum of variants able to flourish (Overbaugh & Bangham, 2001). The choice of antibodies would therefore have been a major influence on the viral mutants detected in the final cloning analysis; it is probable that an alternative antibody or sera combination would have produced very different results. Utilising a MAb specific for a known gp70 epitope may have been more informative, as mutations within the targeted epitope could be easily identified. The C11D8 MAb may have been a useful choice; this neutralising MAb targets the MGPNL motif (Elder, et al., 1987). It is not known which epitope was targeted by the gp70-MAb used in this study or whether it was linear or conformational. As the mutations which arose in the MAb-treated cultures are not confined to the one region, these results cannot be reliably used for epitope-mapping. It is also possible that the mutations present in natural FeLV-C isolates, potentially including D83N and/or N91D, alter Env T-cell epitopes. If this were the case, the advantage these mutations conferred would not be detectable in an antibody-based experimental study such as this.

The inclusion of VNAs in the long-term replication study was based upon the assumption that FeLV-C evolution may be a result of the replicating virus escaping antibody-mediated neutralisation. This hypothesis is not without precedent as there are numerous instances of retroviral antigenic variation and receptor usage alterations being driven by pressure from the host immune response. VNA play a role in the selection and expansion of viral variants in both simple and complex retroviruses, and this has been mimicked successfully *in vitro* in numerous cases (Manrique, et al., 2007; Nakowitsch, et al., 2005; Rwambo, et al., 1990). In the example of equine infectious anaemia virus (EIAV), an *in vitro* model of viral evolution found that 13 viral passages were required to obtain an antibody-escape mutant. This phenotype was conferred by only two altered epitopes in the SU domain (Rwambo, et al., 1990). This is a similar

timespan to that used in this study, indicating it was sufficient to observe antibody-escape mutants. Despite the lack of FeLV-C-associated mutations in these results, development of FeLV-C as a consequence of antibody escape remains a plausible theory. There are numerous variables in this process which could not be replicated in this model, including the broad range of antibodies produced in a competent immune response. Before definitive conclusions can be drawn about the role of VNAs in FeLV-C development, these studies should be repeated using a more extensive range of both polyclonal and monoclonal antibodies.

4.3.2. Analysis of mutations arising during long-term viral replication

A detailed analysis of the mutations that arose during viral replication within FEA cells allows several inferences to be made (Figure 4.3). Firstly, the rate of mutation appeared consistent across all cultures, indicating viral genetic drift occurred at similar rates regardless of the presence of VNAs. Additionally, few mutations were identified more than once, indicating a single FeLV genome had not emerged as the dominant viral subpopulation in any culture. However the limited capacity of the *env* selection method (cloning as opposed to deep sequencing) must be taken into account as it is possible that the amplification of more *env* sequences, or the use of alternative techniques, may have produced different results. Amplification and cloning of *env* genes may not provide a sufficiently broad picture of the genomes present in the culture. It remains possible that dual-tropic virus strains, or those containing mutations indicative of a FeLV-C phenotype were present in some cultures but were not detected during this analysis. The results presented herein therefore represent a "snapshot" of the viral genomes present at 50 days post-infection. It was not possible to determine whether specific viruses formed prominent subpopulations during the course of infection, indicative of a species with a replicative advantage expanding.

4.3.3. Cell-to-cell transmission in FeLV replication

It was initially hypothesised that the mutations within FeLV Env which arose during escape from antibody-neutralisation may correlate with usage of the FLVCR1 receptor and a FeLV-C viral phenotype. However other mechanisms of antibody avoidance, distinct from receptor-usage alteration, were not investigated. It is possible that FeLV switches to spreading preferentially via cell-to-cell transmission, as opposed to a cell-free infection route, to reduce antibody-mediated neutralisation. It was recently suggested that MLV (and presumably other gammaretroviruses) spreads predominantly via cell to cell transmission, although this is a contentious issue (Jolly, 2011; Sherer et al., 2007); nevertheless the ability or preference of FeLV to spread via these two distinct mechanisms has not been investigated. Therefore the mutations investigated herein were not studied for their effects on viral transmission routes. A switch in the main route of viral transmission may allow the virus to escape immune clearance without altering receptor usage. The concept that cell-to-cell spread allows escape from VNAs has been suggested previously for HIV-1 (Jolly, 2011; Martin & Sattentau, 2009). It is thought this transmission route reduces the exposure of the virus to VNAs, both spatially due to increased viral budding at cell-cell interfaces and temporally by reducing the timespan viral antigens are exposed to circulating VNAs.

In further support of this hypothesis, a recent publication described a neutralisation-resistant mutant of EIAV which had acquired enhanced rates of cell-to-cell transmission (W. Wu et al., 2011). The escape mutant displayed resistance to neutralisation by VNAs; however its cell-free viral titre was significantly lower than that of the parental virus. Despite this, the two viruses displayed similar rates of cell-to-cell transmission, indicating this route may overcome the inefficient cell-free infection rate of the mutant and contribute to its resistance to VNAs. As cell to cell transmission is rapidly being recognized as a major factor in viral kinetics, it is possible that FeLV Env proteins which appear less efficient in traditional receptor-binding or single-cycle pseudotype assays may be successfully utilised for this method of viral expansion. Future work may be directed towards investigating the effects of the D83N and N91D mutations upon cell-to-cell transmission rates, and determining whether this

route of viral infection is enhanced by the presence of the D83:D91 motif. However this would require methods able to differentiate cell-to-cell and cell-free viral spread which was out-with the scope of this project. Virions able to bind receptors whilst displaying budding defects may potentially be used, as they would be expected to be limited to a cell-to-cell route of transmission.

4.3.4. Additional factors influencing viral evolution

The experimental data presented here indicate that genetic drift, rather than selective pressure provided by VNAs, induced the *env* mutations observed in viruses from FEA cells. However there are numerous alternative mechanisms for the observed mutations, for example some mutations may have arisen at sites as a result of structural biochemistry. There is evidence that adenine-thymine tracts (consisting of four consecutive A or T nucleotides) are associated with "bends" in the nascent DNA strand, and are more likely to be mutated through misincorporation (Svarovskaia, Cheslock, Zhang, Hu, & Pathak, 2003). As 13 of the 59 mutations (~22%) observed in the FEA long-term replication study occurred in AT-rich tracts (see Appendix 8.3 for nucleotide sequence data) it is possible these are due to this phenomenon, rather than either neutralisation escape or receptor tropism expansion as originally predicted. In addition, some mutations may have arisen as the result of apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) activity. APOBECs mutate retroviral genomes by deaminating the cytosines during first-strand DNA synthesis, eventually causing an accumulation of G to A mutations in the genome. However there was minimal evidence of APOBEC activity in the *env* sequences analysed in this study; of the 59 mutations identified only 7 were G-to-A transitions (~12%). Additionally, only 3 (~5%) are found within likely targets for feline APOBECs (AGG or GGG motifs) (Geret et al., 2011). This is in accordance with other reports that APOBECs exhibit weak restriction of FeLV in natural infections (Geret, et al., 2011; Munk et al., 2008).

In comparison, approximately 50% of the mutations which arose in this study are A to G hypermutations. The causative factor for this unexpectedly high occurrence is not known, however these nucleotide substitutions may be due to double-stranded RNA adenosine deaminases (dsRADs). These cellular enzymes

deaminate adenosine bases within dsRNA, producing an inosine which is then converted to guanosine during reverse transcription (Polson, Crain, Pomerantz, McCloskey, & Bass, 1991). However dsRADs tend to induce “strings” of such transitions, altering approximately 50% of the adenosine bases within the targeted region (Hajjar & Linial, 1995; Nishikura et al., 1991), rather than the isolated single nucleotide polymorphisms which were observed in this study. Although retroviruses do not produce a dsRNA complex at any point during their replication cycle, hypermutation attributed to dsRADs has been reported in both HIV-1 and simple avian retroviruses (Felder et al., 1994; Hajjar & Linial, 1995; Sharmeen, Bass, Sonenberg, Weintraub, & Groudine, 1991).

Continuing viral replication past 50 days post-infection would be unlikely to have produced different results; there were multiple reasons to limit the experiment to this period. In practicality, there were time constraints and a limited amount of each antibody preparation was available. Regardless of these factors, re-infection of naïve cells only occurs until a cell line is chronically infected. At this point, downregulation or masking of the cognate receptor would occur. Although infectious virions would continually be released reinfection and proviral integration would not occur, preventing the reverse transcription stage of the retroviral life cycle during which the majority of mutations are incorporated. Were the study to be continued after this point, the viruses sampled would have been transcribed from established proviruses and would no longer represent a differentiating population. Proviral replication, occurring during cellular mitosis, plays only a minor role in retroviral mutation rates. It is therefore unlikely that continuing the experiment for a longer duration would have altered the experimental outcome.

4.3.5. In vivo models of FeLV-A to -C evolution

There have been no recent attempts to reproduce the FeLV-A to –C conversion process using *in vivo* feline infections. However, a recent publication describes an experimentally-infected cat which displayed FeLV reactivation after approximately 8 years of clinical latency (A. K. Helfer-Hungerbuehler, et al., 2010). As this cat was inoculated with FeLV-A(Glasgow-1) this is a fairly accurate *in vivo* comparison to the studies presented in this chapter. The disease

manifestation, being non-regenerative anaemia and multicentric B-cell lymphoma, is indicative of FeLV-C development. Three variant viruses were identified within this cat at the time of necropsy. Although the *env* genes were predicted to be of subgroup A, receptor usage was not experimentally analysed. As the determinants of FLVCR1 usage have not been elucidated it should not be assumed these supposedly FeLV-A viruses could not utilise alternative receptors; to claim that these strains are not FeLV-C due to a low genetic identity to FeLV-Sarma is inaccurate.

Evidence that these variants are either FeLV-C or dual-tropic comes from an analysis of the Env amino acid sequences (GenBank Accession numbers EU359303 to EU359305). This reveals the presence of polymorphisms conserved between the FeLV-C components of the primary isolates detailed in Chapter 3 (Figure 3.1). These residues are S94, P102, P104, M132, Y138, and P177. Notably, one Env variant also contains the D83N mutation. The presence of these mutations in an *in vivo* study such as this supports the hypothesis that FeLV-C arises from an FeLV-A isolate through the stepwise accumulation of mutations, gradually altering the receptor-binding properties of the SU domain and eventually resulting in a FeLV-C virus solely able to utilise FLVCR1.

4.3.6. The role of receptor availability in retroviral evolution

The studies utilising MDTF-huFLVCR1/2 cells addressed the possibility that a switch in retroviral receptor usage will only be induced if the novel receptor is the solely functional protein available. This is the case for the pathogenic avian leukosis virus, which acquires an expanded receptor tropism when cultured in the presence of soluble non-functional receptors (Melder, Pankratz, & Federspiel, 2003). This alters the subtype classification of the virus, in a manner similar to FeLV. Therefore receptor availability represents a significant potential driving force for retroviral evolution, indicating the use of FEA cells expressing all 3 cognate receptors may have inadvertently decreased the likelihood of FeLV-C evolving in the initial study. If receptor availability is the main determinant of viral evolution, the presence of VNAs would also have had minimal effect on the mutations identified in the study with FEA cells.

It must also be noted that although evolution of novel receptor usage allows the virus to access novel cellular niches and spread further throughout the host, in the case of FeLV this represents an evolutionary *cul-de-sac*, as both FeLV-B and –C do not transmit to naïve hosts in nature. The concept that receptors targeted during chronic viraemia within a host may be distinct from those required for transmission between hosts has been suggested for other retroviruses including HIV (Overbaugh & Bangham, 2001). However, as HIV requires both a receptor (CD4) and co-receptor (either CXCR4 or CCR5) for successful cell entry (reviewed in (Philpott, 2003)), this allows the virus a higher level of variation regarding receptor choices and binding affinities than FeLV, which is constrained by the fact it must retain a threshold binding affinity to THTR1 until a chronic infection is established.

4.4 Conclusions

The most significant drawback to these experiments was the inadequate length of time available; as many retroviral-associated pathologies only arise after years of infection it remains possible that either VNAs or receptor expression play significant roles in FeLV-C evolution. Similarly, the VNA neutralisation studies rely upon the breadth of antibodies present in the pooled feline serum, which would be unique across individual hosts. Finally, it must be highlighted that the cloning method utilised for assessing the Env proteins (isolated after long-term viral culture) would heavily influence the results.

During these studies, an *in vitro* model of FeLV-A to –C evolution was utilised to characterise the roles of both receptor availability and VNAs in this process. FeLV-A mutant viruses displaying differential initial abilities to bind to THTR1 and FLVCR proteins were included, however no association between any one FeLV-A genotype and the development of FeLV-C or dual-tropism was found. Using feline cells permissive to FeLV-A, -B and –C in a long-term viral replication assay, mutations were identified within the Cdom of SU which affected viral titre and infection rates. However these arose through viral genetic drift and were not associated with the presence of VNAs. Non-functional Env proteins

which could not pseudotype gammaretroviral virions were also isolated, although the cause of this non- functionality was not apparent.

Further studies with selective cell lines, which would expand low-titre viral variants able to utilise FLVCR1/2, indicated these variants were either absent or present at a concentration below $1:10^{-5}$ (vol/vol) within the FeLV-A virions. These results indicate receptor availability plays a minimal role in the development of FeLV-C in infected cats. Viruses appearing phenotypically as FeLV-C did not arise under any of the experimental conditions, indicating either extensive incubation periods (>50 days) or other unidentified pressures are required for this to occur. Before any definitive conclusions can be drawn about the role of VNAs in FeLV-C development, this study should be repeated using a more extensive range of both polyclonal and monoclonal antibodies and a more thorough approach to profiling the final *env* sequences should be utilised.

5. Are endogenous feline leukaemia viruses really endogenous?

5.1 Introduction

The aim of the studies detailed in this chapter was to characterise novel virus isolates which appeared phenotypically to comprise solely FeLV-B infections. This would allow investigation into whether inter-host transmission of FeLV-B may occur without the presence of FeLV-A "helper" viruses. It was hypothesised that such events may represent horizontal transmission of transcriptionally-active endogenous FeLV proviruses, posing the question of whether such enFeLV elements are truly endogenous. Further studies regarding horizontal enFeLV transmission are discussed in Chapter 6.

Endogenous retroviruses (ERVs) arise after a proviral integration event occurs within a germline cell or during early embryogenesis. The resultant provirus is maintained at this locus in every cell of the adult host. "Ancient" ERVs are therefore fixed Mendelian elements within the genome of the species, whereas more recently integrated ERVs may display polymorphism between individuals. There are numerous FeLV-related endogenous elements (enFeLV) within the domestic cat genome (Benveniste & Todaro, 1975; Koshy, et al., 1980; Okabe, et al., 1976; Soe, et al., 1983), most of which are polymorphic (Koshy, et al., 1980; Roca, et al., 2005) and many have intact LTRs (Soe, et al., 1983; Soe, et al., 1985). The U3 region of the LTRs differs significantly between endogenous and exogenous FeLV genomes (Berry, et al., 1988; Casey, et al., 1981; Okabe, et al., 1978), hence this domain is often analysed to determine the nature of FeLV proviruses (Tandon, et al., 2008). As enFeLV elements are generally mutated and non-functional, they do not form infectious virions (Soe, et al., 1985), although expression of short transcripts has been observed (Busch, et al., 1983; McDougall, et al., 1994; Niman, et al., 1980).

Recently, full length enFeLV elements with intact open reading frames (ORFs) have been characterised (GenBank Accession numbers AY364318 and AY364319) (Roca, et al., 2004). These endogenous elements possess identical 5' and 3' LTRs

and are present in only 9 - 15% of domestic cats (Roca, et al., 2005) indicating they are relatively recent additions to the feline genome. It was hypothesised that if transcription and packaging of these full-length enFeLV genomes occurred *in vivo,* then they may be transmitted between hosts.

FeLV-B arises through recombination events between the *env* genes of FeLV-A and enFeLV transcripts (Neil, et al., 1991; Overbaugh, Riedel, et al., 1988; Stewart, et al., 1986). The recombination event leading to FeLV-B formation is hypothesised to take place within a virion that has co-packaged two distinct FeLV transcripts, however to date this has not been observed directly. Thus FeLV-B is always found alongside FeLV-A, which is generally regarded as the "helper" virus required for FeLV-B transmission (O. Jarrett & Russell, 1978; Sarma & Log, 1973). The recombination event leads to an alteration of the receptor usage of the virus, as this is determined by the receptor-binding domain (RBD) located within the translocated SU-encoding region (Bae, et al., 1997; Rigby, et al., 1992). FeLV-A strains utilise a thiamine transporter protein (feTHTR1) for cell entry (Mendoza, et al., 2006) whilst FeLV-B utilises an inorganic phosphate-sodium symporter (fePit1) (Rudra-Ganguly, et al., 1998; Takeuchi, et al., 1992). As the RBD of FeLV-B is encoded by an enFeLV region of the recombinant genome, infections caused by transmission of enFeLV-encoded virions would present as subgroup B only in interference assays. Interference assays, a method of classifying viruses according to their receptor usage, rely upon the fact that infection with a retrovirus leads to down-regulation or masking of the receptor upon the cell surface, preventing re-infection by a virus of the same interference group (R.A. Weiss, 1993).

In this study, a group of FeLV field isolates were examined for the presence of variants that displayed the FeLV-B phenotype alone. Two isolates were identified from the sera of naturally infected cats, and the viral genomes were sequenced to identify their proviral origins. Although there was evidence for recombination between endogenous and exogenous transcripts in the genomes of both these isolates, viruses of purely endogenous origin were not identified. Additionally, one isolate co-packaged a defective exogenous FeLV-A genome alongside the functional recombinant. It is predicted that these ostensibly-FeLV-B isolates may be transmitted between hosts without the presence of FeLV-A. It

appears that enFeLV elements may contribute more to FeLV transmission and pathogenesis than previously suspected, despite the lack of enFeLV horizontal transmission.

The overall aim of the studies detailed in this chapter was to characterise novel virus isolates which appeared phenotypically as purely FeLV-B infections. This would allow investigation into whether inter-host transmission of FeLV-B may occur without the presence of FeLV-A "helper" viruses and whether endogenous FeLV proviruses were actively circulating within the host population.

5.2 Results

5.2.1. The field isolates FeLV-2518 and -4314 are phenotypically subgroup B

FEA cells were infected with a panel of 300 FeLV field isolates, which were then classified by interference assay (R.A. Weiss, 1993) into their respective subgroups (initial assays conducted by Matthew Golder, University of Glasgow). Two isolates, designated FeLV-2518 and FeLV-4314, displayed the FeLV-B phenotype alone with no evidence of FeLV-A co-infection. Although FeLV-B infection is highly associated with FeLV-induced lymphomas (Sheets, et al., 1993; Tsatsanis, et al., 1994) and leukaemias (O. Jarrett, et al., 1978; Tzavaras, et al., 1990), the clinical history and disease status of these hosts was not available, therefore the disease manifestation of these FeLV-B isolates is unknown.

To confirm these viruses were replication-competent, cell-free filtered supernatant from the infected FEA cells was used to infect HEK293T cells. Immunoblots of cell-free virions from both FEA and HEK293T cells were conducted to detect both the p27 (CA) and gp70 (SU) proteins, confirming the cells were persistently infected (Figure 5.1). These results indicated that both isolates produced a full-length envelope glycoprotein.

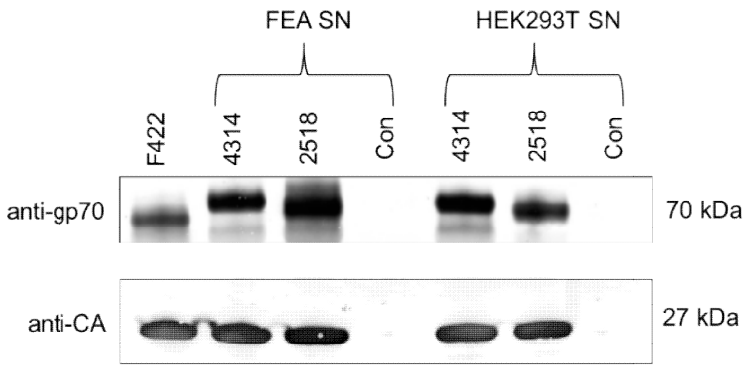


Figure 5.1: FeLV-2518 and -4314 can infect both FEA and HEK293T cells. Cell-free supernatant from FeLV-2518, -4314 and uninfected (Con) FEA and HEK293T cells was harvested, concentrated by ultracentrifugation, separated by SDS-PAGE and probed with anti-CA (lower) and anti-gp70 (upper) antibodies. F422 is a mixed-subgroup isolate of FeLV used as a positive control.

The original interference assays indicated both FeLV-2518 and -4314 were able to superinfect cells chronically infected with either FeLV-A or –C, but not FeLV-B; i.e., they were only able to utilise the Pit1 receptor. To confirm these results, HEK293T cells chronically infected with the novel isolates were transfected with the lacZ expression vector (pMFG) and MLV Gag-Pol construct (pCMVi), producing MLV(FeLV) *lacZ* pseudotypes. Titration of these particles upon a range of MDTF cells expressing FeLV receptors (described in Chapter 3) confirmed the ability of these Env proteins to utilise hPit1 alone as an entry receptor (Figure 5.2). These results also confirmed that the FeLV-B phenotype had been maintained during transmission to naïve HEK293T cells.

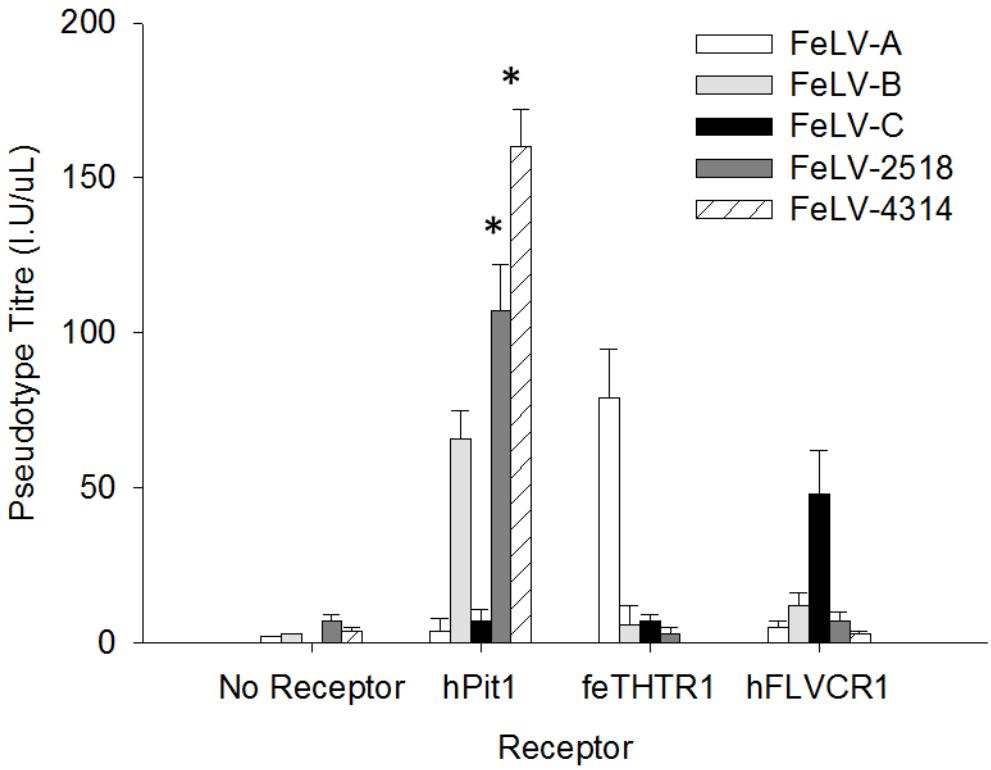


Figure 5.2: FeLV-2518 and -4314 Env proteins mediate cellular entry through the Pit1 receptor.

MLV(FeLV) *lacZ* pseudotypes were titrated on MDTF cells expressing a functional receptor for each subgroup of FeLV. Values represent the mean +/- SEM of three independent experiments. The increase in FeLV-2518 and -4314 titre during infection of hPit1-expressing cells as compared to other cell lines is statistically significant (Students unpaired *T* test, $p<0.001$).

5.2.2. FeLV-2518-infected cells downregulate both THTR1 and Pit1

Interference assays and single-cycle pseudotype assays indicate the receptor usage of gammaretroviruses. These methods rely upon binding of the novel SU protein to a cognate receptor which is known to be expressed in the target cell. In contrast, reverse interference assays demonstrate which receptor/s are downregulated within cells infected with the novel isolate, indicated by their resistance to superinfection by sarcoma viruses pseudotyped with Env proteins of a known interference group. Transformation, induced by the sarcoma virus

genome, indicates successful infection and indirectly confirms the present of the required receptor in the indicator cell surface.

Reverse interference assays were conducted using FEA cells infected with both FeLV-2518 and -4314 to indirectly confirm their FeLV-B phenotype. These results confirmed the expectations that both viruses had induced downregulation of Pit1 expression, the FeLV-B receptor (Figure 5.3). However, FeLV-2518 infection had additionally induced downregulation of THTR1, the FeLV-A receptor.

(A)

Cell Line	Sarcoma Pseudotype (Env)					
		A	B	C	2518	4314
	FEA FeLV-A		X	X	X	X
	FEA FeLV-B	X		X		
	FEA FeLV-C	X	X		X	X
	FEA FeLV-2518			X		
	FEA FeLV-4314	X		X		
	FEA	X	X	X	X	X

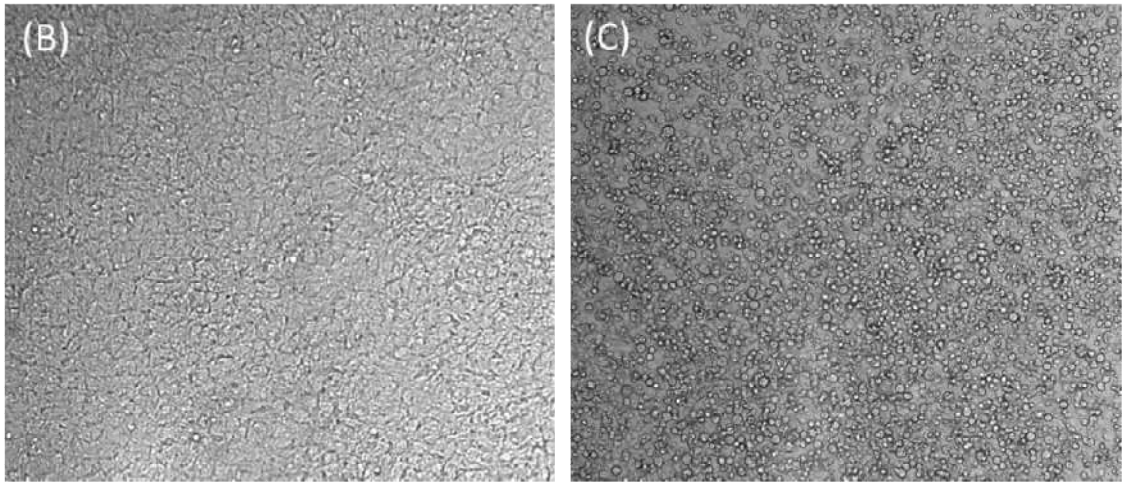


Figure 5.3: Receptor downregulation by novel FeLV-B isolates.

(A) Summary of reverse interference assay results; X indicates transformation was observed and hence the required receptor was present upon the cell surface. Trends are representative of three independent experiments.

(B) Representative photomicrograph of untransformed cells (x100).

(C) Representative photomicrograph of transformed cells following infection with murine sarcoma virus (x100).

Further evidence for interference by receptor down-regulation was provided when chronically infected FEA cells were infected with MLV(FeLV) *lacZ* pseudotypes possessing the FeLV-A, -B or –C Envs. A decrease in lacZ titre would indicate a lowered surface expression of the respective cognate receptor. Accordingly, when FEA cells were infected with subgroup A, the titre of FeLV-A Env-bearing pseudotypes fell markedly compared with the titre of -B and -C Env-bearing pseudotypes. Conversely, prior infection with FeLV-B reduced the subsequent susceptibility to FeLV-B Env-bearing pseudotypes while FeLV-C infected cells were resistant to infection with FeLV-C, but not FeLV-A or –B Env bearing pseudotypes. These assays confirmed the decrease of both Pit1 and THTR1 which was observed previously in FeLV-2518 infected FEA cells (Figure 5.4). Cells infected with FeLV-4314 displayed lowered expression of the Pit1 protein alone.

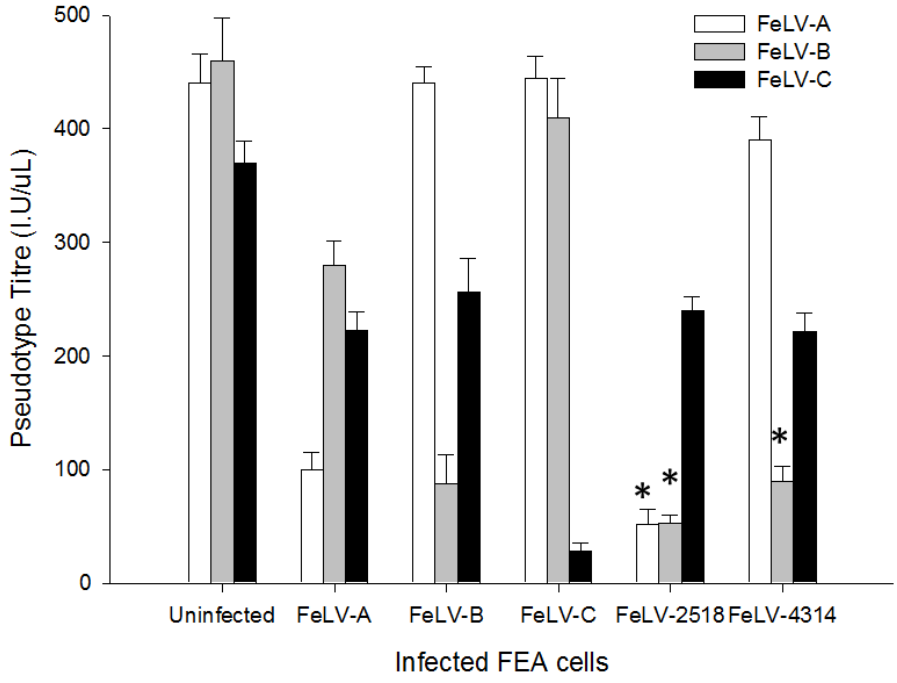


Figure 5.4: FEA cells infected with FeLV-2518 are resistant to both FeLV-A and –B, while FeLV-4314 infected cells are resistant to superinfection with FeLV-B alone.

MLV (FeLV) *lacZ* pseudotypes were titrated upon a range of FEA cells infected with the Glasgow-1 (A), Gardner-Arnstein (B) and Sarma (C) strains of FeLV, or the novel strains -2518 and -4314. Titres were calculated 72 hours post-infection. Values represent the mean +/- SEM of three independent experiments.

The decreases in titre marked with asterisks are statistically significant ($p<0.001$).

5.2.3. FeLV-4314 and -2518 are not encoded by single enFeLV transcripts

As the FeLV-4314 and -2518 viruses contained full-length Env proteins (Figure 5.1) and appeared to be solely of the FeLV-B subgroup, it was predicted the *env* genes were of endogenous origin and these isolates may be the products of transcriptionally active enFeLV proviruses. PCRs were conducted to amplify endogenous *env* and U3 regions from cell-free virus and proviruses, respectively ("FeLV enEnv" and "FeLV en-U3", PCR details included in Appendix 8.2). However enFeLV *env* could not be detected in either FeLV-2518 or -4314 viral cDNA, indicating an endogenous *env* was not encoding the full-length Env protein detected earlier. In support of this, endogenous LTRs were only detected in gDNA from feline cells, indicating transmission of an enFeLV provirus to the HEK293T cells had not occurred.

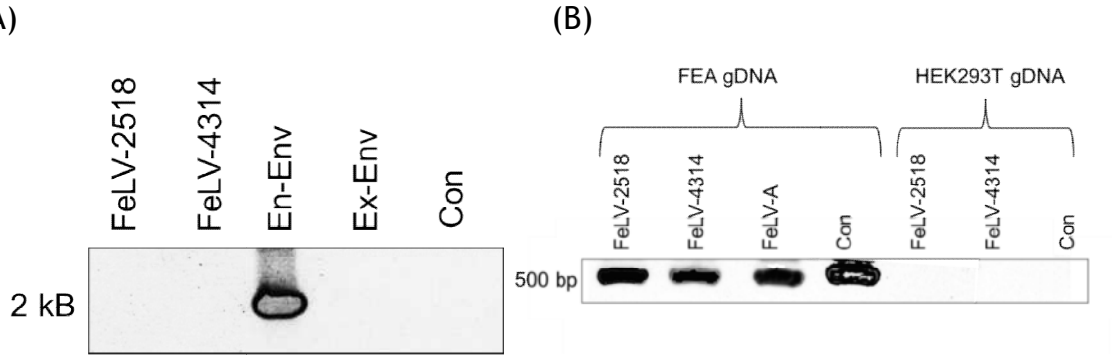


Figure 5.5: EnFeLV transcripts are not present in FeLV-2518 and -4314.
(A) Virus was pelleted from the supernatant of cells chronically infected with FeLV-2518 and -4314 and used to prepare cDNA. cDNAs were then screened for the presence of enFeLV transcripts by PCR with primers specific for enFeLV *env*. Plasmids containing endogenous and exogenous *env* clones (En-Env and Ex-Env, respectively) were included to confirm primer specificity. Con = no template control. (B) Genomic DNA from HEK293T and FEA cells chronically infected with FeLV-2518, -4314 and A (Glasgow-1) were screened for the presence of enFeLV LTR sequences using PCR with enFeLV LTR-specific primers. Genomic DNA from uninfected FEA (Con) and from FEA cells chronically infected with FeLV-A or

uninfected HEK293T (Con) were included to confirm primer specificity. Products indicate the presence of enFeLV LTRs in feline gDNA only.

5.2.4. A defective exogenous FeLV env gene is present within FeLV-2518

The lack of evidence for the involvement of enFeLV in these isolates indicated an exogenous genome was present, despite the fact earlier assays did not indicate a functional subgroup A virus was present in either case. A PCR was conducted with exogenous FeLV-specific primers, to amplify exogenous *env* transcripts from viral cDNA ("FeLV Env", PCR detailed in Appendix 8.2). Products could not be detected from FeLV-4314 templates; however FeLV-2518 cDNA produced a ~1kB amplicon (Figure 5.6). This could be reproduced using cellular gDNA from both infected HEK293T and FEA cells, indicating it was also present in proviral form.

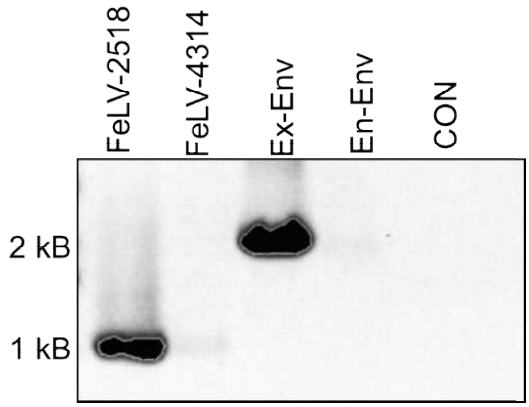


Figure 5.6: Exogenous *env* PCR reveals a truncated FeLV-A *env* transcript within FeLV-2518 virions, termed FeLV-2518(A).
Viral RNA was isolated from chronically infected and uninfected (CON) FEA cells, and screened by RT-PCR to detect exogenous FeLV *env* transcripts. Plasmids containing endogenous and exogenous *env* clones (En-Env and Ex-Env, respectively) were included to confirm primer specificity.

Cloning and sequence analysis of the ~1kB amplicon revealed it to be an exogenous FeLV *env* transcript, with an internal ~900bp deletion spanning the SU/TM cleavage site, hydrophobic membrane anchor and the majority of the TM

domain (see Figure 5.11 for a schematic of the defective genome). It also contained a premature termination codon, preventing the final 37 amino acids from being translated. This defective exogenous FeLV genome was termed FeLV-2518(A); the Env amino acid sequence is detailed in Appendix 8.4 (C). Although the absence of a hydrophobic TM region suggests this peptide would be soluble, it was not detected within supernatant from FeLV-2518-infected cultures. However the FeLV-A RBD within this peptide was intact. Given the unexpected downregulation of THTR1 that was observed in FeLV-2518-infected cells, it could be speculated that this peptide binds to immature THTR1 proteins within the cell and prevents display of the functional receptor upon the cell surface.

5.2.5. FeLV-4314 and -2518 contain exogenous LTRs

To confirm the presence of this exogenous FeLV genome in the virions of FeLV-2518, a PCR to specifically amplify the U3 region of exogenous FeLV LTRs was conducted upon genomic DNA from both FEA and HEK293T cells ("FeLV ex-U3", detailed in Appendix 8.2. Surprisingly, amplicons of the expected size (~500bp) were observed from both FeLV-2518 and -4314 templates (Figure 5.7). Sequence analysis confirmed these were integrated exogenous FeLV U3 motifs.

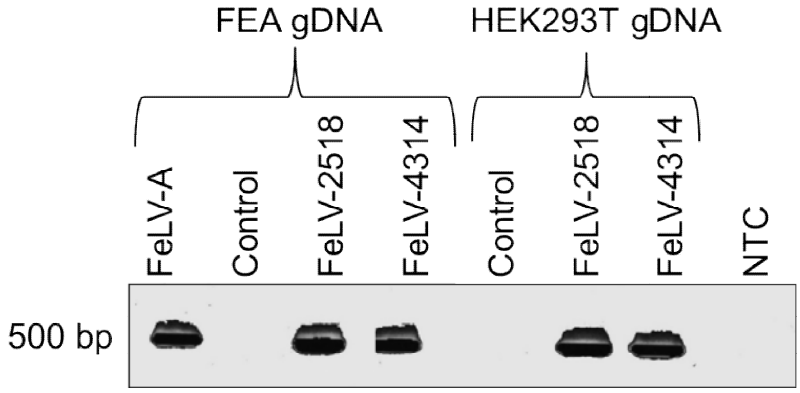


Figure 5.7: Exogenous LTRs are present in FeLV-2518 and -4314.
Genomic DNA was isolated from infected FEA and HEK293T cells and screened by PCR for the detection of exogenous U3 proviral motifs. DNA from FeLV-A-infected and uninfected (control) cells were included to confirm primer specificity. A water-only no template control (NTC) was also included.

5.2.6. Non-FeLV retroelements are not present in either isolate

Although exogenous LTRs were present in both virus isolates, the lack of either full length exogenous or endogenous FeLV *env* transcripts suggested an alternative endogenous retrovirus may be contributing to viral transmission. To investigate potential involvement of the endogenous feline retrovirus, RD-114, immunoblots against the RD-114 capsid protein and a PCR to detect RD-114 *env* transcripts were conducted ("RD-114 Env", PCR detailed in Appendix 8.2). These indicated RD-114 was not involved in the transmission or replication of these novel isolates (Figure 5.8).

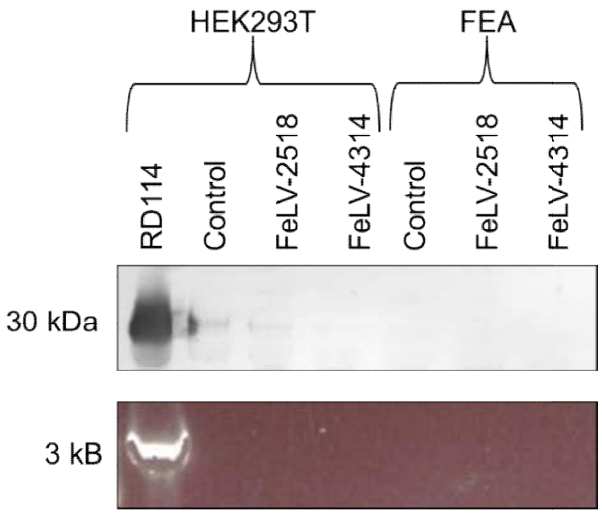


Figure 5.8: The endogenous virus RD114 is not present in FeLV-2518 or -4314.

Cell-free supernatant from chronically infected and uninfected (control) HEK293T and FEA cells was harvested, concentrated by ultracentrifugation and separated by SDS-PAGE before being probed for the presence of the RD114 capsid protein (upper panel). Viral RNA was also isolated and screened by RT-PCR for the presence of RD114 *env* transcripts (lower panel). Positive control samples were obtained by transfection of HEK293T cells with the RD-114 infectious molecular clone, pS3C3 (Reeves, et al., 1985).

In addition to RD-114, there exists in the domestic cat genome a putatively-functional retrovirus termed FcEV (*Felis catus* endogenous virus) (van der Kuyl,

et al., 1999). To ensure this endogenous retroviral element was not contributing to viral transmission, a PCR for FcEV Env transcripts in viral cDNA was conducted ("FcEV Env", PCR detailed in Appendix 8.2). Amplicons were sequenced to ensure specificity of the PCR. There was no indication this virus contributed to transmission or replication of FeLV-2518 or -4314 (Figure 5.9).

Figure 5.9: FcEV transcripts are not present in FeLV-2518 and -4314.
Genomic DNA was isolated from chronically infected and uninfected (control) HEK293T and FEA cells and screened by PCR for the presence of proviral FcEV *env* sequences. Amplicons were not observed in FeLV-2518 or -4314 infected non-feline cell lines.

5.2.7. FeLV-4314 and -2518 possess recombinant genomes

As transcripts of endogenous retroviral elements (enFeLV, FcEV or RD114) were not present in FeLV-2518 or -4314 isolates, it was concluded that these viruses must consist of recombinant FeLV genomes. Prototype FeLV-B viruses are of recombinant origin, however the isolation of this subgroup without the additional presence of a FeLV-A virus has not been documented previously. An enFeLV-*env*-specific sense primer and an antisense primer specific for a highly conserved region within the U3 of both enFeLV and exogenous FeLV, were therefore selected to amplify recombinant FeLV *env* genes from the gDNA of infected HEK293T cells ("FeLV-Recombinant", PCR details in Appendix 8.2). Amplicons of the expected size, 2.2kB, were observed from both FeLV-2518 and -4314 templates (Figure 5.10). Cloning and sequence analysis revealed both products to be recombinant *env* sequences with exogenous FeLV LTRs. This

correlates with the seemingly-conflicting results produced in other PCR assays, being the presence of exogenous U3 motifs without an accompanying exogenous *env* in both FeLV-2518 and -4314. It is presumed these recombinant FeLV *env* genes encode the full-length Env glycoproteins observed in Figure 5.1. It is not known which of the viral genomes found within FeLV-2518 virions contributes the remaining viral proteins.

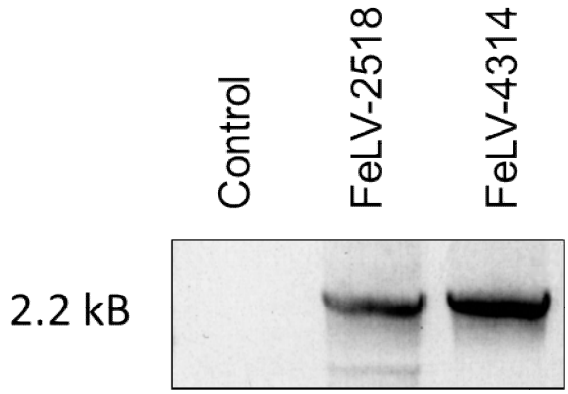


Figure 5.10: FeLV-4314 and -2518 contain recombinant *env* genes.
Genomic DNA was isolated from chronically infected and uninfected (control) HEK293T cells and screened by PCR for the presence of recombinant *env* proviruses.

A multiple sequence alignment was constructed to compare the nucleotide sequences of FeLV-2518 and -4314 with both exogenous and endogenous FeLV *env* genes and LTRs. The genomes of FeLV-A(Glasgow-1) (GenBank Accession M12500), FeLV-A(Rickard) (GenBank Accession AF052723), FeLV-A(FAIDS) (GenBank Accession M18247), enFeLV (AY364318) and enFeLV (AY364319) were compared with the FeLV-2518 and -4314 products. The 3' recombination breakpoints were identified as the site at which the nucleotide identity switched between endogenous and exogenous genes (see Appendix 8.4 (A)). These were accompanied by short regions of very high sequence conservation which presumably facilitated the recombination event between the exogenous and endogenous RNA strands.

The 3' recombination breakpoint of FeLV-4314 was found to be approximately 200bp downstream of the SU/TM cleavage site. It consisted of a 21bp stretch

(encoding the SISALEK motif within TM), conserved between both the prototype FeLV-A(Glasgow-1) and enFeLV viral genomes. Upstream there were 12 randomly-distributed mutations, encoding 7 amino acid substitutions, which distinguish this from the full-length enFeLV genome AY364318 (Roca, et al., 2004). This indicated that although the contributing enFeLV locus is not definitively known it is likely to be a recently-endogenised retrovirus. It is hypothesised that the majority of the TM domain, and the accompanying U3 region of the 3' LTR, are derived from an exogenous FeLV-A genome that is no longer present within the isolate.

In comparison, the 3' recombination breakpoint of the FeLV-2518 genome is an 18 nucleotide stretch encoding the WTSDFC motif, ~100bp upstream of the SU/TM cleavage site. This recombination site has been identified previously in FeLV-B isolates (Boomer, Gasper, Whalen, & Overbaugh, 1994) indicating it may be a preferential region for RT strand transfer during DNA synthesis. Within the endogenously-derived region of FeLV-2518, there is only one nucleotide difference (causing a K to I substitution) when compared to the AY364318 genome, again indicating a recently-endogenised provirus is the most probable source of this region. Sequence comparison of FeLV-2518 and the defective FeLV-2518(A) *env* gene revealed homologous stretches both within the 3' region of *env* and across U3. This provides evidence for FeLV-2518(A) being the source of the exogenous regions within the functional FeLV-2518 genome. However numerous mutations were observed clustered within the region immediately downstream of the stop codon of FeLV-2518(A). This genetic drift may have occurred since the recombination event that formed the functional FeLV-2518 viral genome, as there was no longer selective pressure upon the exogenous genome to retain functionality.

Using a series of oligonucleotides conserved between exogenous and endogenous FeLV sequences, the whole viral genome of both FeLV-2518 and -4314 was sequenced from viral cDNA. Genome sequence contigs were created for each virus (Appendix 8.4 (A)). This allowed identification of the 5' recombination breakpoints, identified by the alteration from an exogenous sequence to that of an endogenous FeLV element (Figure 5.11 for a schematic of the overall genome structures).

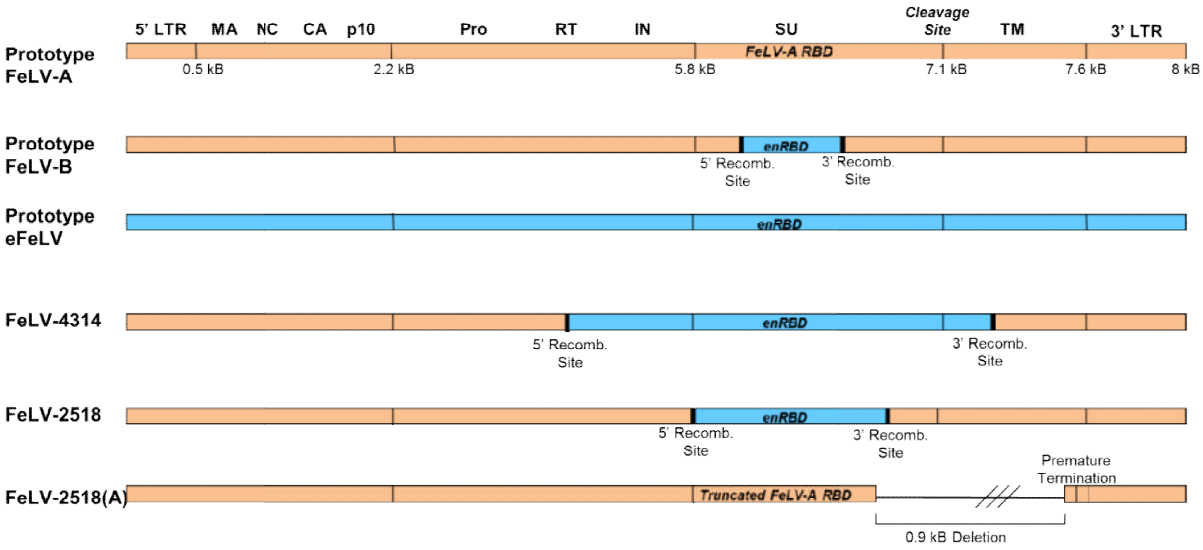


Figure 5.11: The genomes of FeLV-2518 and -4314.

Viral RNA was isolated from chronically infected HEK293T cells and the genomes of FeLV-2518 and -4314 were determined and annotated by the assembly of multiple contigs, resulting from overlapping PCRs. Recombination breakpoints between endogenous and exogenous transcripts were identified and compared to the previously-described FeLV-B isolates. Nucleotide and amino acid sequences are detailed in Appendix 8.4..

FeLV-2518 contains a 5' recombination breakpoint within the signal peptide of *env*, whereas the 5' recombination site of FeLV-4314 is found within the *pol* gene, specifically within the RT ORF. The fact that multiple transcripts were not identified within the FeLV-2518 viral cDNA at any point suggests FeLV-2518(A) possesses an identical sequence to that of FeLV-2518 outside the *env* gene, and hence the two genomes could not be distinguished. This supports the conclusion that the now-defective FeLV-2518(A) is the parental virus of the functional recombinant FeLV-2518.

5.2.8. Alteration of the LTRs may alter the pathogenic potential of enFeLV

As the Gag and Pol polyproteins of endogenous and exogenous FeLV genomes are highly conserved (Berry, et al., 1988; Roca, et al., 2004), substituting the majority of the *env* gene of FeLV-A with that of enFeLV would exert a similar phenotypic effect as the transmission of a wholly endogenous enFeLV to a novel host. It is not known whether this event would be detrimental; as ERVs rarely display pathogenicity towards their wildtype host (Miyazawa et al., 2010; Wilson, 2008) an adverse effect would be unlikely. The most commonly-cited example of pathogenic endogenously-derived retroviruses is that of AKR mice, which are not a naturally-occurring breed (Fan, 1997). However the acquisition of exogenous LTRs to an otherwise-endogenous FeLV genome would be expected to significantly alter the pathogenic potential of the isolate, as these regions contain differential promoter and enhancer elements (Berry, et al., 1988).

To support this theory, bioinformatics software MatInspector (Genomatix) (Cartharius, et al., 2005; Quandt, et al., 1995) was used to screen the exogenous and endogenous FeLV LTRs for potential transcription factor (TF) binding sites. This program has been used previously to accurately map regulatory elements of retroviral U3 regions (Kwon, Lee, Greenhalgh, & Cho, 2011). Parameters were set to detect only those transcription factor binding sites which displayed maximum conservation between the input sequence/s and the prototype binding sequence (matrix similarity of 1.00). Although this increases the accuracy of the predictions, this method also increases the probability that additional TF binding sites will remain undetected. The results (summarised in Table 5.1) indicated that the LTRs would be recognised by different TFs and therefore may be differentially expressed *in vivo*. Three potential TF binding sites were predicted within the enFeLV U3 region, whereas 5 and 6 sites were predicted for FeLV-2518 and -4314, respectively. Additionally, only one TF binding site was conserved between all three motifs (myeloid zinc-finger protein MZF1).

Table 5.1: The U3 regions of endogenous and exogenous LTRs contain differential TF binding sites.

The MatInspector program (Cartharius, et al., 2005; Quandt, et al., 1995) was used to screen U3 domains for potential TF binding sites with a matrix similarity value of 1.00. The sequence described is the motif possessing TF binding potential; capital letters indicate the core binding sequence.

U3 Region	Transcription Factor	Protein Family	Binding Sequence
enFeLV	Myeloid zinc finger protein MZF1	Myeloid zinc finger 1 factors	aaGGGGaaggg
enFeLV	Pleomorphic adenoma gene 1	Pleomorphic adenoma genes	caGGGGgtcaaaacaaggggaag
enFeLV	Pleomorphic adenoma gene 1	Pleomorphic adenoma genes	gaGGGGgaaaaacatgggtggct
FeLV-4314	Nuclear factor of activated T-cells 5	Nuclear factor of activated T-cells 5	catGGAAaattactcaagt
FeLV-4314	AML1/CBFA2 Runt domain binding site	Human acute myelogenous leukemia factors	atctGTGGttaagca
FeLV-4314	Atp1a1 regulatory element binding factor 6	Two-handed Zn finger homeodomain TFs	ctgctGTTTcagc
FeLV-4314	SF1 steroidogenic factor 1	Vertebrate steroidogenic factor	gtttCAAGgccactg
FeLV-4314	Myeloid zinc finger protein MZF1	Myeloid zinc finger 1 factors	gtGGGGattgg
FeLV-4314	Zinc finger / POZ domain transcription factor	ZF5 POZ domain zinc finger	ctgtgcGCGCgcttt
FeLV-2518	Nuclear factor of activated T-cells 5	Nuclear factor of activated T-cells 5	tatGGAAaattactcaagt
FeLV-2518	AML1/CBFA2 Runt domain binding site	Human acute myelogenous leukemia factors	atctGTGGttaagca
FeLV-2518	Atp1a1 regulatory element binding factor 6	Two-handed Zn finger homeodomain TFs	ctgctGTTTcagc
FeLV-2518	Myeloid zinc finger protein MZF1	Myeloid zinc finger 1 factors	gtGGGGattgg
FeLV-2518	Zinc finger POZ domain transcription factor	ZF5 POZ domain zinc finger	ctgtgcGCGCgcttt

In support of these results, a manual comparison of the known TF binding sites present in the FeLV-A U3 region (Fulton, Plumb, Shield, & Neil, 1990; A. K. Helfer-Hungerbuehler, et al., 2010) also indicates the endogenous counterpart possesses differential TF binding capacities. Although the leukaemia virus factor B (LVb) site, CAT and TATA boxes are intact in both endogenous and exogenous LTRs, in contrast the simian virus 40 core enhancer (CORE), nuclear factor 1 (NF1), glucocorticoid response element (GRE) and FeLV-specific binding motif (FLV1) domains are highly mutated in endogenous sequences (Figure 5.12).

Figure 5.12: The U3 regions of endogenous and exogenous FeLV proviruses display differential TF binding motifs.

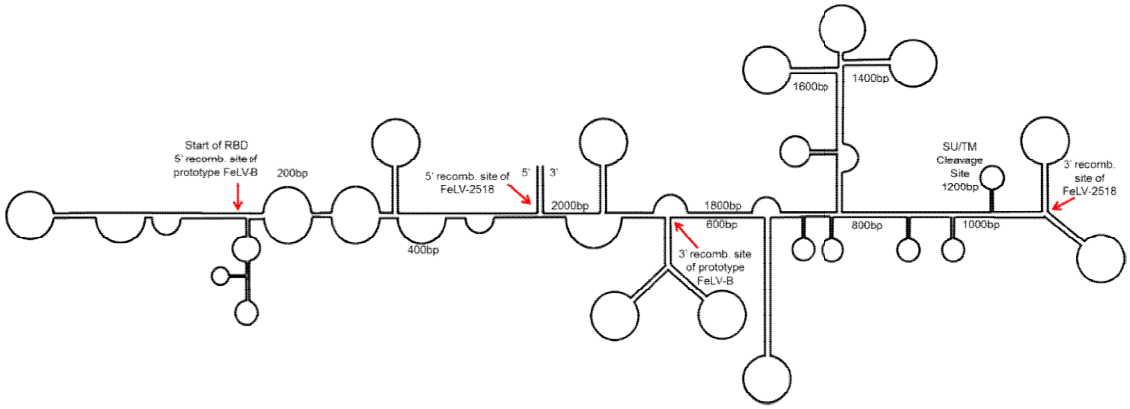
A multiple sequence alignment of the U3 motifs from FeLV-2518, -4314 and two recently-endogenised enFeLV elements was screened for elements of the enhancer framework previously described in FeLV LTRs.

Although this analysis cannot be extrapolated without supportive experimental data, it does provide preliminary evidence that the acquisition of novel exogenous LTRs would significantly alter the transcriptional activity and pathogenic potential of a full-length enFeLV provirus. The switch in LTRs may have allowed FeLV-4314 to outgrow the exogenous FeLV-A which was presumably present originally in the host. Given time, this may also occur with FeLV-2518, as there is no evidence that the defective FeLV-2518(A) genome is required for transmission or replication of this recombinant variant.

5.2.9. The RNA structure of the RBD displays increased propensity for recombination

According to the published literature, FeLV-4314 is both the first strain of FeLV-B isolated without the presence of a FeLV-A "helper" virus, and the first described recombinant FeLV possessing a 5' recombination breakpoint within the RT ORF. However the majority of FeLV-B isolates described to date contain recombination sites within a central region of the SU domain (Ahmad & Levy, 2010; Sheets, et al., 1992). To investigate why this region appears preferentially subject to such high rates of recombination, bioinformatics software programs mFold (Zuker, 2003; Zuker & Jacobson, 1998), Alifold (Hofacker, Fekete, & Stadler, 2002) and RNAStructure Version 5.3 (Reuter & Mathews, 2010) were used to model the secondary structures within FeLV *env* RNA. This aimed to identify potential recombination "hotspots" which may exist in either the endogenous or exogenous *env* genes. Apart from the 5' leader sequences, retroviral dimeric RNA is not thought to form interstrand structures within virions (Murti, Bondurant, & Tereba, 1981; Paillart, Marquet, Skripkin, Ehresmann, & Ehresmann, 1996), therefore monomeric sequences were used in these analyses. The original structural predictions are included in Appendix 8.5; Figure 5.13 displays schematics of the resulting models, redrawn for ease of reference.

(A)



(B)

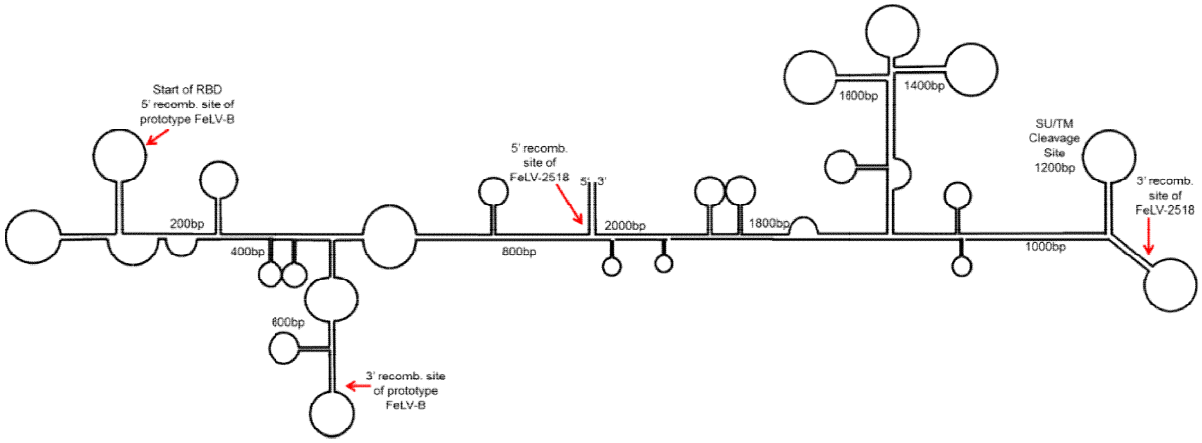


Figure 5.13: The predicted RNA secondary structures in (A) exogenous and (B) endogenous FeLV *env* transcripts.

Schematics are based upon the predicted structures from the mFold and RNAstructure webservers. Original files are included in Appendix 8.5.

This bioinformatics analysis indicated that the overall RNA secondary structure was conserved between exogenous and endogenous *env* RNA transcripts. The region upstream of the RBD forms a helix which is structurally conserved between exogenous and endogenous *env* sequences. Reverse transcriptase tends to stall at the termini of RNA helices to facilitate RNA "unwinding", hence such regions are common sites for recombination events (Galetto & Negroni, 2005). The presence of this conserved helix immediately prior to the RBD-encoding RNA may therefore contribute to the high incidence of recombination sites within the 5' sequence of the RBD. In comparison, the sections encoding the respective RBDs form highly divergent short unstable stem loops, which do not interact with the more conserved sequences. The relatively high proportion of single-stranded

RNA in this region would decrease the probability of further recombination occurring within this sequence. This supports observations that the majority of FeLV-B *env* genes described to date bear RBDs from enFeLV *envs*.

The sequence downstream of the RBD is once again highly conserved between exogenous and endogenous FeLV. This results in almost identical predicted secondary structures forming in the regions encoding the TM of both enFeLV and FeLV-A *env*. As there are numerous stable helices within this region, recombination may occur and remain undetected due to the high primary sequence conservation.

This preliminary *in silico* analysis suggests that the high rate of recombination within the 5' region of *env* is due to secondary structures within the RNA which facilitate increased RT strand transfer within this region. It would be of interest to determine accurately the frequency of recombination and whether it additionally occurs in the highly conserved TM domain; however time and resource limitations made this impossible at this point.

5.3 Discussion

The results presented within this chapter provide evidence that strains of FeLV appearing phenotypically as purely subgroup B may be circulating in the domestic cat population. Two strains of this nature were isolated and found to be replication-competent in multiple cell lines, without the required presence of a viable FeLV-A helper virus. Genetic characterisation of these field isolates revealed they are both recombinant viruses, possessing mostly endogenously-derived *env* gene sequences and in the case of FeLV-4314, a significant portion of an endogenous *pol* gene. FeLV-2518 also packaged a defective exogenous genome. The expression of an Env-related peptide from this genome appeared to induce downregulation of THTR1 within cells, presumably by intracellular peptide-receptor binding prior to display on the cell surface.

5.3.1. FeLV-4314 and -2518 represent distinct recombination events

There are 12 nucleotide differences between the exogenous U3 domains of FeLV-2518 and -4314, making it unlikely that they arose from a conserved parental virus. Thus a recombination event resulting in a mostly-endogenous viral *env* gene with exogenous LTRs appears to have occurred separately on at least two occasions in naturally infected hosts, and manifests as an FeLV-B infection without a co-infection of FeLV-A. This is the first reported identification of a 3' recombination breakpoint being identified within a FeLV-B TM domain, and a 5' site identified within RT, as the majority of recombination sites span a central 250bp region of SU (Sheets, et al., 1992). There are rare reports of recombination sites being identified in the *pol* gene, although these are further downstream than the position described here (Overbaugh, Riedel, et al., 1988; Pandey, et al., 1991).

The high sequence identity of the endogenous portions of both FeLV-2518 and -4314 to the recently-endogenised enFeLV proviruses (Roca, et al., 2004) indicates that retroelements such as these are transcriptionally active and contribute to FeLV-B formation. Additional evidence for this comes from the fact that both FeLV-2518 and -4314, as well as the full-length enFeLV genomes (Roca, et al., 2004) contain the central-SU C11D8 epitope (MGPNL) (Elder, et al., 1987; Nunberg, Rodgers, et al., 1984) which is not found in defective enFeLV elements such as CFE-6 (McDougall, et al., 1994; Sheets, et al., 1993). Thus the CFE-6 provirus and other previously-described enFeLV may no longer be suitable choices for FeLV-B genetic comparisons. For example, it was recently stated that geographically diverse FeLV-B isolates display a range of identical non-synonymous substitutions when compared to CFE-6, providing evidence they are preferentially selected for during FeLV-B generation (Ahmad & Levy, 2010). A more probable scenario exists wherein recently-endogenised enFeLV loci are more likely to be transcriptionally active and thus are the main contributors to FeLV-B genomes.

5.3.2. RNA recombination in gammaretroviruses

Recombination occurs during reverse transcription when the RT enzyme switches from the initially reverse-transcribed "donor" template to an alternative "acceptor" RNA strand (Worobey & Holmes, 1999). Synthesis of the nascent DNA strand continues, resulting in a novel recombinant product. Thus two cycles of infection are required for recombination between exogenous genomes to occur: (1) the initial infection of a cell by two distinct viruses, the transcripts from which are later co-packaged, and (2) the subsequent infection of a naïve target cell by the heterozygous virions, at which point reverse transcription produces the recombinant genome and subsequent provirus. In the example of FeLV-B formation, it is endogenous expression of enFeLV transcripts that contributes the second genome, rather than a distinct secondary viral particle.

It is not known which enFeLV loci contribute to FeLV-B formation or whether this varies between hosts. As the intact full-length enFeLV elements are polymorphic between domestic cat genomes (Roca, et al., 2005), it is probable that defective mutated elements contribute in some cases of FeLV-B development. Early studies upon enFeLV *gag* genes indicated they were highly mutated and thus assumed to be defective; however both the packaging signal and leader sequences were intact (Berry, et al., 1988). It is therefore feasible that co-packaging of defective endogenous RNA transcripts alongside exogenous genomes may occur even in host cats lacking the full-length, putatively-functional enFeLV loci. It must be noted that co-packaging of this nature has not been observed directly, although co-packaging of both FeLV and MLV RNAs has been observed *in vitro* and results in novel recombinant genomes (Pandey, et al., 1991; Yin & Hu, 1997). There is also the notable case of AKR mice, which develop leukaemia following development of a replication-competent virus from recombination events between three endogenous elements. Interestingly, in this case a specific locus (*Bxv-1)* contributes the LTRs which directly influence the pathogenic potential of the final virus (Coffin, Stoye, & Frankel, 1989). Therefore the formation of FeLV-B, arising through recombination between exogenous and endogenous transcripts in infected animals, is not without parallels.

HIV has an estimated recombination rate of approximately 1.4×10^{-5} per site per generation (Neher & Leitner, 2010) whereas MLV recombines at least 10-fold less (Simon-Loriere & Holmes, 2011). Therefore the frequency of recombination varies significantly across the retroviral genera, and is influenced by both the processivity and fidelity of the RT enzyme and the presence of structural motifs within viral RNA (Simon-Loriere & Holmes, 2011). RT pause sites include highly stable structures such as the termini of dsRNA helices. These aid strand transfer events, inducing recombination, by stalling the RT enzyme and increasing the probability strand transfer will occur during synthesis of the antisense DNA strand (Lanciault & Champoux, 2006; Simon-Loriere & Holmes, 2011). This pausing is also thought to allow the RNaseH function of the RT enzyme to further degrade the donor strand, thereby increasing interactions between the acceptor RNA and the nascent DNA (Roda et al., 2002).

There are conflicting opinions as to whether RNA recombination offers advantages to retroviruses. It is often assumed to allow generation of functional viruses from potentially nicked and damaged genomes, and therefore heightens replication fidelity by effectively purging deleterious mutations which would otherwise render the genome non-functional (Temin, 1991). In the case of HIV, intergenic domains tends to possess highly stable RNA structures, thus decreasing the probability that recombination will produce deleterious viral progeny by nucleotide misincorporation and the introduction of frame-shift mutations (Simon-Loriere, Martin, Weeks, & Negroni, 2010). In contrast, recombination allows complementation between functional and defective viral genomes, which decreases the overall fitness of the viral population. It has been suggested that recombination may merely be a “mechanistic by-product” arising due to the nature of the retroviral life cycle (Simon-Loriere & Holmes, 2011); as two strand transfer events are required for a successful infection, the RT enzyme may have evolved to possess low processivity and template affinity (Temin, 1993). In the case of FeLV, recombination producing a novel subgroup (FeLV-B) may increase viral fitness by expanding receptor tropism and allowing continued viraemia.

5.3.3. Exogenous LTRs may alter the properties of enFeLV viral strains

The 3' LTR is often a contributing factor in the virulence and oncogenic potential of retroviruses. Promoter and enhancer-like elements, including the CCAAT (Grosschedl & Birnstiel, 1980) and Goldberg-Hogness boxes (Corden, et al., 1980; Proudfoot, 1979), are located within the U3 region and may activate proto-oncogenes downstream of the site of insertion (Fan, 1997; L. S. Levy, Lobelle-Rich, & Overbaugh, 1993). There are numerous examples of specific FeLV U3 domains, usually containing short repeats either upstream or within the enhancer regions, being associated with heightened pathogenesis. In some cases, the repeats form novel TF binding sites, leading to higher rates of viral replication and an accelerated disease progression (Finstad, Prabhu, Rulli, & Levy, 2004; Prabhu, et al., 1999). These include cases of FeLV-related multicentric lymphoma (Athas, Choi, Prabhu, Lobelle-Rich, & Levy, 1995; Chandhasin, et al., 2004) and acute myeloid leukaemia (Hisasue et al., 2009; Matsumoto et al., 1992; Nishigaki et al., 1997). These repeated motifs are not found in the LTRs detailed within this study; however it would be of interest to determine the clinical manifestation of FeLV-2518 and -4314 infection and characterise how this is influenced by the exogenous LTRs they contain.

There is also recent evidence that the U3 region may be directly involved in FeLV pathogenesis; a short positive-sense RNA transcript is produced from the FeLV U3 region *in vitro* and is thought to activate the NFκB signalling pathway (Abujamra et al., 2006; Forman, Pal-Ghosh, Spanjaard, Faller, & Ghosh, 2009). It is not known if this pathway and/or the transcript itself contribute to FeLV-induced diseases, although the NFκB pathway is associated with cancer progression (Okamoto, Sanda, & Asamitsu, 2007). The stimulation of this pathway may increase cell proliferation and therefore heighten the opportunity for retroviral activation of proto-oncogenes (Forman, et al., 2009). Supporting evidence for a direct role of gammaretroviral LTRs in disease progression comes from the fact that the U3 domain activates the AP-1 signalling pathway (Abujamra, Faller, & Ghosh, 2003; S. K. Ghosh & Faller, 1999; Weng, Choi, & Faller, 1995). Notably enFeLV LTR expression does not exert these effects (S. K. Ghosh, Roy-Burman, & Faller, 2000). Therefore the effective replacement of

endogenous LTRs with those from an exogenous viral strain would almost certainly alter the pathogenic potential of the virus. Similar effects have been described in MLV, wherein a switch in LTR sequences between distinct strains predictably alters the resulting disease spectrum (Chatis, Holland, Hartley, Rowe, & Hopkins, 1983; Fan, 1997).

5.3.4. The potential roles of the defective FeLV-2518(A) genome

It is not known if the truncated exogenous Env peptide encoded by FeLV-2518(A) plays a role in the transmission and/or replication of FeLV-2518. Were this protein secreted, it may either prevent FeLV-A infection through competitive receptor-binding, or enhance infection with other viruses in a similar manner to that of FeLIX. However gp70-specific immunoblots did not indicate it was present in cell-free supernatant. The fact that decreased surface expression of feTHTR1 in FeLV-2518-infected cells was observed with two distinct techniques (reverse interference assays and lacZ-pseudotype infection) indicates binding between the 2518(A) Env peptide and the receptor occurs intracellularly and prevents both peptide secretion and feTHTR1 display. Thus the predicted behaviour of 2518(A) Env is similar to that of the described protective enFeLV Env peptide (McDougall, et al., 1994), in that it may reduce cellular susceptibility to superinfection by downregulating surface expression of FeLV cognate receptors. A similar mechanism is seen in mice wherein endogenously expressed polytropic and xenotropic MLVs (the Rmcf and Rmcf2 resistance genes) interact with the XPR1 receptor (Jung, et al., 2002; T. Wu, et al., 2005).

However it remains possible that low levels of the 2518(A) Env peptide are secreted and were not detectable. As it would be predicted to form soluble RBD-like proteins, it was initially suggested that it would possess similar properties to FeLIX, an endogenously-encoded Env peptide required for FeLV-T infection of T-lymphocytes (Anderson, et al., 2000; Lauring, et al., 2001). FeLIX consists of the N-terminal 273 residues of the Pit1-RBD; comparatively the 2518(A) Env peptide possesses the initial 295 residues of FeLV-A RBD. FeLIX is thought to restore infectivity to non-infectious gammaretroviruses by “priming” the otherwise non-functional SU proteins, allowing fusion of the viral and cellular membranes to

occur. The ability of other soluble gammaretroviral RBDs to restore infectivity to mutant viruses has also been characterised (Lavillette, et al., 2001; Lavillette, Ruggieri, Russell, & Cosset, 2000). However the amino acid sequence of FeLV-2518 Env is predicted to be wholly functional, therefore the 2518(A) Env peptide would presumably not be required to induce fusion. More importantly, 2518(A) Env does not possess a functional N-terminal fusion motif. This motif, consisting of a SPHQ sequence, couples the receptor-binding properties to the fusion machinery of gammaretroviral Env proteins. Proteins with mutations in the fusion motif are able to bind receptors but cannot mediate cellular entry (Barnett & Cunningham, 2001); this is likely to be the case concerning 2518(A) Env which contains a SPPQ motif. Comparatively, the full-length FeLV-2518 genome encodes a presumably-functional SPHQ, indicating 2518(A) Env is likely to be obsolete for fusion mediation even if it binds to feTHTR1.

It can therefore be presumed that the lack of a fusion motif in 2518(A) Env peptides would make it unable to aid infection by defective virions. Conversely, it may be able to bind THTR1 intracellularly and prevent future FeLV-A superinfection. Finally, it is not known whether 2518(A) Env forms part of the Env trimer complex present on released FeLV-2518 virions. Heterodimerisation of gammaretroviral Env proteins has been observed *in vitro* (Dewannieux & Collins, 2008) and results in decreased infectivity of the released virions. Thus it is possible FeLV-2518 virions contain heterotrimeric Env complexes, although the lack of a conjugated TM domain makes it difficult to predict the stability of 2518(A) Env peptides. Future experiments may be directed towards unravelling the respective contributions of the FeLV-2518 and 2518(A) Env proteins to the receptor-binding and cellular entry processes.

5.4 Conclusions

It must be noted that the interference assays detailed in this Chapter do not directly measure downregulation of receptor expression upon a cell surface; rather these are indirect measurements of viral entry which is assumed to be a consequence of altered receptor availability. As in Chapter 3, MDTF receptor expression levels were assumed to be approximately equivalent and therefore

would not restrict infection by either of the novel viruses studied. Equally, Env incorporation in the pseudotype virions was assumed to be equivalent.

Although enFeLV horizontal transmission events were not identified, two FeLV field isolates (FeLV-2518 and -4314) were isolated which presented as FeLV-B without FeLV-A co-infection. These viral genomes are recombinants possessing unusual recombination breakpoints. It is hypothesised that in the case of FeLV-4314, the acquisition of an exogenous LTR allowed the recombinant virus to achieve higher rates of transcription due to the U3 promoter and enhancer elements, possibly contributing to it outgrowing the exogenous virus that was originally present. In the case of FeLV-2518, a defective FeLV-A genome is also packaged and maintained throughout infection of naïve cells, potentially causing a decrease in functional THTR1 expression.

These studies describe the first isolation of FeLV viruses displaying the B subgroup phenotype without the presence of a fully functional helper FeLV-A virus. These results may shed some light upon the biology of FeLV-B formation. Since both of these ostensibly subgroup B viruses occurred naturally, it is likely that enFeLV expression contributes to FeLV pathogenesis more than was assumed previously.

6. The functionality of endogenous FeLV elements

6.1 Introduction

An ERV is a heritable retroviral element present at fixed loci within the genomes of all individuals within a species. Therefore all retroelements, including solo LTRs and defective retroviral genetic relics, represent ancient retroviral infections by a once-functional exogenous virus which may or may not have a related viral descendant currently circulating within the host community. In recent years, advancements in genomics and bioinformatics have resulted in a significant increase in the identification of recently-endogenised ERVs; these elements are recognised by their high degree of polymorphism between individuals of the species and the presence of intact retroviral motifs within the endogenous provirus. Although the acquisition of mutations and deletions ensures "ancient" ERVs are defective, in the case of these recent genomic integration events insufficient evolutionary periods have elapsed for this to be the case. This leads to the question of whether recently-endogenised proviruses possess the capacity to function as replication-competent exogenous viruses. It can be assumed their expression would not exert a pathogenic effect, as natural selection would ensure a highly pathogenic retrovirus would not become an established retroelement within its host germline. In contrast, although there are examples of retroviral genes being co-opted by the host for their beneficial effects (Varela, Spencer, Palmarini, & Arnaud, 2009), the polymorphic nature of these retroelements indicates they do not perform an essential role in the hosts' biology. Such recent endogenisation events have been described in diverse species, including koalas (Tarlinton, Meers, & Young, 2006), mule deer (Elleder et al., 2012), laboratory-derived mice strains (Kwon, et al., 2011; Lee, Horiuchi, Itoh, Greenhalgh, & Cho, 2011; Ribet et al., 2008) and significantly, in particular breeds of the domestic cat (Roca, et al., 2005; Roca, et al., 2004).

The description of intact enFeLV proviruses, identified within a feline genomic library, indicates that FeLV is continually invading the host genome. As exogenous FeLV infection has pathogenic consequences for the host, the study of these proviruses represents an opportunity to investigate the mechanisms

involved in the endogenisation process, which would be expected to attenuate the virus and allow it to become an established genetic element as opposed to a horizontally transmissible, pathogenic retrovirus (Oliveira, Satija, Kouwenhoven, & Eiden, 2007). Alternatively, if these endogenous elements are transcriptionally active and have retained their ability to transmit horizontally, this leads to the question of how they are being maintained in the genome considering their pathogenic potential.

The two genomic regions which differ significantly between endogenous and exogenous FeLV are the *env* gene and the U3 domain within the LTRs. It can therefore be predicted that differences in the functionality of enFeLV compared to exogenous FeLV-A would be ascribed to either of these regions. Given the essential function of Env in mediating retroviral infection and the large extent to which exogenous FeLV Env proteins have been studied, the initial studies described in this chapter were aimed towards characterising potential functionality of enFeLV Env. However the presence of a functional *env* gene would not automatically indicate an endogenous provirus was truly replication-competent; the provirus must additionally be transcriptionally active. Although published descriptions of enFeLV expression in host tissues have provided inconclusive results to date (Busch, et al., 1983; Kidney, Ellis, Haines, & Jackson, 2001; McDougall, et al., 1994; Niman, et al., 1980), the presence of a tentatively endogenous functional RT enzyme in FeLV-4314 (Chapter 5) displaying high nucleotide conservation with the AY364318 and AY364319 proviruses (Roca, et al., 2004) indicates these enFeLV genomes are transcriptionally active, although the tissue specificity and level of transcription is unknown.

Finally, it must be noted that for a complete viral replication cycle to occur an intact *gag-pol* ORF such as that found in AY364318 and AY364319 would not suffice. Genomic retroviral RNA is present in a dimeric form which binds to the nucleocapsid (NC) component of the Gag polyprotein, driving construction of the viral core. The dimerisation and packaging signals (Ψ) are found within *cis*-acting secondary structures within the 5' region of the viral RNA (Prats, et al., 1990). As these elements encompass the 5' LTR and overlap with the *gag* ORF, the primary nucleotide sequence of this region will have a significant effect upon

the transmission potential of a virus by potentially altering the intramolecular structures which the RNA is able to attain. Notably, subgenomic mRNA is not selectively packaged as the Ψ packaging signal is located downstream of the splice donor site (Maurel & Mougel, 2010). Therefore *env*-encoding spliced transcripts would not suffice for enFeLV transmission despite potentially encoding functional glycoproteins; an intact leader and 5' *gag* sequence is essential for this process.

The aims of the studies discussed in this chapter were to assess endogenous Env protein functionality and conduct an *in silico* analysis of 5' RNA structural motifs of enFeLV elements. This would identify potential factors which may restrict horizontal transmission of otherwise-intact enFeLV elements.

6.2 Results

The primary block to horizontal transmission of whole enFeLV proviruses would be expected to be the lack of a functional Env protein. Despite possessing an intact *env,* the enFeLV provirus AY364318 was identified within a feline genomic library (Roca, et al., 2005; Roca, et al., 2004) and experimental expression has not been described. The ability of wholly enFeLV Env proteins to pseudotype viral cores and/or mediate cellular entry has therefore not been elucidated, partly due to the lack of a cloned enFeLV *env* construct. Initial experiments aimed to clone full-length *env* genes from the feline embryonic fibroblast (FEA) cell line.

6.2.1. Multiple intact enFeLV env genes are present in the feline genome

A PCR was conducted to specifically amplify enFeLV *env* genes from both uninfected FEA cells and those infected with FeLV-2518 and -4314 ("FeLV enEnv" PCR, detailed in Appendix 8.2). Amplicons were cloned into the pVR1012 expression vector and amino acid sequences were determined. Five full-length *env* genes with intact ORFs were identified (clones Env-1 to -5). An additional

two *env* genes were isolated by the method described above by other laboratory members and are included in subsequent analyses (Env-6 and-7, Figure 6.1).

```
          1
AY364318  MEGPTHPKPS KDKTFSWDLM ILVGVLLRLD VGMANPSPHQ VYNVTWTITN LVTGTKANAT SMLGTLTDAF
enFeLV-1  .......... .......... .......... .......... .......... .......... ..........
enFeLV-2  .......... .......... .......... .......... .......... .......... ..........
enFeLV-3  .......... .......... .......... .......... .......... .......... ..........
enFeLV-4  .......... .......... .......... .......... .......... ....A..... ..........
enFeLV-5  .......... .......... .......... .......... .......... .......... ..........
enFeLV-6  .......... .......... .......... .......... .......... .......... ..........
enFeLV-7  .......... .......... .......... .......... .......... .......... ..........

         71
AY364318  PTMYFDLCDI IGNTWNPSDQ EPFPGYGCDQ PMRRWQQRNT PFYVCPGHAN RKQCGGPQDG FCAVWGCETT
enFeLV-1  .......... .......... .......... .......... .......... .......... ..T.......
enFeLV-2  .......... .......... .......... .......... .......... .......... ...A......
enFeLV-3  .......... .......... .......... .......... .......... .......... ..........
enFeLV-4  ..I....... .......... ....R..... .......... .......... .......... ..........
enFeLV-5  .......... .......... .......... .......... .......... Q......... ..........
enFeLV-6  .......... .......... .......... .......... .......... .......... ..........
enFeLV-7  .......... .......... .......... .......... .......... .......... ..........

        141
AY364318  GETYWRPTSS WDYITVKKGV TQGIYQCSGG GWCGPCYDKA VHSSKTGASE GGRCNPLILQ FTQKGRQTSW
enFeLV-1  .......... .......... .......... .......... ....I..... .......... ..........
enFeLV-2  .......... .......... .......... .......... ....I..... .......... ..........
enFeLV-3  .......... .......... .......... .......... .......... .......... ..........
enFeLV-4  .....K.... .......... .......... .......... ....I..... .......... ..........
enFeLV-5  .......... .......... .......... .......... ....I..... .......... ..........
enFeLV-6  .......... .......... .......... .......... ....I..... .......... ..........
enFeLV-7  .......... .......... .......... .......... ....I..... .......... ..........

        211
AY364318  DGPKSWGLRL YRSGYDPIAL FSVSRQVMTI TPPQAMGPNL VLPDQKPPSR QSQIESRVTP HHSQGNGGTP
enFeLV-1  .......... .......... .........V .......... .......... .......... ..........
enFeLV-2  .......... .......... .......... .......... .......... .......... ..........
enFeLV-3  .......... .......... .......... .......... .......... .......... ..........
enFeLV-4  .......... .......... .......... .........P .......... ........I. ..P.......
enFeLV-5  .......... .......... .......... .......... .......... .......... ..........
enFeLV-6  .......... .H........ .......... .......... .......... .......... ..........
enFeLV-7  V......... .......... .......... .......... .......... .......... ..........

        281
AY364318  GITLVNASIA PLSTPVTPAS PKRIGTGNRL INLVQGTYLA LNVTNPNKTK DCWLCLVSRP PYYEGIAVLG
enFeLV-1  .......... .......... .......... .......... .......... .......... ....E.....
enFeLV-2  .......... .......... .......... .......... .......... .......... ....E.....
enFeLV-3  .......... .......... .......... .......... .......... .......... ....E.....
enFeLV-4  .......... .......... .......... .......... .......... .......... ..........
enFeLV-5  .......... .......... .......... .......... .......... .......... ..........
enFeLV-6  .......... .......... .......... .......... F......... .......... ....E.....
enFeLV-7  .......... .......... .......... .......... .......... .......... ..........

        351
AY364318  NYSNQTNPPP SCLSDPQHKL TISEVSGQGL CIGTVPKTHQ ALCKKTQKGH KGTHYLAAPS GTYWACNTGL
enFeLV-1  .......... .......... .......... .......... ...N...... .......... ..........
enFeLV-2  .......... .......... .......... .......... ...N...... .......... ..........
enFeLV-3  .......... .......... .......... .......... ...N...... .......... ..........
enFeLV-4  .......... ..A....... .......... .......... ...N...... .........N ..........
enFeLV-5  .......... .......... .......... .......... .......... .......... ..........
enFeLV-6  .......... .......... .......... .......... .......... .......... ..........
enFeLV-7  .......... .......... ......R... .......... ...N...... .......... ..........

        421
AY364318  TPCISMAVLN WTSDFCVLIE LWPRVTYHEP EYIYSHFENK PRFKRDPISL TVALMLGGIT VGGMAAGIGT
enFeLV-1  .......... .......... .......... .......... ......S... .......... ..........
enFeLV-2  .......... .......... .......... .......... .......... .......... ..........
enFeLV-3  .......... .......... .......... .......... .......... .......... ..........
enFeLV-4  .......... ........T. .......... .......... .......... .....Q.... ..........
enFeLV-5  .......... .......... .......... .......... .......... .......... ..........
enFeLV-6  .......... .......... .......... .......... .......... .......... ..........
enFeLV-7  .......... .......... .......... .......... .......... .......... ..........

        491
AY364318  GTAALLETAQ FRQLQMAMHT DIQALEESIS ALEKSLTSLS EVVLQNRRGL DILFLQEGGL CAALKEECCF
enFeLV-1  .......... .......... .......... .......... .......... .......... ..........
enFeLV-2  .......... .......... .......... .......... .......... .......... ..........
```

```
enFeLV-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........
enFeLV-4  ..........  ..........  ..........  ..........  ..........  ..........  .T........
enFeLV-5  ..........  ..........  ..........  ..........  ..........  ..........  ..........
enFeLV-6  ..........  ..........  ..........  ..........  ...F......  ..........  ..........
enFeLV-7  ........V.  ..........  ..........  ..........  .......Q..  ..........  ..........

        561
AY364318  YADHTGLVRD NMAKLRERLK QRQQLFDSQQ GWFEGWFNKS PWFTTLISSI MGPLMILLLI LLFGPCILNR
enFeLV-1  ..........  ......G...  ..........  ..........  ..........  ..........  .F........
enFeLV-2  ..........  ..........  ..........  ..........  ..........  ..........  .F........
enFeLV-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........
enFeLV-4  ..........  ..........  ..........  ..........  ..........  ..........  .........Q
enFeLV-5  ..........  ..........  ..........  ..........  ..........  ..........  ..........
enFeLV-6  ..........  ..........  ..........  ..........  ..........  ..........  ..........
enFeLV-7  ..........  ..........  ..K.......  ..........  ..........  ..........  ..........

        631
AY364318  LVQFVKDRIS VVQTLVLTQQ HQRLGQCDSD QPYHPSZ
enFeLV-1  ..........  ..........  ..........  .......
enFeLV-2  ..........  ..........  ..........  .......
enFeLV-3  ..........  .........L  ..........  .......
enFeLV-4  ..........  ..........  Y.........  .......
enFeLV-5  ..........  ..........  ..........  .......
enFeLV-6  ..........  ..........  ..........  .......
enFeLV-7  ..........  ..........  ..........  .......
```

Figure 6.1: The feline genome contains multiple intact enFeLV *env* genes.
A multiple protein sequence alignment of the seven intact enFeLV *env* genes cloned from gDNA of FEA cells was constructed using the ClustalW algorithm. Provirus AY364318 was used as the reference sequence (Roca, et al., 2004). Dots indicate conserved sites.

Comparisons between the predicted amino acid sequences of the 7 cloned enFeLV Env and the published intact Env AY364318 revealed multiple non-synonymous mutations in each ORF, although the overall sequence conservation was extremely high. This supports previous observations that FeLV retroelements are highly polymorphic within the feline genome (Koshy, et al., 1980; Roca, et al., 2005). Although Env-1, -2, and -5 were isolated from FEA cells infected with FeLV-4314 and -2518, none of the Envs possessed 100% sequence identity to the endogenous portions of these viruses, indicating the parental enFeLV element had not been identified. This was expected as these recombinant viruses arose within naturally hosts, which would possess a genome distinct from that of FEA cells.

6.2.2. The majority of intact enFeLV env genes are non-functional

To investigate the ability of these Env proteins to pseudotype MLV virion cores, MLV(FeLV) *lacZ* pseudotypes were produced by transient transfection of HEK293T cells. Virions were concentrated and analysed by SDS-PAGE to ascertain the level of Env incorporation (Figure 6.2).

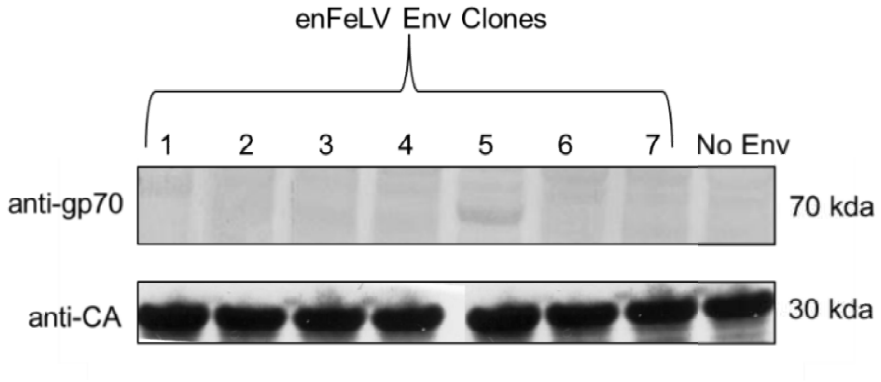


Figure 6.2: The majority of enFeLV Env amplified from FEA cells are not incorporated into viral pseudotype particles.
MLV(FeLV) *lacZ* pseudotypes bearing the novel enFeLV Env proteins were concentrated by ultracentrifugation, separated by SDS-PAGE and immunoblotted. Membranes were screened with either anti-FeLV gp70 MAb (upper) or anti-MLV CA MAb (lower).

These results indicated only enFeLV Env-5 was able to pseudotype MLV viral cores. There remains a small possibility that the other Env proteins were present upon viral particles and were not specifically recognised by this anti-gp70 MAb. This is unlikely as previous experiments indicated this antibody bound specifically to FeLV-B Env (Figure 3.7) which possesses similar epitopes to enFeLV Env within the RBD. Additionally the high degree of sequence conservation between these endogenous Envs makes it unlikely that Env-5 possessed a unique epitope which is lacking in the remaining six.

The RBD, fusion motif, fusion peptide and R peptide are intact within all seven ORFs, therefore virions possessing these glycoproteins would be predicted to be infectious. To further investigate the functionality of these novel proteins, pseudotypes were corrected for equivalent RT activity and titrated upon the range of receptor-expressing MDTF cells described earlier. The results indicated only one of the seven enFeLV Env proteins (Env-5) was able to mediate cellular entry (Figure 6.3). This supports observations that this was the sole endogenously-encoded glycoprotein incorporated into virions. As expected, enFeLV Env-5 utilised the hPit1 receptor, thus appearing phenotypically as a FeLV-B strain.

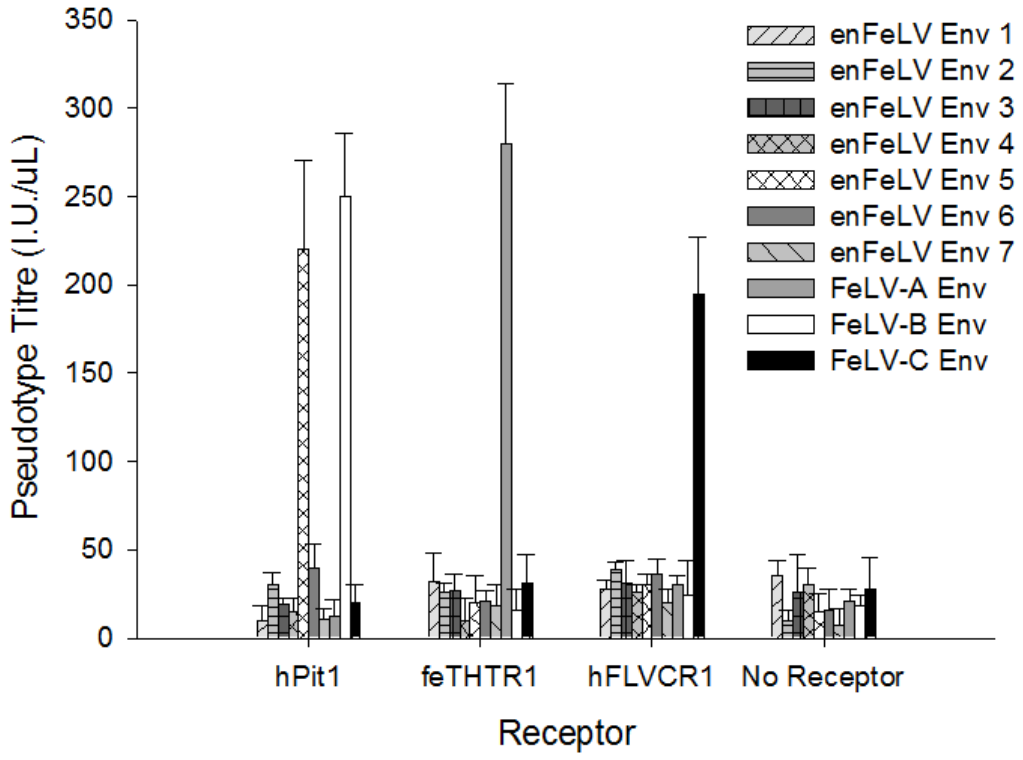


Figure 6.3: enFeLV Env-5 utilises hPit1 for infection.

HEK293T cells were used to prepare MLV (FeLV) *lacZ* pseudotypes bearing the seven enFeLV Envs or reference subgroup A (Glasgow-1), B (Gardner-Arnstein) or C (Sarma) Envs. The viral pseudotypes were then titrated upon MDTF cells expressing huPit1, feTHTR1, huFLVCR1 or vector only. 72 hours post-infection, cells were stained for lacZ expression and counted manually. Values represent the mean +/- SEM of three independent experiments.

The lack of incorporation of the remaining six enFeLV Env proteins could not be explained, as they possessed all the motifs and domains required for a functional gammaretroviral Env protein. It is possible these proteins are translated within the cell and are either degraded or incorrectly trafficked, preventing budding of intact virions. To investigate this possibility, immunofluorescence using the anti-gp70 MAb was performed to detect intracellular Env. HEK293T cells were transiently transfected to produce MLV(FeLV) *lacZ* pseudotypes, before being treated with both methanol and paraformaldehyde to allow visualisation of intracellular and membrane-associated proteins. The results indicated enFeLV Env-2, -3, -5, -6, and -7 were translated within cells (Figure 6.4). In contrast, enFeLV Env -1 and -4 could not be detected, indicating an unidentified block to translation exists for these proteins. As the gp70-MAb was of the same preparation as that used for the previously-described immunoblots, this experiment also confirmed the specificity of the antibody and hence the lack of incorporation of the Env proteins into viral particles (excluding Env-5).

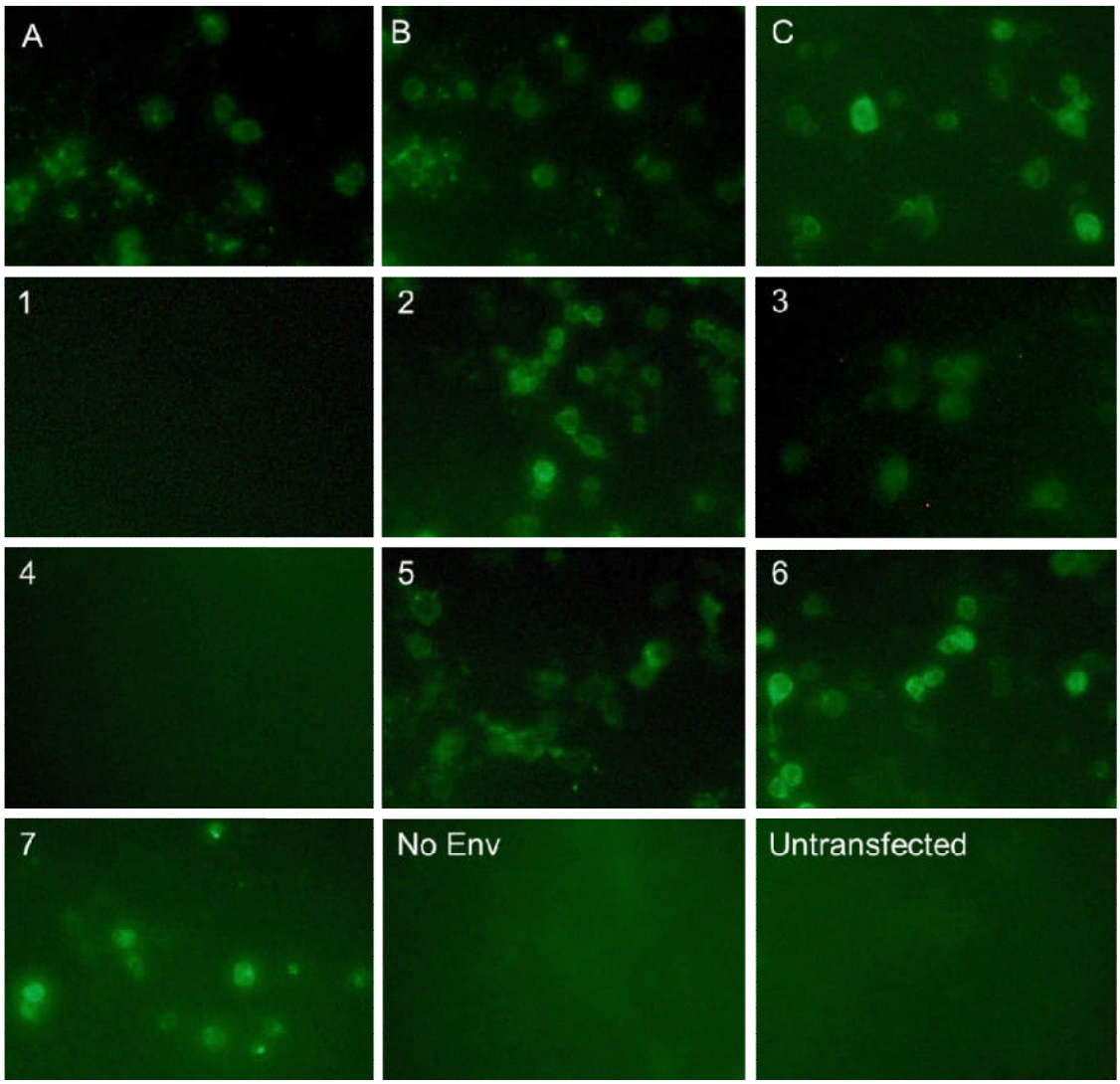


Figure 6.4: enFeLV Envs-2, -3, -6 and -7 are expressed in transfected cells. HEK293T cells transfected with enFeLV expression vectors (enFeLV-1 to -7) were processed for immunofluorescence using an anti-gp70 MAb. Untransfected cells and cells transfected with vector only (No Env) were included to assess background fluorescence, while FeLV-A, -B and –C Envs were included as positive controls for antibody specificity.

Mutations within the signal peptide may alter protein trafficking, preventing an otherwise-functional Env from being displayed at the cell surface. However this peptide is intact in both the functional Env-5 as well as in Env-2, -3, -6 and -7 which are not released from transfected cells. Similarly, a mutated SU/TM cleavage site may prevent Env incorporation into virions, however this motif is conserved between all enFeLV Envs studied. Finally, as Env-1 and -4 do not possess any shared mutations the lack of protein synthesis in these cases cannot

be attributed to any single specific residue. At this point it is not known what difference(s) between the Env proteins prevent synthesis of Env-1 and -4, nor what causes the subsequent lack of incorporation of Env-2, -3, -6 and -7 into virions.

These studies indicate that intact enFeLV *env* genes are present in the domestic cat genome at a higher copy number than previously assumed, and a minority of these genes encode functional glycoproteins able to pseudotype gammaretroviral virions. It is possible that retroviral *env* genes of low infectivity are able to form endogenous elements preferentially, a possibility which has been raised previously (Oliveira, et al., 2007). However if *env* genes such as that encoding Env-5 were located within a full-length transcriptionally active enFeLV provirus, they may be able to mediate cellular infection through the fePit1 receptor and be transmitted horizontally between hosts as an exogenous FeLV-B infection. The assumption that such *env* genes are located within full-length enFeLV genomes is highly plausible. The high degree of sequence conservation between the intact *env* genes described herein and those described in the literature (Roca, et al., 2004) indicates they are likely to be recent endogenisation events.

6.2.3. EnFeLV env transcripts are not detectable in feline tissues

There are numerous examples of host-mediated silencing of ERVs through methylation of the proviral DNA (Feenstra, Fewell, Lueders, & Kuff, 1986; Gimenez et al., 2009; Groudine, Eisenman, & Weintraub, 1981; Harbers, Schnieke, Stuhlmann, Jahner, & Jaenisch, 1981; Hsieh & Weinstein, 1990), an effect which is ablated during the *in vitro* cloning process. Therefore the presence of a replication-competent provirus does not necessarily indicate transcription will occur in host tissues. Such epigenetic methods of retroviral silencing are especially relevant when considering genomically intact proviruses resulting from recent integration events (Lee, et al., 2011), however a genome-wide analysis of the methylation status of ERVs within the domestic cat has not been conducted. Although expression of full-length genomic enFeLV mRNA has also not been investigated, short enFeLV *env*-derived transcripts have been identified previously in lymphoid and placental tissues (Busch, et al., 1983;

McDougall, et al., 1994). To characterise the transcription profile of intact enFeLV *env* genes, whole RNA was extracted from numerous tissue samples from 3 domestic cats (referred to as cats 623, 624 and 625). These cats had been negative control animals for a previous experimental study and were confirmed to be retrovirus-negative. Tissue samples had been snap-frozen in liquid nitrogen during necropsy and maintained at -80°C until RNA purification. Eluted RNA was DNase-treated to remove potential gDNA contamination. A two-step RT-PCR was then conducted to amplify enFeLV *env* transcripts ("FeLV enEnv", PCR detailed in Appendix 8.2) (Figure 6.5). A PCR for the detection of GAPDH transcripts was conducted to confirm the integrity of the RNA ("GAPDH", PCR detailed in Appendix 8.2).

Although the RNA was of a high quality in the majority of samples, enFeLV *env* transcripts could not be detected in any sample using this method. Unfortunately a positive control tissue, such as placenta, was not available for comparison; however as the enFeLV *env* PCR produced the desired amplicons in earlier assays upon gDNA, it is highly probable that the lack of products in this case is indicative of a lack of transcripts. It remains possible that they were present at levels below the detection sensitivity of this PCR, and thus may have been detectable using either nested or quantitative PCR techniques. Alternatively, they may be expressed preferentially in other tissues or cells that were not studied here, such as peripheral blood monocytes.

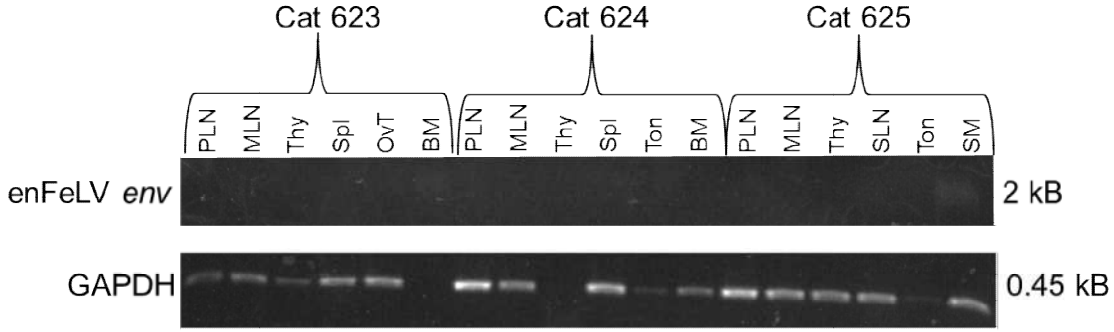


Figure 6.5: enFeLV *env* transcripts are not expressed in domestic cat tissues. Whole RNA was extracted, DNase-treated and screened with two-step RT-PCR for the presence of both enFeLV *env* (upper) and GAPDH transcripts (lower). Tissues studied included popliteal lymph node (PLN), mesenteric lymph node (MLN), thymus (Thy), spleen (Spl), ovarian tract (OVT), bone marrow (BM), tonsils (Ton), submandibular lymph node (SLN), and synovial membrane (SM).

6.2.4. *In silico* modelling of MLV RNA structural elements

The fact that enFeLV *env* transcripts were not detected in this study does not necessarily indicate enFeLV horizontal transmission is prevented by a lack of genomic mRNA expression. Transcription of whole enFeLV genomes in certain tissues is required for recombinant FeLV-B genomes to form, a process which occurs in approximately 40% of chronic FeLV-A infections (O. Jarrett, et al., 1978). A currently unidentified block may exist which prevents transmission of full-length enFeLV viral transcripts in the absence of an exogenous genome. As the LTRs and *env* gene are the main genomic regions differing between endogenous and exogenous FeLV, and the studies described herein had confirmed specific enFeLV Env proteins were functional (Env-5), it was inferred that the enFeLV LTRs act to restrict the horizontal transmission of enFeLV genomes.

The 5' LTR and adjoining leader sequence of the *gag* ORF forms RNA secondary structures (collectively referred to as the packaging signal, Ψ), which allow both dimerisation of the RNA monomers and encapsidation of the genomic RNA by the viral structural proteins. Production of a recombinant FeLV-B genome during reverse transcription would require the formation of a heterodimeric RNA genome composed of an exogenous and endogenous FeLV transcript. It can therefore be assumed that the respective Ψ elements of these viral genomes are able to interact. However transmission of a wholly enFeLV virion in the absence of an exogenous counterpart would require formation of an enFeLV RNA homodimer.

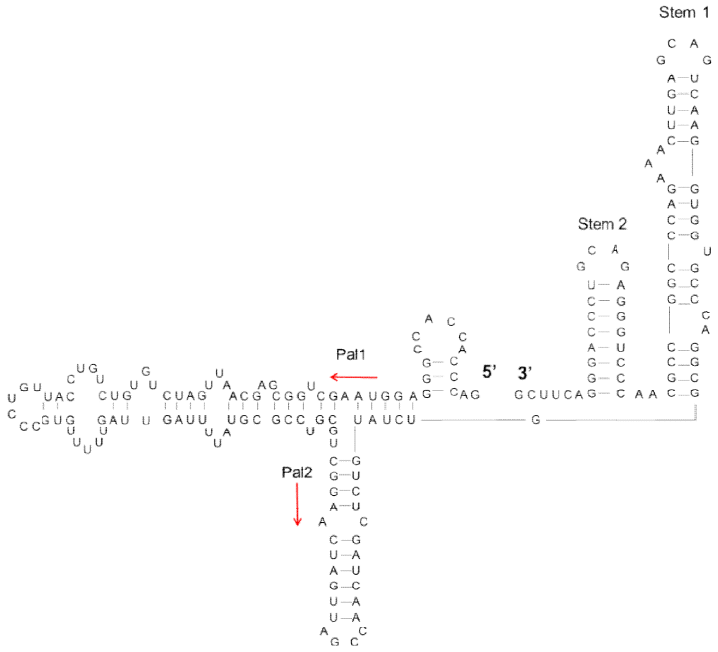
Ideally, *in vitro* dimerisation assays would be utilised to quantify the ability of enFeLV and FeLV-A RNA to form both homo- and heterodimers. However the lack of a full-length enFeLV molecular clone made this impossible. *In silico* modelling of RNA secondary structures was therefore performed to investigate the dimerisation and packaging potential of both enFeLV and exogenous FeLV-A leader sequences. Although early studies of enFeLV LTRs indicated that they possessed intact packaging signals within the U5 region (Berry, et al., 1988), the

importance of RNA secondary structure conservation during successful RNA packaging has since been recognised. Given the high degree of divergence between exogenous and enFeLV genomes in the neighbouring U3 region, it cannot be assumed that enFeLV forms a functional packaging signal despite possessing a conserved U5 domain.

The minimal dimerisation active sequence (MiDAS) of murine sarcoma virus is composed of a stretch of 170 nucleotides within the 5' LTR (Badorrek & Weeks, 2005). The RNAStructure, mFold and Alifold programs were used to produce models of the RNA secondary structures in both monomeric and dimeric forms of this RNA domain (see Chapter 2 for details of these programs). The ability of a program to accurately predict the known structural elements of importance within MLV RNA was the determining factor for the use of that program for the subsequent FeLV models.

The original models produced by the Alifold program are included in Appendix 8.6. This program produced a highly accurate replication of the structures which contribute to MLV packaging and dimerisation (Badorrek & Weeks, 2005; Miyazaki, Irobalieva, et al., 2010) (Figure 6.6). These include two hairpins culminating with GACG "tetraloops" within each monomer (Stem 1 and 2). In the dimeric RNA genome, these stems form intermolecular "kissing-loops" via hydrogen bonds which form between the unpaired cytosine and guanosine bases of the two genomes. These then stabilise the tertiary structure of the dimeric RNA. MLV also possesses palindromic sequences (Pal-1 and -2) which form loose stem-loops in the RNA monomer. This region undergoes a structural rearrangement during dimerisation to form an extended intermolecular RNA helix (Badorrek, Gherghe, & Weeks, 2006; Badorrek & Weeks, 2005; De Tapia, Metzler, Mougel, Ehresmann, & Ehresmann, 1998; Miyazaki, Garcia, et al., 2010). Although the primary nucleotide sequences of MLV and FeLV packaging signals are not highly conserved, their ability to cross-package alternative gammaretroviral genomes indicates that these secondary structural elements are conserved throughout the genus and are able to interact functionally (Doty, Sabo, Chen, Miller, & Abkowitz, 2010; Metais et al., 2010).

(A)



(B)

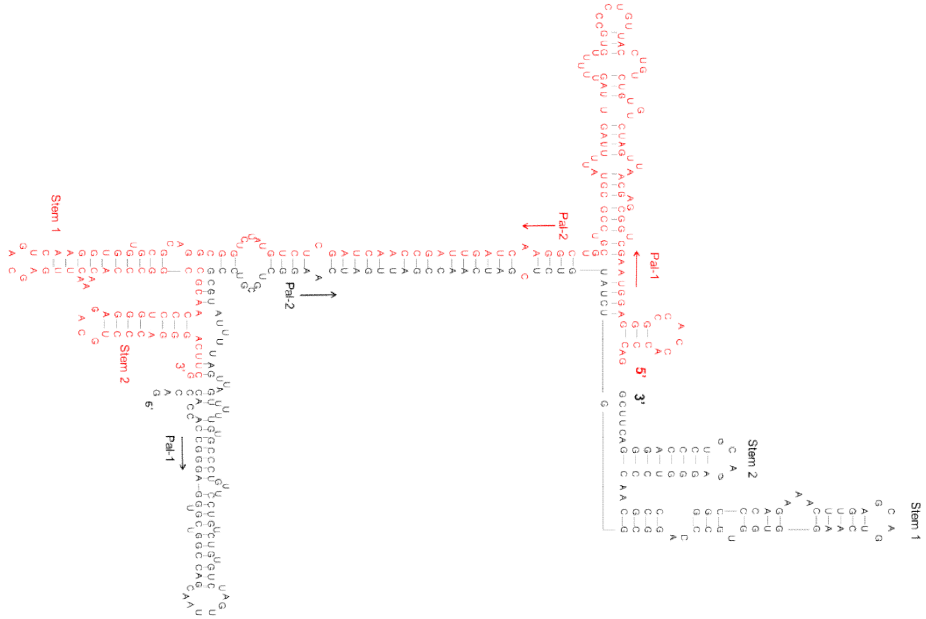


Figure 6.6: The Alifold program accurately reproduces the known structural elements within the MLV leader sequence.

The Alifold webserver was used to predict the RNA secondary structures within the minimal dimerisation activation sites (MiDAS) of (A) monomeric MLV RNA and

(B) dimeric MLV RNA genomes. All structures are conserved with those described in the literature with consideration to both dimerisation (Badorrek & Weeks, 2005) and NC binding elements (Miyazaki, Garcia, et al., 2010). The second genome in (B) is presented in red for ease of reference.

Given the accuracy of the MLV models, the Alifold program appeared likely to produce accurate models of FeLV RNA, for which structures that are experimentally-verified are not available for comparison. Additionally, Alifold includes phylogenetic relationships of input sequences in the resulting models (Gruber, et al., 2008), a factor which is not considered in the mFold or RNAStructure servers. It has been estimated that algorithms for prediction of RNA structures correctly identify only 60% of helices when a single input sequence is used (Dowell & Eddy, 2004; Mathews et al., 2004), therefore the capacity of Alifold to uptake multiple sequences in the form of a FASTA alignment would drastically increase the accuracy of resulting models.

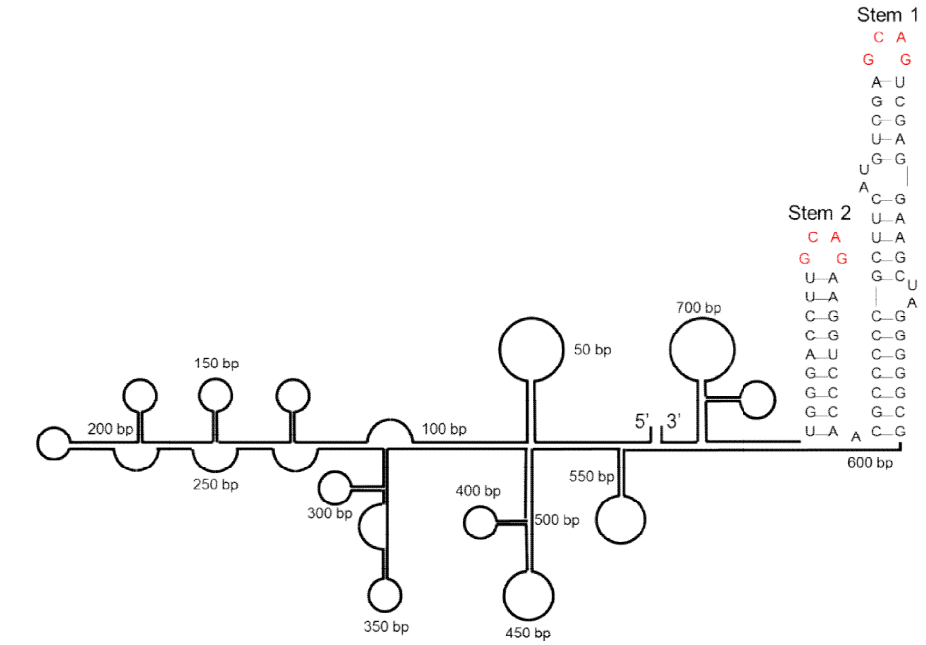
6.2.5. RNA structural elements are intact in FeLV-A RNA homodimers

Predicted RNA secondary structures were generated using the Alifold webserver (Gruber, et al., 2008). In each case a 800bp region was modelled, consisting of the 5' LTR and the *gag* leader sequence from both the FeLV-A(Rickard) and FeLV-FAIDS genomes. Although the large amount of research upon MLV has led to the MiDAS being accurately mapped to a short 170bp region, it cannot be assumed that sequences external to this motif do not contribute to dimerisation and packaging in FeLV, therefore the entire LTR was included in these analyses.

Models of both monomeric and dimeric exogenous RNA were produced (Figure 6.7). The resulting dimeric structure was highly symmetric and contained four GACG tetraloops, indicating "kissing" loops would be able to form during tertiary folding via intermolecular hydrogen bonds. A structural rearrangement was also predicted to occur during dimerisation; a lengthy 5' region which formed numerous intramolecular stem-loops in the monomer formed multiple intermolecular helices and short stem-loops in the dimer. This is similar to the events concerning the palindromic sequences within the MLV models, although

the primary sequences are not conserved between the two viruses. Four prototypic NC binding domains (consisting of an unbound 5' UCUG 3' motif) were identified in the RNA dimer. Collectively, the presence of these predicted structures in the models supports indirect observations that FeLV-A forms functional homodimeric RNA genomes during viral replication.

(A)



(B)

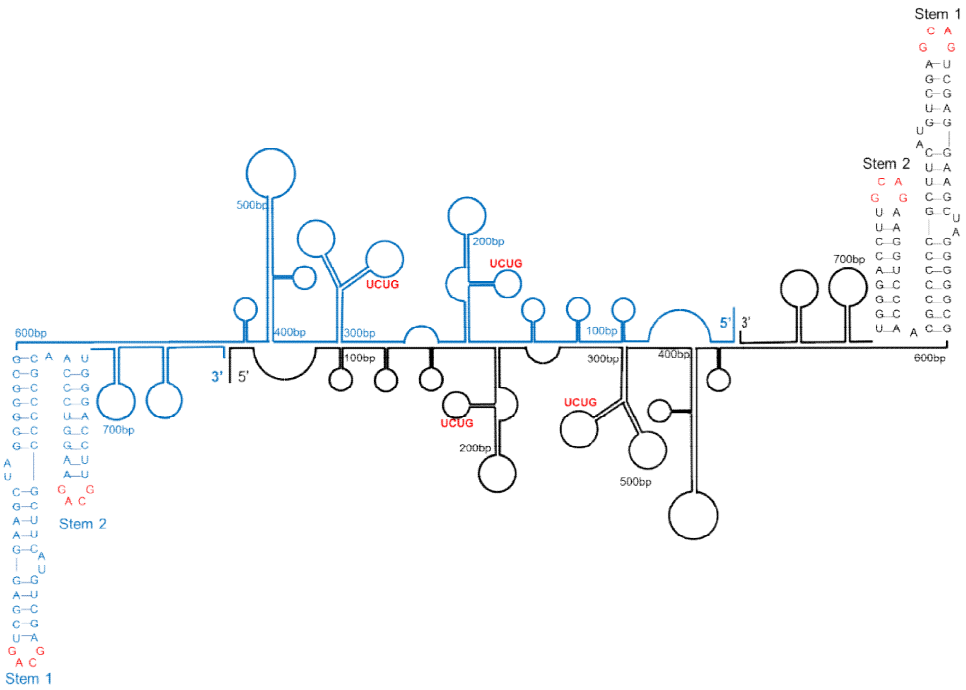


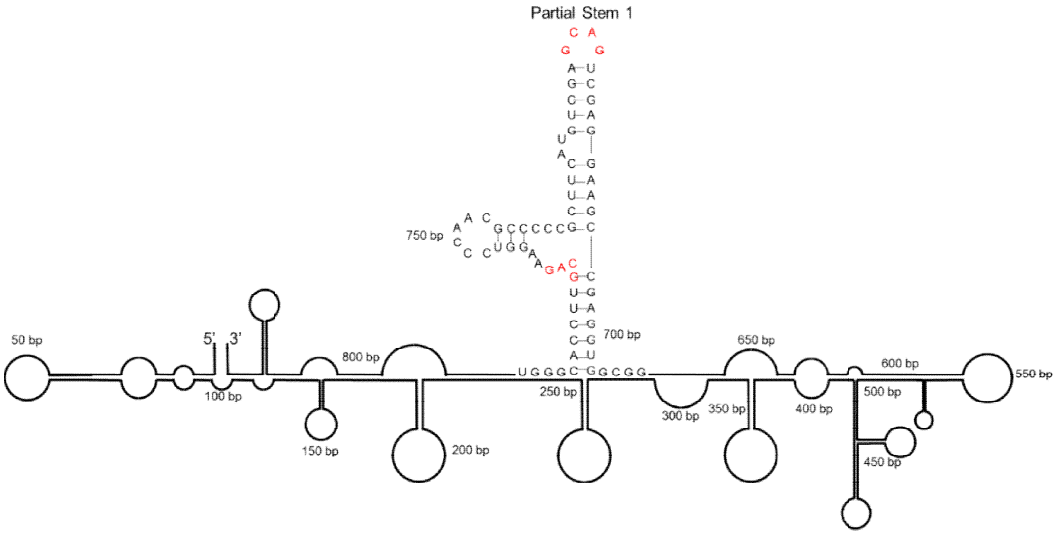
Figure 6.7: The leader sequence of FeLV-A RNA homodimers is predicted to form the required structural elements for viral replication.

Models were produced of the structures within (A) FeLV-A monomeric RNA (free energy of -316 kcal/mol) and (B) homodimeric RNA genomes (free energy of -987.7 kcal/mol). The second genome in (B) is presented in blue for ease of reference.

6.2.6. EnFeLV RNA homodimers may be non-functional

The RNA secondary structures within the 5' LTR and leader sequences of intact enFeLV proviruses, AY364319 and AY364318 were then modelled. Importantly, the free energy predicted for these structures is similar to that of the exogenous models (approximately -990 kcal/mol for the dimeric form), indicating they are equally likely to form in a physiological environment and are of similar stability. The monomeric form of enFeLV RNA was predicted to contain only a single GACG tetraloop, as the nucleotides correlating to Stem 2 form a short loose stem with an extended loop. However within the homodimeric form, one of the two genomes was rearranged at this region into the prototypic 2-stem loop structures (presented in black in Figure 6.8[B]). Although it is possible weak "kissing" loops may form between the partial Stem 1 in genome A and full-length Stem 1 in genome B, the significant disruption in Stem 1 of A as well as the lack of overall symmetry would sterically hinder this conformation. Additionally, only three NC-binding motifs are present in the enFeLV homodimeric RNA. Together these findings indicate that there is likely to be differential exposure of the predicted functional elements required for packaging of endogenous homodimeric RNA into nascent virions.

(A)



(B)

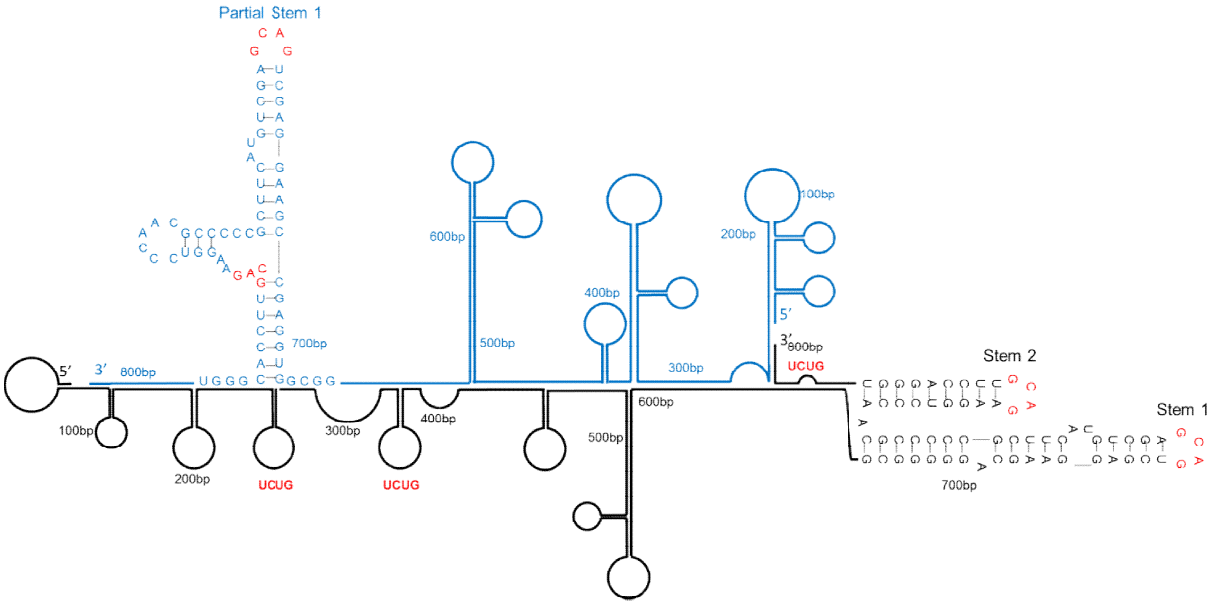


Figure 6.8: The enFeLV RNA leader sequence is not predicted to form the structures required for viral packaging.

Models were produced of the secondary structures within (A) enFeLV monomeric RNA (free energy of -256.3 kcal/mol) and (B) enFeLV homodimeric RNA genomes (free energy of -988 kcal/mol). The second genome in (B) is presented in blue for ease of reference.

6.2.7. Heterodimeric RNA genomes have regained functionality

A model was produced of the structures within a heterodimeric RNA genome. This was composed of a single exogenous FeLV-A (presented in black in Figure 6.9) and an accompanying endogenous FeLV leader sequence (presented in blue in Figure 6.9). This model possessed a similar free energy to both the dimeric structures described above. In this heterodimeric genome, a significant rearrangement of both monomeric RNAs had occurred. Firstly, the exogenous RNA presented only a single GACG tetraloop (Stem 1). Although this was composed of a different primary sequence to the Stem 1 in the monomer, it was of a similar length and stability indicating it would be able to partake in kissing loop formation. The endogenous RNA strand had also undergone structural alterations and presented in the heterodimer with two complete stem-loops, in contrast to both the monomeric and homodimeric forms. Similarly to the exogenous homodimeric RNA, this heterodimer also contained four NC binding motifs.

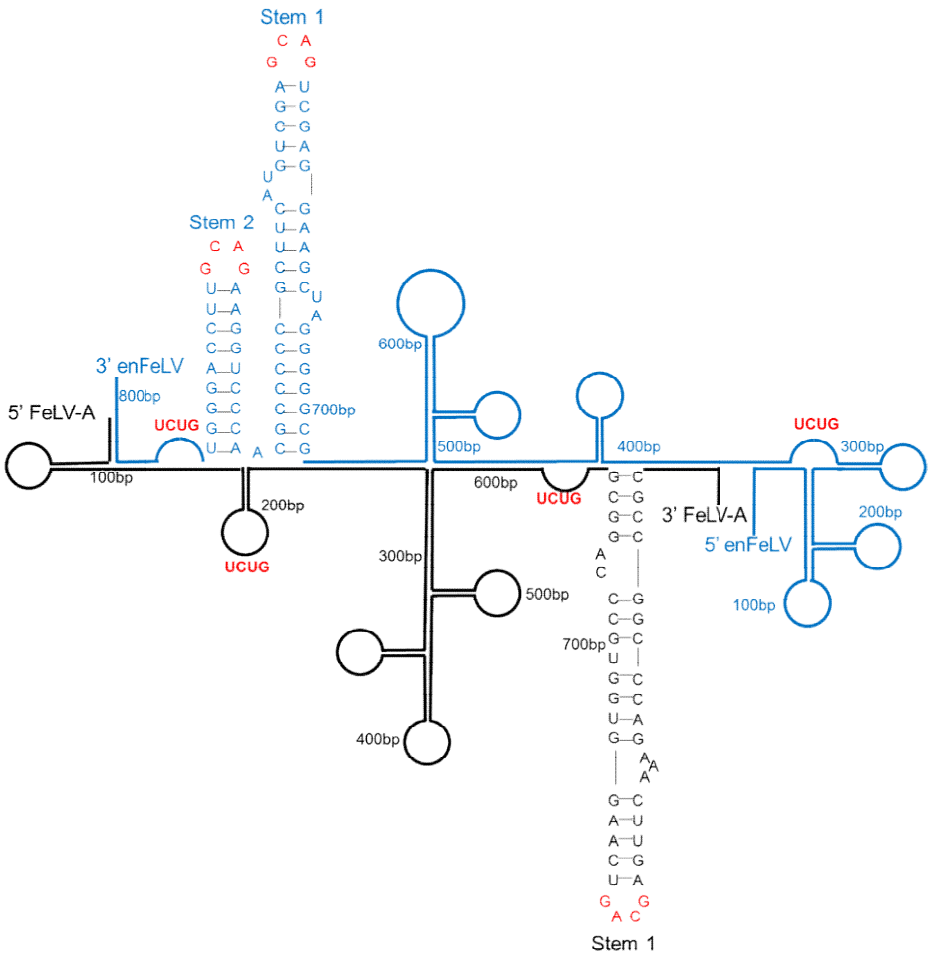


Figure 6.9: Heterodimeric RNA genomes are predicted to contain the structural elements required for packaging.

A model of the secondary structures within an exogenous/endogenous heterodimeric RNA genome was produced (free energy of -988.5 kcal/mol). Similar to the exogenous homodimer, and in contrast to the endogenous homodimer, this genomic RNA would be packaged into nascent virions. The exogenous RNA sequence is presented in black, whereas the endogenous sequence is presented in blue.

Together these results support a model wherein recently-endogenised enFeLV proviruses, although possessing intact ORFs and encoding potentially functional Env proteins, are unable to spread in a horizontal manner due to their inability to efficiently dimerise and be packaged by viral NC proteins. This restriction is mediated by the presence of a significantly non-conserved U3 domain within their 5' LTR, despite the primary sequence of both the U5 region and *gag* ORF being conserved with that of the functional exogenous viruses. During FeLV-A

infection, *in silico* models predict that the presence of the exogenous genome alongside the expression of an endogenous transcript causes a rearrangement of the secondary structures within the RNA leader sequences. This leads to the formation of a stable heterodimeric RNA genome, within which accessible stem-loop structures required for tertiary RNA folding have been restored. The transmission of this genomic RNA to a naïve cell, during which recombination events may occur throughout reverse transcription, leads to the prototypic FeLV-B infection seen in domestic cats.

6.3 Discussion

With the advancement of molecular biology techniques, the decreasing cost of whole genome sequencing and increasing power of bioinformatics software there are now examples of full-length ERVs in the genomes of multiple species that are predicted to be functional. The presence of retroelements such as the proviruses AY364318 and AY364319, thought to have become endogenised recently (Roca, et al., 2005; Roca, et al., 2004), is therefore not unique to the genome of the domestic cat, however FeLV represents one of few currently-circulating retroviruses which exist in both endogenous and exogenous replication-competent forms. Yet pathogenicity and the ability to vertically transmit throughout multiple generations are presumably mutually exclusive properties, suggesting a degree of viral attenuation must accompany the endogenisation process (Oliveira, et al., 2007). As recent genomic integration events are marked by a high degree of conservation between the novel endogenous provirus and the exogenous counterpart, the study of these elements allows accurate identification of the mutations contributing to this process.

6.3.1. The majority of intact enFeLV Env proteins are non-functional

In the case of FeLV, there are two main regions which are significantly non-conserved between endogenous and exogenous genomes; the *env* gene and U3 region of the LTR. In the studies described in this chapter, both regions were analysed for their effects upon the transmission potential and functionality of

enFeLV proviruses. The results indicate that although intact *env* ORFs are present within the feline genome, only a minority of the corresponding proteins are able to pseudotype viral cores. Non-conserved mutations within the remaining Env proteins prevent either protein translation or correct trafficking and incorporation into viral particles.

It is possible FeLV genomes encoding non-functional Env proteins preferentially endogenise within the feline genome, although a potential mechanism for this selection process is not clear. A gene encoding a defective Env protein may require less host-mediated silencing, as transcripts will have a limited capacity for successful translation. Additionally, recombinant FeLV-B Env proteins arising from these transcripts may possess lowered infectivity and be unable to spread easily. In this case even an enFeLV transcript that is expressed widely will not necessarily contribute to pathogenic FeLV-B genomes. However this hypothesis suggests the functionality of Env-5 is acquired through a gain-of-function mutation. As there is only one mutation unique to Env-5 (R121Q), it would be of interest to specifically back-mutate this residue and assay its effect upon both Env incorporation and receptor usage.

Alternatively, rather than selection for the endogenisation of already-defective enFeLV *env* genes, a more probable scenario is that the non-functional enFeLV ORFs independently acquired a range of non-conserved mutations which depleted their respective infectivity after the integration event. The comparative prevalence of intact *env* ORFs encoding defective Env proteins may therefore be evidence for attenuation of otherwise-functional enFeLV, a process which would increase the probability of the retroelement becoming established within the species genome. In the case of Env-1 and -4, the extent of mutations prevent initial protein synthesis, however expression of Env-2, -3, -6 and -7 could be detected within transfected cells. It is possible these proteins are not present upon virions due to a lack of association with the *gag*-encoded structural proteins; this correlates to their presence within the cytoplasm but not in the secreted virus preparations. Successful recruitment of Env glycoproteins by the gammaretroviral Gag polyprotein requires an intermediate stage during which the proteins colocalise within cytoplasmic vesicles, prior to the final virus assembly (Sandrin, Muriaux, Darlix, & Cosset, 2004). It is possible these Env

proteins each possess distinct mutations which alter their intracellular trafficking and prevent successful association with Gag. However mutations conferring this phenotype are generally mapped to the cytoplasmic tail (Sandrin, et al., 2004). Although the blocked Env proteins -2, -3, -6 and -7 do not contain excessive mutations in this region, there are scattered mutations throughout this domain which are not present in the functional Env-5. Future work should be aimed towards assessing the impact of these mutations upon protein trafficking and Env incorporation.

It is possible that these apparently non-functional enFeLV Env proteins were present within virions at extremely low levels, below the detection sensitivity of either the anti-gp70 immunoblot or the pseudotyping system. If this is the case, this indicates that they are either incorporated at too low a level to initiate cellular entry via hPit1, or utilise a distinct cognate receptor. Given the high degree of conservation these proteins displayed with the functional Env-5 (which utilised hPit1), this is unlikely. However specific FeLV-B isolates may utilise either hPit1 or the orthologue hPit2, and it has been suggested that the proportion of endogenously-derived residues within FeLV-B Env affects the ability of the protein to utilise Pit2 (Boomer, et al., 1997; Nunberg, Williams, et al., 1984). The determinants for hPit2 usage were tentatively mapped to the VRB region within the SU domain, whereas hPit1 usage maps to the VRA motif (Boomer, et al., 1997). Although the ability to utilise hPit2 was not directly investigated in these studies, it is unlikely that the non-functional Envs are able to utilise this receptor as MDTF wildtype cells express the murine homologue of Pit2 (mPit2) and these cells were not susceptible to infection with any enFeLV pseudotypes. It remains possible that these proteins are synthesised at levels undetectable with a standard immunoblot, and are able to utilise the feline homologues of Pit which were not included in these analyses. If cloned constructs encoding these receptors were available, it would be of interest to target the mutations present within the VRB motif of these Env proteins and screen for an altered ability to bind to fePit1 and -2.

6.3.2. Non-functional packaging signals may prevent horizontal transmission of enFeLV

The interpretation of the RNA structural *in silico* models produced in this analysis relies upon the assumption that the conserved 2-stem-loop structure is the primary determinant of RNA dimer functionality; i.e., whether the correct tertiary structure can be obtained and is subsequently recognised by the viral structural proteins. The validity of this assumption is supported by the fact that gammaretroviral RNAs are packaged by heterologous viral proteins promiscuously, despite the fact that the primary sequence of their corresponding leader motifs are not necessarily conserved (Doty, et al., 2010; Metais, et al., 2010; Patience, Takeuchi, Cosset, & Weiss, 1998; Suling, Quinn, Wood, & Patience, 2003; Torrent, Gabus, & Darlix, 1994). The final hypothesis that enFeLV RNA homodimers are restricted from inclusion in nascent virions due to their inability to form functional "kissing" loops is therefore highly plausible. Although it remains possible that the partial stem-loop observed in the enFeLV homodimeric RNA is able to participate in intermolecular binding, further support for this interpretation is provided by the observation that although each "kissing" loop supports dimerisation individually, maximum stability of the RNA dimer requires the presence of both kissing loops (Ly & Parslow, 2002; Oroudjev, Kang, & Kohlstaedt, 1999). This suggests exogenous homodimeric RNA genomes will preferentially be formed, regardless of the presence of endogenous transcripts, as this was the only dimeric structure which possessed four complete GACG tetraloops. Combined with the low levels of enFeLV transcription (Busch, et al., 1983; Kidney, et al., 2001; McDougall, et al., 1994; Niman, et al., 1980), this presumably contributes to the prevention of FeLV-B developing in all domestic cats chronically infected with FeLV-A.

Once a dimeric RNA genome has undergone tertiary folding, it must then interact with the NC proteins to ensure selectivity is maintained during the packaging process. This occurs prior to the protease-mediated cleavage of NC from the Gag polyprotein and is distinct from the subsequent "coating" of the RNA with NC, which eventually forms the viral core particle (Rein, 1994). In the prototype gammaretrovirus MLV, NC proteins bind to eight exposed UCUG motifs with high affinity in the dimeric RNA genome. However these motifs are

sequestered within intramolecular helices in RNA monomers, preventing monomeric RNA encapsidation (D'Souza & Summers, 2004; Dey, York, Smalls-Mantey, & Summers, 2005; Gherghe & Weeks, 2006; S. F. Johnson & Telesnitsky, 2010). Mutations of the guanosine within these motifs reduces packaging 100-fold, therefore the loss of a single NC-binding site may significantly alter the rate of genome encapsidation and final infectious viral titre (Gherghe et al., 2010). As the enFeLV homodimeric RNA genome contains only 3 exposed UCUG motifs this would be expected to exhibit decreased packaging titres compared to either the exogenous homodimeric or heterodimeric RNA, both of which present with four unpaired UCUG motifs. Additionally, the overall secondary structure and long-range tertiary interactions of viral RNA also contribute to the specificity of NC recognition; it is not merely due to the presence of exposed UCUG motifs (Gherghe, et al., 2010). Thus the combined effect of the altered stem-loop structures and the decrease in potential NC-binding sites in enFeLV homodimeric RNA may significantly decrease the transmission potential of virions containing this genome.

It must be highlighted that co-packaging and dimerisation of heterogeneous FeLV genomes has not been directly observed, although this has been documented in numerous other gammaretroviruses (Mikkelsen, Rasmussen, & Pedersen, 2004; S. V. Rasmussen, Mikkelsen, & Pedersen, 2002; Suling, et al., 2003; Villanueva, Campbell, & Roth, 2003). Alternative mechanisms must therefore be considered for both the lack of enFeLV horizontal transmission and FeLV-B formation. For example, the degree of epigenetic silencing of retroviral elements within the feline genome has not been investigated and may prevent expression of otherwise functional ERVs. During processes involving demethylation (such as cellular differentiation) a low level of enFeLV transcription may occur, leading to temporal and spatial determination of FeLV-B formation. Equally, FeLV-B genomes may arise through homologous recombination of host DNA rather than through strand-transfer events during reverse transcription. However viral genetic recombination is more likely to occur during the packaging of two unique genomes within a single virion, rather than during homologous recombination within host DNA after infection by two distinct particles (Katz & Skalka, 1990). Thus if chimaeric viral genomes are routinely observed, such as that which occurs during FeLV-B formation, this provides indirect evidence for genomic

dimerisation of the two parental viruses. It can therefore be assumed FeLV-B arises after dimerisation of two heterogeneous RNA transcripts and a subsequent RT strand transfer event during reverse transcription. In the absence of an exogenous FeLV-A genome, the enFeLV genomic RNA monomers may form homodimers which these models indicate are unable to form stable “kissing” loops and achieve the tertiary structure required for virion packaging.

6.4 Conclusions

The conclusions which can be drawn from these experimental results (specifically describing the expression and functionality of enFeLV Env proteins) have a central caveat which assumes the reactivity of each Env with the gp70 antibody is e qual and consistent. As described within the text, it is unlikely that any Env has lost the epitope in question, however it cannot be ruled out. The lack of positive control reagents also means that only preliminary interpretation of these results is possible (for example, placenta was not available for RT-PCR studies and this is the only tissue definitely established to routinely express enFeLV *env* transcripts). Regarding the *in silico* RNA structural analysis, although the results are intriguing it must be stressed that definitive conclusions cannot be drawn until corresponding experimental data has been obtained.

The results presented in this chapter suggest a novel mechanism of viral restriction, wherein intact proviral genomes are unable to transmit in an exogenous manner due to the inability of their dimerisation and packaging signals to form the required RNA secondary structures. In the case of FeLV, these ERVs are rescued by the presence of an FeLV-A transcript which initiates refolding of the RNA and formation of a heterodimeric genome, eventually resulting in FeLV-B formation. As an increasing number of putatively replication-competent ERVs are currently being isolated from numerous species, this mechanism may be relevant to other retroviruses in addition to FeLV.

As this hypothesis is primarily supported by simulations of RNA folding, these results are preliminary and require validation with experimental data before any final conclusions can be drawn. The possibility that enFeLV full-length transcripts are transmitted between hosts at extremely low levels, presenting as

FeLV-B sole infections, cannot be excluded at this stage. Ideally, *in vitro* RNA binding assays using a range of transcripts, including both the leader sequences alone and whole viral genomes, should be conducted to accurately assess the dimerisation potential of these viral genomes.

7. Concluding Remarks

Although protective vaccines have been developed for FeLV, this virus remains a serious pathogen both for domestic and wild felids. The pathogenic mechanisms of infection remain poorly understood and treatment options for those hosts which display chronic viraemia and FeLV-associated disease are limited. In addition to these unresolved issues, the relevance of FeLV as a model for human diseases, including HIV-AIDS and cancer progression, means that significant further research is required to understand this intriguing retrovirus. Although many aspects of FeLV have been previously investigated, the recent advancements of molecular biology now allow these areas to be revisited and viewed from a renewed perspective. The studies discussed in this thesis focussed broadly upon the evolution of novel subgroups FeLV-B and –C which tend to display heightened pathogenicity from their parental FeLV-A virus.

There are many aspects which determine collectively whether a FeLV infection is significantly pathogenic and whether novel subgroups arise within the host. The majority of studies to date, including those discussed herein, have focussed upon the LTRs and the Env protein as both these elements play a significant role in determining whether a productive infection occurs within a host. Whether the resulting chronic infection proves to be significantly pathogenic over time depends upon additional factors including the integration site, degree of resulting immunosuppression (which were not investigated within this research) and the ability of Env to utilise alternative receptors. Finally, it must also be considered whether virus binding to the cognate receptor depletes the wildtype function of that cellular protein, such as in the case of PRCA arising during FeLV-C infection (Quigley, et al., 2004). Therefore additional research into a number of areas is required before the development of novel FeLV subgroups can be fully understood. The studies described within this thesis had four main research aims: (1) to identify Env determinants which predispose FeLV-A to FeLV-C conversion; (2) to investigate the potential role of the host humoral response in FeLV-C evolution; (3) to determine the potential for inter-host FeLV-B transmission without the additional presence of FeLV-A; and (4) to characterise the functionality of enFeLV elements and the possibility of enFeLV horizontal

transmission between hosts. The results, although intriguing and informative, highlight the degree of further research required in this field before definitive conclusions can be reached.

As the protective correlates of vaccination have not been definitively established and no currently-available vaccine provide sterilising immunity, the development of FeLV-C remains a possibility in all domestic cats. Given the severe mortality and morbidity of this subgroup, it is of utmost importance to ascertain which subgroups of FeLV-A may preferentially evolve into FeLV-C and which factors contribute to this process. These results support a model wherein FeLV-A isolates containing the D83:D91 motif possess heightened replication rates and an increased ability to bind to both THTR1 and FLVCR1, compared to the prototype FeLV-A strains not known to lead to FeLV-C (Chapter 3). Over time, it is predicted that viral subpopulations would arise as the virus acquires mutations through genetic drift. These would gradually increase the SU-FLVCR1 binding affinity, eventually resulting in dual-tropic "intermediate" viruses able to mediate cellular entry through a range of receptors (Shalev, et al., 2009). Prototype FeLV-C isolates would eventually develop, at which point the virus would be predicted to have lost THTR-binding ability and rely solely upon FLVCR1 for entry. This model supports previous observations that the primary sequences of FeLV-C isolates display low conservation, reflecting their independent evolution from different FeLV-A isolates despite inducing the prototypic PRCA disease symptoms (Brojatsch, et al., 1992).

In a parallel scenario to the development of FeLV-C, alterations in receptor usage induced by the gradual acquisition of mutations has been observed for HIV-1; this may reflect a common mechanism of evolution for retroviruses. This further highlights the relevance of FeLV research and its applications to general retrovirology. However in the case of HIV-1 this switch in receptor usage is partially due to an escape from host immunity; this could not be replicated in *in vitro* models (Chapter 4). The ability of the virus to continuously replicate in the face of an immune response determines whether FeLV is able to infect bone marrow cells, resulting in the secondary viraemia and a subsequent chronic infection. It is therefore logical that a virus able to circumvent this immunity would display an increased probability of inducing disease and potentially

developing into FeLV-C. Although these studies did not indicate that the presence of VNAs increased the appearance of mutations associated with FeLV-C, this remains a plausible theory given the numerous other examples of retroviral evolution mediating antibody-escape.

The fact that the mutations studied in Chapter 3 were detected in multiple independent FeLV isolates indicates an epitope in this region may be commonly targeted by the host immune response. However these mutations did not confer neutralisation resistance (Chapter 4), although this should be interpreted with caution as the use of a broader range of antibodies may have produced different results. A hypothesis not investigated herein is that these mutations alter a T-cell epitope. As T cell epitope recognition differs broadly between individuals, escape from CTL responses by a viral variant is unlikely to result in a viral strain which has a replicative advantage in a secondary host (Overbaugh & Bangham, 2001). This explanation supports the observations that FeLV-C cannot be transmitted between hosts, however it does not explain why these mutations occurred in multiple independent field samples from presumably unrelated hosts. As B cell epitopes are broadly recognised between individuals of a species, it is more probable these mutations play a role in immune evasion through this mechanism.

These results also indicated that a selective pressure in the form of receptor-availability does not drive evolution of FeLV-C. This is in direct contrast to the opinion that VNAs and receptor availability on target cells are the main forces acting during retroviral evolution (Overbaugh & Bangham, 2001). However it must be noted that the broad expression profile of THTR1 makes it unlikely that a virion would encounter a cell which solely expresses FLVCR1. This theory may be physiologically irrelevant to FeLV, whilst holding true for other retroviruses which utilise receptors with non-overlapping expression profiles.

The low amino acid conservation between Env proteins of individual FeLV-C isolates, documented in these studies and those of others (Adema, 2003; Brojatsch, et al., 1992), indicates it may be the overall structure rather than the acquisition of a defined RBD which determines the success of FLVCR1-SU binding. This model is applicable to other retroviruses; gibbon ape leukaemia virus

(GALV), amphotropic MLV and FeLV-B all utilise Pit homologues and yet do not display high amino acid conservation between their Env proteins, indicating their tertiary structures may be analogous (L. Pedersen, Johann, van Zeijl, Pedersen, & O'Hara, 1995; Tailor & Kabat, 1997; Tailor, Nouri, & Kabat, 2000; Tailor et al., 1993). These results therefore support suggestions that the SU binding site within a receptor is not a predetermined invariable factor in retroviral cellular entry (Tailor, et al., 1993). Future work should be aimed towards determining the relative binding positions of FeLV-A and–C Env proteins to both THTR1 and FLVCR1. The increase in affinity of SU for FLVCR1 predicted to occur during FeLV-C evolution may be accompanied by a switch in the receptor domains involved in binding.

Similar to FeLV-C, FeLV-B development in a host significantly alters the disease association. The development of lymphomas and/or leukaemias in a domestic cat confers an increased rate of mortality and morbidity and limited treatments are available. Although FeLV-B is not thought to occur in endangered wild felids due to their lack of enFeLV elements, it appears that FeLV-B isolates may be transmissible between hosts without co-transmission of FeLV-A (Chapter 5). The isolates FeLV-2518 and -4314 are therefore of significant interest and may represent a novel pathogen for endangered felids, previously assumed to only be at risk of FeLV-A infection. Had time and resources allowed, it would have been of interest to investigate the clinical implications of FeLV-2518 and/or -4314 and compare these to a prototypic FeLV-A/B infection. It has been previously noted that FeLV-B variants associated with thymic lymphoma possess further upstream 3' recombination junctions than those associated with other diseases (Ahmad & Levy, 2010). Thus the proportion of the viral genome originating from an endogenous element appears to influence the disease progression during infection, supporting the notion that ERVs are preferentially selected for their altered virulence (Oliveira, et al., 2007).

Instances of enFeLV horizontal transmission were not identified in these studies, despite the prediction that it would be these infections presenting as FeLV-B alone. Given that FeLV-4314 contains the majority of enFeLV-derived *env* and *pol* genes and the *gag* ORF is highly conserved between enFeLV and exogenous genomes, the only remaining portion of the FeLV-4314 genome which remains

ostensibly exogenous are the LTRs. The analysis of the transcription factor binding sites contained within the respective LTRs indicates that enFeLV virions would not exert similar pathogenic effects to FeLV-4314 or -2518, as exogenous LTRs contain significantly more intact promoter elements.

The *in silico* analysis of recombination sites within FeLV-B genomes (Chapter 5) may also have implications for other viral research. Given that FeLV-2518 and -4314 were produced during natural infections, these results provide an *in vivo* comparative model for observations regarding increased recombination at the termini of RNA helices *in vitro*. Retroviral recombination contributes significantly to genetic variation in all retroviruses and may alter neutralisation susceptibility, receptor-utilisation or replication kinetics (Simon-Loriere & Holmes, 2011). However accurate identification of recombination breakpoints may prove difficult within a highly homogeneous viral population; FeLV-B therefore provides a unique model wherein the contributing genomes differ significantly in two distinct areas (U3 and *env*). This allows accurate recombination-site mapping and the subsequent correlation between predicted RNA secondary structures. To the best of our knowledge, this analysis is the first to link RNA structural elements with specific phenotypically-distinct subgroups of FeLV.

The investigations into the functionality of enFeLV proviruses (Chapter 6) raise some intriguing propositions. The discovery that full-length intact Env ORFs are more prevalent than previously observed was partially expected, given the lack of feline genomic research conducted to date and the knowledge that enFeLV elements are polymorphic (McDougall, et al., 1994; Roca, et al., 2004). However the inability of these proteins to express *in vitro* indicate an intact ORF may not be indicative of a functional Env. Only one of seven proteins identified was able to pseudotype viral cores and mediates cellular entry (enFeLV Env-5). The remaining proteins displayed differential levels of expression within the cellular cytoplasm; mutations conferring these properties could not be identified. Had time allowed, it would have been of interest to identify which mutation/s within each Env was responsible for the respective defects observed.

These preliminary results also provide further evidence as to the conservation of RNA secondary structures in the packaging and dimerisation domains of gammaretroviruses. As the majority of gammaretroviral RNA structural work conducted to date is concerned with MLV, the study of another virus such as FeLV would complement these results and highlight similarities and differences. However *in vitro* analyses of both the dimerisation and packaging abilities of enFeLV RNA homodimers and FeLV-A/enFeLV heterodimers are required to provide definitive answers to whether this is the block preventing enFeLV horizontal transmission.

The novel suggestion that altered RNA structures provide a mechanism of restriction for potentially-functional endogenous retroelements supports the general theory that endogenous retroviral elements are by their nature inoffensive to the host (Lower, 1999; Oliveira, et al., 2007). An actively pathogenic endogenous retrovirus would by its very nature be purged from the host over evolutionary time, although a relic such as a solo LTR may remain. The exception to this premise is the example of FeLIX. Despite being endogenously encoded, this peptide rescues defective FeLV-T strains and allows infection of T lymphocytes (Anderson, et al., 2000). However given the fact that FeLV-T is only rarely reported, it is probable that the majority of enFeLV peptides confer a protective advantage to hosts. This is the alternative explanation for the maintenance of their functionality and expression (McDougall, et al., 1994). In contrast, it has also been suggested that the likelihood of persistence versus deletion or inactivation of the retroelement (through, for example, recombinational deletion during meiosis, resulting in a solo LTR genomic relic) may simply reflect the gene density and recombination rate of the genomic area in which it is integrated (Katzourakis, Pereira, & Tristem, 2007). Nonetheless, the general observation that ERVs are non-pathogenic and display lowered infectivity compared to their exogenous counterparts is supported by these results, which indicate apparently-functional enFeLV elements may be restricted by their inability to form the required RNA structures for efficient viral transmission.

Overall, the studies discussed in this thesis raise intriguing hypotheses applicable to both FeLV and general retrovirology, and highlight the necessity of further research upon this significant feline pathogen.

8. Appendices

8.1 Buffers and solutions

Bromophenol Blue Protein Loading Buffer

0.25M Tris-HCl; 2.5% (w/v) SDS; 10% (w/v) glycerol, 1% (1/v) 2-mercaptoethanol; 0.02% (w/v) bromophenol blue; pH 6.8.

Phosphate-Buffered Saline

137mM NaCl; 2.7mM KCl; 8mM Na_2HPO_4; 2mM KH_2PO_4; pH 7.3.

DNA Electrophoresis Loading Buffer (10x concentration)

0.1M EDTA; 15% (w/v) glycerol; 0.05% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol.

Tris-Borate EDTA (TBE) Buffer (10x concentration)

890mM Tris Base; 2mM di-sodium EDTA; pH 8.3.

Tris-Glycine Buffer

25mM Tris Base (7.9); 192mM glycine; 0.1% (w/v) SDS; pH 8.3.

Luria Broth (LB)

10% (w/v) bactotryptone; 5% (w/v) yeast extract; 85mM NaCl; pH 7.0.
1.5% agarose included for LB agar plates.

Super Optimal Broth for Catabolite Repression (SOC Broth)

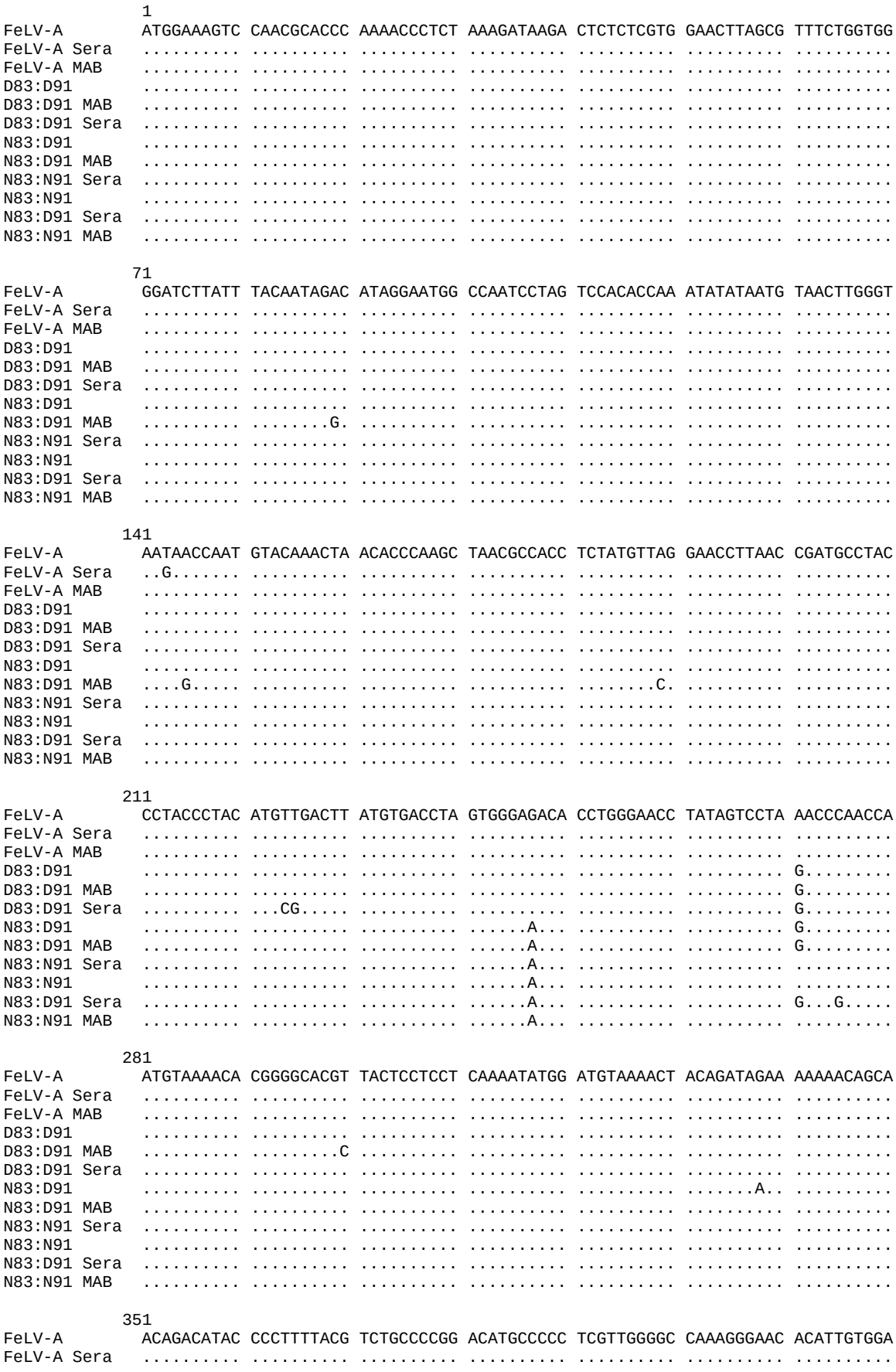
20mM glucose, 10mM $MgCl_2$, 10mM $MgSO_4$; 2.5mM KCl; 10mM NaCl; 2% (w/v) bactotrypone; 0.5% (w/v) yeast extract.

8.2 List of Primers

FeLV Env	Sense:	5’ GGTCGACATGGAAGGTCCAACGCACCCAAAA 3’
	Antisense:	5’ GGGGTCGACTGGAATCATACATTTAATTGGAAAT 3’
FeLV SU	Sense:	5’ CGATCTAGAATGGAAAGTCCAACGCACCCAAAA 3’
	Antisense:	5’ CATGGATCCGCTGTGTACACATATTCGGGTTGATG 3’
FeLV enEnv	Sense:	5’ GGTCGACATGGAAGGTCCAACGCACCCAAAA 3’
	Antisense:	5’ CGCGGCCGCTTAGCTGGGGTGATACGGTTGGT 3’
FeLV en-U3	Sense:	5’ GAGCTAGCAATGCGACTCAGACCAACCGTATCA 3’
	Antisense:	5’ GGTACCCGGGGCGGTCAAGTCTCGGCAAAG 3’
FeLV ex-U3	Sense:	5’ GAGCTAGCAATACGATCCGGACCGACCATG 3’
	Antisense:	5’ GGTACCCGGGGCGGTCAAGTCTCGGCAAAG 3’
RD-114 Env	Sense:	5’ GTTTGACGACCCCCGCAAGGCTAT 3’
	Antisense:	5’ GGGCCAGCACCATGGCATGTACAACA 3’
FcEV Env	Sense:	5’ ATTCCACCCTCACACCAGAATC 3’
	Antisense:	5’ TTGAGTTAGGACCAAGGCCTG 3’
GAPDH	Sense:	5’ CCTTCATTGACCTCAACTACAT 3’
	Antisense:	5’ CCAAAGTTGTCATGGATGACC 3’
FeLV-Recombinant	Sense:	5’ AAGACTAGACGTGGGAATGGCC 3’
	Antisense:	5’ AATTTTCCATACCTTGTGAAATGG 3’

All PCRs were conducted using a high-fidelity commercially prepared mastermix (Roche), which included 200uM of each dNTP and 0.2U/uL of polymerase enzyme. Primers were added to a final concentration of 0.5uM each, with 50ng (plasmid) or 200ng (genomic DNA) of template DNA per reaction. Cycling parameters consisted of an initial denaturation (94°C, 2 minutes), followed by 35 cycles of denaturation-annealing-extension (94°C, 30 seconds; 50°C, 30 seconds; 72°C, 1 minute/kB of template DNA). A final extension period of 5-10 minutes at 72°C was included before reactions were stored at 4°C. In some cases the annealing temperature was altered to increase reaction specificity.

8.3 Nucleotide mutations arising in long-term FeLV cultures



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FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  ..G....... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

           421
FeLV-A        GGGGCACAAG ATGGGTTTTG TGCCGCATGG GGATGTGAGA CCACCGGAGA AGCTTGGTGG AAGCCCACCT
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... ......G... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

           491
FeLV-A        CCTCATGGGA CTATATCACA GTAAAAAGAG GGAGTAGTCA GGACAATAGC TGTGAGGGAA AATGCAACCC
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

           561
FeLV-A        CCTGGTTTTG CAGTTCACCC AGAAGGGAAG ACAAGCCTCT TGGGACGGAC CTAAGATGTG GGGATTGCGA
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......C.. .......... .......... ..........
D83:D91 Sera  .........A .....T.... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... ..G....... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

           631
FeLV-A        CTATACCGTA CAGGATATGA CCCTATCGCT TTATTCACGG TGTCCCGGCA GGTATCAACC ATTACGCCGC
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    ....G..... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

           701
FeLV-A        CTCAGGCAAT GGGACCAAAC CTAGTCTTAC CTGATCAAAA ACCCCCATCC CGACAATCTC AAACAGGGTC
FeLV-A Sera   ....A..... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......C.. .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........
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           771
FeLV-A       CAAAGTGGCG ACCCAGAGGC CCCAAACGAA TGAAAGCGCC CCAAGGTCTG TTGCCCCCAC CACCATGGGT
FeLV-A Sera  .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91      .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB  .......... .......A.. .......... .......... .......... .......... ..........
D83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:D91      .......... .......... .......... .......... .......... C......... ..........
N83:D91 MAB  .......... ......G... .......... .......... .......... .......... ..........
N83:N91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91      .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB  .......... .......... .......... .......... .......... .......... ..........

           841
FeLV-A       CCCAAACGGA TTGGGACCGG AGATAGGTTA ATAAATTTAG TACAAGGGAC ATACCTAGCC TTAAATGCCA
FeLV-A Sera  .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB   .......... .......... .......... .......... ........G. .......... ..........
D83:D91      .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB  .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:D91      .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB  .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91      .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB  .......... .......... .......... .......... .......... .......... ..........

           911
FeLV-A       CCGACCCCAA CAAAACTAAA GACTGTTGGC TCTGCCTGGT TTCTCGACCA CCCTATTACG AAGGGATTGC
FeLV-A Sera  .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91      .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB  .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:D91      .......... ....G..... .G........ .......... .......... .......... ..........
N83:D91 MAB  ...G...... .......... .......... ......G... .......... .......... ..........
N83:N91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91      .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB  .......... .......... .......... .......... .......... .......... ..........

           981
FeLV-A       AATCTTAGGT AACTACAGCA ACCAAACAAA CCCCCCCCCA TCCTGCCTAT CTACTCCGCA ACACAAACTA
FeLV-A Sera  .......... .......... .......... .......... .........C .......... ..........
FeLV-A MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91      .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB  .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:D91      .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB  .......... .......... .....G.... .......... .......... .......... ..........
N83:N91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91      .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera .......... .......... .T........ .......... .......... .......... ..........
N83:N91 MAB  .......... .......... .......... .......... .......... .......... ..........

          1051
FeLV-A       ACTATATCTG AAGTATCAGG GCAAGGAATG TGCATAGGGA CTGTTCCTAA AACCCACCAG GCTTTGTGCA
FeLV-A Sera  .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91      .......... .......... .......... .......... ......T... .......... ..........
D83:D91 MAB  .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:D91      .......... .......... .......... .........G .......... .......... ..........
N83:D91 MAB  .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91      .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera .......... .......... .......... .......... .......... .......... .........G
N83:N91 MAB  .......... .......... .......... .......... .......... .......... ..........

          1121
FeLV-A       ATAAGACACA ACAGGGACAT ACAGGGGCGC ACTATCTAGC CGCCCCCAAC GGCACCTATT GGGCCTGTAA
FeLV-A Sera  .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91      .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB  .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:D91      .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB  .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera .......... .......... .......... .......... .......... .......... ..........
N83:N91      .......... .......... .......... .......... .......... .......... ..........
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N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... G......... .......... ..........

          1191
FeLV-A        CACTGGACTC ACCCCATGCA TTTCCATGGC GGTGCTCAAT TGGACCTCTG ATTTTTGTGT CTTAATCGAA
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .C........ ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... ........T. .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... ........G. .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .A........ .......... .......... ..........

          1261
FeLV-A        TTATGGCCCA GAGTGACTTA CCATCAACCC GAATATGTGT ACACACATTT TGCCAAAGCT GTCAGGTTCC
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ....C.....
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

          1331
FeLV-A        GAAGAGAACC AATATCACTA ACGGTTGCCC TTATGTTGGG AGGACTTACT GTAGGGGGCA TAGCCTCGGG
FeLV-A Sera   .......... .......... .......... .......... .......... .......... .....G....
FeLV-A MAB    .......... .......... .......... .......... .....G.... .......... .....G....
D83:D91       .......... .......... .......... .......... .......... .......... .....G....
D83:D91 MAB   .......... .......... .......... .......... .......... .......... .....G....
D83:D91 Sera  .......... .......... .......... .......... .......... .......... .....G....
N83:D91       .......... .......... .......... .......... .......... .......... .....G....
N83:D91 MAB   .......... .......... .......... .......... .......... .......... .....G....
N83:N91 Sera  .......... .......... .......... .......... .......... .......... .....G....
N83:N91       .......... .......... .......... .......... .......... A......... .....G....
N83:D91 Sera  .......... .......... .......... .......... .......... .......... .....G....
N83:N91 MAB   .......... .......... .......... .......... .......... .......... .....G....

          1401
FeLV-A        GGTCGGAACA GGGACTAAAG CCCTCCTTGA AACAGCCCAG TTCAGACAAC TACAAATGGC CATGCACACA
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... ...C...... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... ......C... .......... ...G...... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .G........ .......... .......... ..........

          1471
FeLV-A        GACATCCAGG CCCTAGAAGA ATCAATTAGT GCCTTAGAAA AGTCCCTGAC CTCCCTTTCT GAAGTAGTCT
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .....C.... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

          1541
FeLV-A        TACAAAACAG ACGGGGCCTA GATATTCTAT TCTTACAAGA GGGAGGGCTC TGTGCCGCAT TGAAAGAAGA
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
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N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......G.. .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

            1611
FeLV-A        ATGTTGCTTC TATGCGGATC ACACCGGACT CGTCCGAGAC AATATGGCCA AATTAAGAGA AAGACTAAAA
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  ..A....... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

            1681
FeLV-A        CAGCGGCAAC AACTGTTTGA CTCCCAACAG GGATGGTTTG AAGGATGGTT CAACAAGTCC CCCTGGTTTA
FeLV-A Sera   .......... .....G.... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .G........ .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... A......... .......... .......... ..........
N83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

            1751
FeLV-A        CAACCCTAAT TTCCTCCATT ATGGGCCCCT TACTAATCCT ACTCCTAATT CTCCTCTTCG GCCCATGCAT
FeLV-A Sera   .......... .......... .........G .......T.. .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .........C .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .....G.... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   .......... ..........  .C........ .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... .........C

            1821
FeLV-A        CCTTAACCGA TTAGTACAAT TCGTAAAAGA CAGAATATCT GTGGTACAGG CTTTAATTTT AACCCAACAG
FeLV-A Sera   .......... .......... .......... .......... .......... .......... ..........
FeLV-A MAB    .......... .......... .......... .......... .......... .......... ..........
D83:D91       .......... .......... .......... .......... .......... .......... ..........
D83:D91 MAB   .......... .......... .......... .......... .......... .......... ..........
D83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:D91       .......... .......... .......... .......... .......... .......... ..........
N83:D91 MAB   ..C....... .......... .......... .......... .......... .......... ..........
N83:N91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91       .G....... .......... .......... .......... .......... .......... ..........
N83:D91 Sera  .......... .......... .......... .......... .......... .......... ..........
N83:N91 MAB   .......... .......... .......... .......... .......... .......... ..........

            1891
FeLV-A        TACCAACAGA TAAAGCAATA CGATCCGGAC CGACCATGA
FeLV-A Sera   .......... .......... .......... .........
FeLV-A MAB    .......... .......... .......... .........
D83:D91       .......... .......... .......... .........
D83:D91 MAB   .......... .......... .......... .........
D83:D91 Sera  .......... .......... .......... .........
N83:D91       .......... .......... .......... .........
N83:D91 MAB   .......... .......... .......... .........
N83:N91 Sera  .......... .......... .......... .........
N83:N91       .......... .......... .......... .........
N83:D91 Sera  .......... .......... .......... .........
N83:N91 MAB   .......... .......... .......... .........
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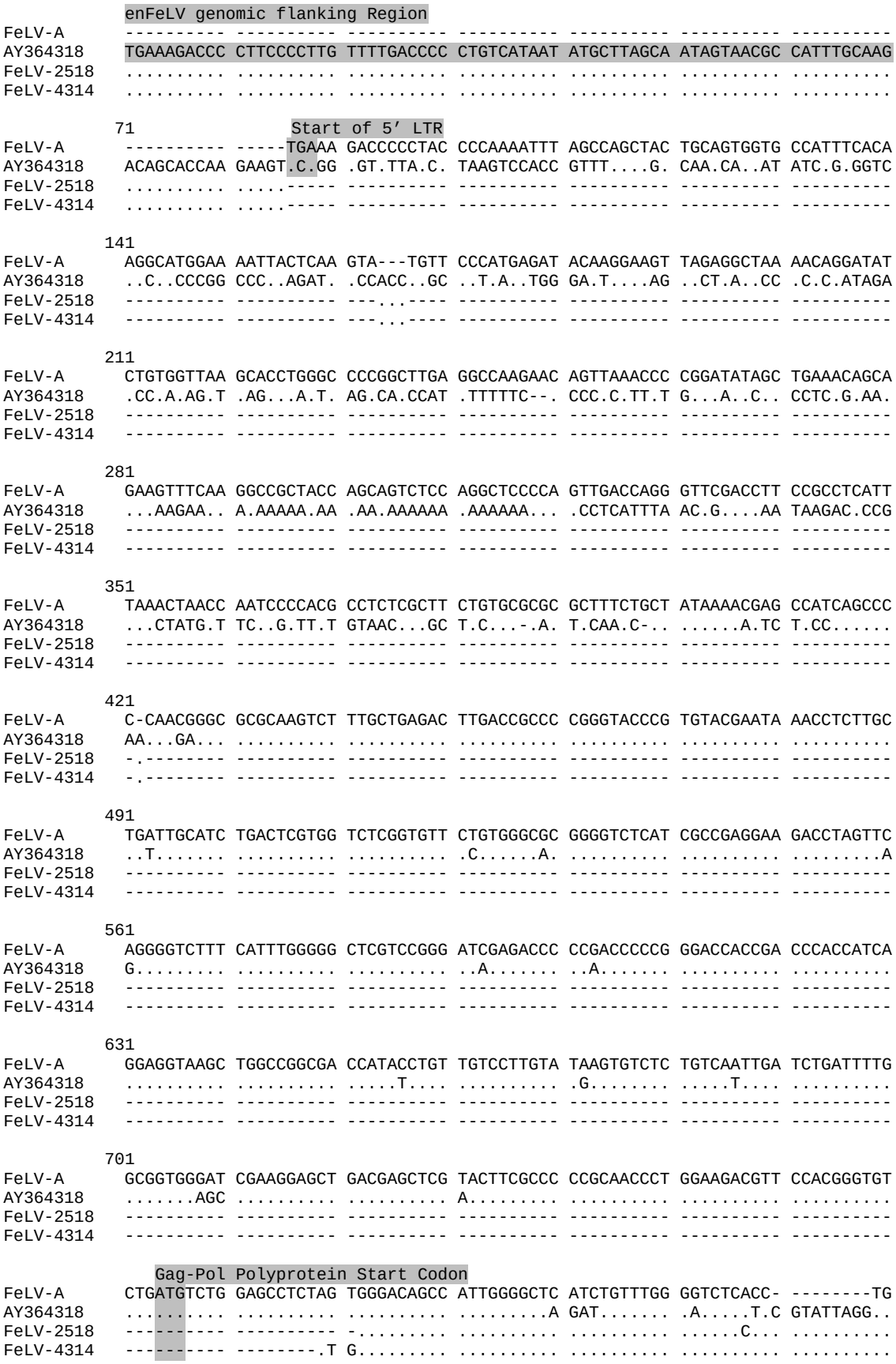
8.4 Genome sequences of FeLV-2518 and -4314

(A) Whole genome nucleotide alignment



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          841
FeLV-A      AATACAGGGT GTTGATCGGA GACGAGGGAG CCGGACCCTC AAAGTCTCTT TCTGAGGTTT CATTTTCGGT
AY364318    .......... .......... .......... .......... ..G.....C. .......... ..........
FeLV-2518   .......... .......... .......... .......... ..G....... .......... ..........
FeLV-4314   .......... .......... .......... .......... ........C. .......... ..........

          911
FeLV-A      TTGGTACCGA AGCCGCGCGG CACGTCTTGT CATTTTTTGT CTGGTTGCGT CTTTTCTTGT CCCTTGTCTA
AY364318    ......T... .......... .......... ....C..... ..T....... ....C..... ...C......
FeLV-2518   ........A. .......... .......... .........C .......... .......... ..........
FeLV-4314   ........A. .......... .......... .......... .......... .......... T.........

          981
FeLV-A      ACCTTTTTAA TTGCAGAAAC CGTCATGGGC CAAACTATAA CTACCCCCTT AAGCCTCACC CTTGATCACT
AY364318    .......... ........G. .......... ......G... .......... G......... ..CA.C....
FeLV-2518   .......... .......... .......... ..G....... .......... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... ..........

         1051
FeLV-A      GGTCTGAAGT CCGGGCACGA GCCCATAACC AAGGTGTCGA GGTCCGGAAA AAGAAATGGA TTACCTTATG
AY364318    .......G.. ..A......G ....G...T. .G........ A......... .......... ....AC.G..
FeLV-2518   .......... .......... ........T. ....G..... .......... .......... ..........
FeLV-4314   .......... .......... ........T. .......... .......... .......... ..........

         1121
FeLV-A      TGAGGCCGAA TGGGTGATGA TGAATGTGGG CTGGCCCCGA GAGGGAACTT TTTCTCTTGA TAACATTTCC
AY364318    ...A...... .....A.... .......A.. T......... ..A....... .CA.CA.... C..T.....A
FeLV-2518   .......... .......... .......... .......... ..A....... .......... ..........
FeLV-4314   .......... .......... .......... .........G ..A....... .......... ..G.......

         1191
FeLV-A      CAGGTTGAGA AAAAGATCTT CGCCCCGGGA CCGTATGGAC ACCCCGACCA GGTTCCTTAC ATTACCACAT
AY364318    .....C...G .G.GA..... .........G ..A....... ....A..T.. AA.C.....T ........G.
FeLV-2518   .......... .......... .......... ..A....... .......... A......... ..........
FeLV-4314   .......... .......... .......... ..A....... .......... A......... ........G.

         1261
FeLV-A      GGAGATCCTT AGCCACAGAC CCCCCTTCGT GGGTTCGGCC GTTCCTACCC CCTCCCAAAC CTCCCACGCC
AY364318    ........C. .......... ......C.A. .......C.. A.....G... .....T..G. A.....G.A.
FeLV-2518   .......... .......... .......... .......T.. .......... .......... .......A..
FeLV-4314   .......... .......... .......... .......T.. .......... .......... .......A..

         1331
FeLV-A      ---CCTCCCT CAACCTCTCT CGCCGCAGCC CTCCGCCCCT CTTACCTCTT CCCTCTACCC CGTCCTCCCC
AY364318    AGAT.CT..C G.G.....T. .......A.. ...A.....C .CC.T...C. .......... ...T......
FeLV-2518   .......... ........T. .......... .........C .......... .......... ...T.....T
FeLV-4314   .......... ........T. .......... .......... .......... .......... ...T......

         1401
FeLV-A      AAGTCAGACC CTCCCAAACC GCCTGTGTTA CCGCCTGATC CTTCTTCCCC TTTAATAGAT CTCTTAACAG
AY364318    ..AC...... .C.....GG. ......A... ..A..CA... .......... ......T... ..........
FeLV-2518   .......... .C........ .......... .......... .......... ......T... ..........
FeLV-4314   ...C...G.. .C........ .......... .......... ....C..... ......T... ..........

         1471
FeLV-A      AAGAGCCACC TCCCTATCCG GGAGGTCACG GGCCA---CC GCCGTCAGGT CCTAGGACCC CAACCGCTTC
AY364318    .......... .........T ..G....... .....ACA.. .........C .....A.... ....T..C..
FeLV-2518   .......... .......... ..G....... .......... ...A...... .......... ..........
FeLV-4314   .......... .......... ..G....... .......... ...A...... .......... ..........

         1541
FeLV-A      CCCGATTGCA AGCCGGCTAA GGGAACGACG AGAAAACCCT GCTGAAGAAT CTCAAGCCCT CCCCTTGAGG
AY364318    .........C ........GC .A........ ......T..A .....GA... .......... ......A...
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... ..........

         1611
FeLV-A      GAGGGCCCCA ACAACCGACC CCAGTATTGG CCATTCTCAG CCTCAGACCT GTATAACTGG AAGTCGCATA
AY364318    ..A.....A. .....A.... ......C... ........G. ....T..... ...C..T... ..A.T.....
FeLV-2518   ..A....... .......... .......... .......... .T........ .......... ..........
FeLV-4314   ..A....... .......... .......... .......... .T......T. .......... ..........

         1681
FeLV-A      ACCCCCCTTT CTCCCAAGAC CCAGTGGCCC TAACTAACCT AATTGAGTCC ATTTTAGTGA CGCATCAACC
AY364318    .......... ......G... .......... .......... .......... .......... .A.....G..
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... .A........

         1751
FeLV-A      AACCTGGGAC GACTGCCAGC AGCTCTTGCA GGCACTCCTG ACAGGCGAAG AAAGGCAAAG GGTCCTTCTT
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AY364318    ......... ........A. .......A.. ...T...... ..G.CA..G. .G..A..... ......C...
FeLV-2518   ......... ......... ......... ......... ......... ......... .........
FeLV-4314   ......... ......... ......... ......... ......... ......... .........

         1821
FeLV-A      GAGGCCCGAA AGCAGGTTCC AGGCGAGGAC GGACGGCCAA CCCAACTACC CAATGTCATT GATGAGACTT
AY364318    ..A....... ....A..... ......... ......... ....G..G.. .......G.. ..C...G...
FeLV-2518   ......... ......... ......... ......... ......... ......... ..C.......
FeLV-4314   ......... ......... ......... ......... ......... ......... ..C...G...

         1891
FeLV-A      TCCCCTTGAC CCGTCCCAAC TGGGATTTTG CTACGCCGGC AGGTAGGGAG CACCTACGCC TTTATCGCCA
AY364318    ......... ......... ......... ......... ......... ......... .........
FeLV-2518   .T....... ......... ......... ......... ......... ......... .........
FeLV-4314   ......... ......... ......... ......... ......... ......... .........

         1961
FeLV-A      GTTGCTATTA GCGGGTCTCC GCGGGGCTGC AAGACGCCCC ACTAATTTGG CACAGGTAAA GCAGGTTGTA
AY364318    ......G... ......... ......... ......... ......... ......... ...A......
FeLV-2518   ......... ......... ......... ......... ......... ......... .........
FeLV-4314   ......... ......... ......... T......... ......... ......... .........

         2031
FeLV-A      CAAGGGAAAG AGGAAACGCC AGCAGCATTT TTAGAAAGAT TAAAAGAGGC TTACAGAATG TACACTCCCT
AY364318    ......... ......... ...CT....C ......... ......... ......... .........
FeLV-2518   .C....... ......... ......... ......... ......... ......... .........
FeLV-4314   .....A.... ......... ......... ......... ......... ......... .........

         2101
FeLV-A      ATGACCCTGA GGACCCAGGG CAAGCAGCTA GTGTTATACT ATCCTTTATA TACCAGTCTA GCCCAGATAT
AY364318    ......... ......... ..G..T.... .......C.. G........C ......... ....G..C..
FeLV-2518   .C....... ......... .....G.... .......T.. ...T..C... ......... .........
FeLV-4314   ......... ......... .....G.... .......C.. ......C... ......... .........

         2171
FeLV-A      AAGAAATAAG TTACAAAGGC TAGAAGGTCT ACAAGGGTTC ACCCTATCTG ATCTGCTAAA AGAGGCAGAA
AY364318    ......... ......... .......C.. ...G...... ..A..G.... ..T....... .........
FeLV-2518   ......... ........A. .......C.. ......... ......... ......... .........
FeLV-4314   ......... ......... .......C.. ......... ......... ......... .........

         2241
FeLV-A      AAGATATACA ACAAAAGGGA GACCCCAGAG GAAAGGGAAG AAAGATTATG GCAGCGGCAA GAAGAAAGAG
AY364318    ......... ......... ......... ......... ......... .........G .........
FeLV-2518   ......... ......... ......... ......... ......... .........G .........
FeLV-4314   ......... ......... ......... ..G...... ......... .........G .........

         2311
FeLV-A      ATAAAAAGCG CCACAAGGAG ATGACTAAAG TTCTGGCCAC AGTAGTTGCT CAGAATAGAG ATAAAGATAG
AY364318    ......... ...T...... ......... ......... ......... ......... ....G.....
FeLV-2518   ......... ......... ......... ......... ......... ......... .........
FeLV-4314   ......... ......... ......... ......... ......... ......... .........

         2381
FeLV-A      AGAAGAAAGT AAACTGGGGG ATCAAAGGAA AATACCTCTG GGAAAGGACC AGTGTGCCTA TTGCAAGGAA
AY364318    ...G...... ........A. ......... ......... ..G..A.... ......... .........
FeLV-2518   ......G... ......... ......... ......... ..G..A.... ......... .........
FeLV-4314   ......... ........A. ......... ......... ..G..A.... ......... .........

         2451
FeLV-A      AAGGGGCATT GGGTTCGCGA TTGCCCCAAA CGACCCCGGA AGAAACCCGC CAACTCCACT CTCCTCAACT
AY364318    .....A.... ......... ......G... ......... ......... AG........ .........C
FeLV-2518   .G....... ......... ......... ......... ......... ......... ........T.
FeLV-4314   ......... ......... ......... ......AA.. ......... ......... .........

         2521
FeLV-A      TAGGAGATTA GGAGAGTCAG GGCCAGGACC CCCCCCCTGA GCCCAGGATA ACCTTAAAAA TAGGGGGGCA
AY364318    ...A...... ......... ......... ......... ......... ...C.....G .........
FeLV-2518   ......... ......... ......... ......... ......... ......... .........
FeLV-4314   ......... ......... ......... ......... ......... ......... .........

         2591
FeLV-A      ACCGGTGACT TTCCTGGTGG ACACGGGAGC CCAGCACTCA GTACTAACTC GACCAGATGG ACCTCTCAGT
AY364318    ...A.....C ..T....... .T........ .........G .....G.... ......... .........
FeLV-2518   ......... ..T....... ......... ......... .....G.... ......... .........
FeLV-4314   ......... ..T....... ......... ......... .....G.... ......... .........

         2661
FeLV-A      GACCGCACAG CCCTGGTGCA AGGAGCCACG GGAAGCAAAA ACTACCGGTG GACCACCGAC AGGAGGGTAC
AY364318    ......T... ......... .........A ......... .......A.. ......... ........G.
FeLV-2518   ......... ......... ......... ......... ......... ......... .........
FeLV-4314   ......... ......... ......... ......... ......... ......... .........
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         2731
FeLV-A       AACTGGCAAC CGGTAAGGTG ACTCATTCTT TTTTATATGT ACCTGAATGT CCCTACCCGT TATTAGGGAG
AY364318     .......... .......... .......... .......... .......... .......... ..........
FeLV-2518    .......... .......... .......... .......... .......... .......... ..........
FeLV-4314    .......... .......... ........C. .......... .......... .......... .......A..

         2801
FeLV-A       AGACCTATTA ACTAAACTTA AGGCCCAAAT CCATTTTACC GGAGAAGGGG CTAATGTTGT TGGGCCCAAG
AY364318     .......... ..C.....C. .......G.. ......C... ........A. .......... ...A....T.
FeLV-2518    .......... .......... .......... .......... .......... .......... ........G.
FeLV-4314    .......... .......... .......... .......... .......... .......... ........G.

         2871
FeLV-A       GGTTTACCCC TACAAGTCCT TACTTTACAA TTAGAAGAGG AGTATCGGCT ATTTGAGCCC GAAAGTACAC
AY364318     ..C....... .......... C..C..G... C.......A. .......... .........G ......GA..
FeLV-2518    .......... .......... .......... .......... .......... .........A ..........
FeLV-4314    .......... .......... .......... .......... .......... .........A ..........

         2941
FeLV-A       AAAAACAGGA GATGGACATT TGGCTTAAAA ACTTTCCCCA GGCGTGGGCA GAAACAGGAG GTATGGGAAC
AY364318     T......A.G T.......G. .......... .......... ...A...... .......... ....A....T
FeLV-2518    .......... .......... .......... .......... .......... .......... .........T
FeLV-4314    .......... ........C. .......... .......... .......... .......... .........T

         3011
FeLV-A       GGCTCATTGT CAAGCCCCCG TTCTCATTCA ACTTAAGGCT ACTGCCACTC CAATCTCCAT CCGACAGTAT
AY364318     .........C .........A .C........ ......A... ........C. .......... ...G.....C
FeLV-2518    .......... .........A .......... .......... .......... .......... ..........
FeLV-4314    .......... .......... .......C.. .......... .......... .......... ..........

         3081          RT Start Codon
FeLV-A       CCTATGCCCC ATGAAGCATA CCAGGGAATT AAGCCTCATA TAAGAAGAAT GCTAGATCAA GGCATCCTCA
AY364318     ..C....... ...T..T.. ...A...... ..A..C.... ....G..... ...G..C... ..........
FeLV-2518    .......... ...T..G.. .......... .......... .......... .......... ..........
FeLV-4314    .......... ...T..... .......... .......... ..C....... .......... ..........

         3151
FeLV-A       AGCCCTGCCA GTCCCCATGG AATACACCCT TATTACCTGT TAAGAAGCCA GGGACCGAGG ATTACAGACC
AY364318     .......... .......... .........C .......... C..A...... ..A....G.. ..........
FeLV-2518    .......... .......... .......... .......... A......... .......... ..........
FeLV-4314    .......... .......... .......... .......... .......... .......... ..........

         3221
FeLV-A       AGTGCAGGAC TTAAGAGAAG TAAACAAAAG AGTGGAAGAC ATCCATCCTA CTGTGCCAAA TCCATATAAC
AY364318     .......... .......... .......... G..A...... ........C. .......... C.....C...
FeLV-2518    ...A...... .......... .......... ...A...... .......... .......... ..........
FeLV-4314    .......... .......... .......... ...A...... .......... .......... ..........

         3291
FeLV-A       CTCCTTAGCA CCCTCCCGCC GTCTCACCCT TGGTACACTG TCCTAGATTT AAAAGACGCT TTTTTCTGCC
AY364318     .......... .......A.. T......... ........C. ........C. ......T... .........T
FeLV-2518    .......... .......... .......... .......... .......... ...G...... ..........
FeLV-4314    .......... .......... .......... .......... .......... ...G...... ..........

         3361
FeLV-A       TGCGACTACA CTCTGAGAGT CAATTACTTT TTGCATTTGA ATGGAGAGAT CCAGAAATAG GACTGTCAGG
AY364318     .......... .C.......C ........C. .......... .....A.... .....G.... .G........
FeLV-2518    .......... .C.......C .......... .C........ .......... .......... ..........
FeLV-4314    .......... .......... ..G....... .......... .......... .......... ..........

         3431
FeLV-A       GCAGCTAACC TGGACACGCC TTCCTCAAGG GTTCAAGAAT AGCCCCACCC TATTTGATGA GGCCCTGCAC
AY364318     ...A..G... .....T.... ....A..... .......... .......... .......... ......A...
FeLV-2518    .......... .......... ....A..G.. .......... ......G... .......... ..........
FeLV-4314    ...A...... .......... .......G.. .......... .......... .......... ..........

         3501
FeLV-A       TCAGACCTGG CCGATTTCAG GGTAAGGTAC CCGGCTCTAG TCCTCCTACA ATATGTAGAT GACCTCTTGC
AY364318     .......... .A........ ...G.....T ..A...T... .......... ...C...... ........A.
FeLV-2518    ..G....... .......... .......... .......... .......... .......... ..........
FeLV-4314    .......... .......... .......... .......... .......... .......... ..........

         3571
FeLV-A       TGGCTGCGGC AACCAGGACT GAATGCCTGG AAGGGACTAA GGCACTCCTT GAGACTTTGG GCAATAAGGG
AY364318     .......... .....A...C ........A. .......... A......... .......... ..........
FeLV-2518    .......... .......... .......... .......... .......... .......... ..........
FeLV-4314    .......... .......... .......... .......... .......... .......... ..........

         3641
FeLV-A       GTACCGAGCC TCTGCAAAGA AGGCCCAAAT TTGCCTGCAA GAAGTCACAT ACCTGGGGTA CTCTTTAAAA
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AY364318    T......... .......... .......... .......... A......... ....A..... .......G.G
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... ..........

          3711
FeLV-A      GATGGCCAAA GGTGGCTTAC CAAAGCTCGC AAGGAAGCCA TCCTATCCAT CCCTGTGCCT AAAAACTCAC
AY364318    .....T.... .A........ .........G ..A....... .T..C..... .......... ......C...
FeLV-2518   .......... .......... .........G ..A....... .......... .......... ......C...
FeLV-4314   .......... .......... .........G ..A....... .......... .......... ......C...

          3781
FeLV-A      GACAAGTAAG AGAGTTCCTT GGAACTGCAG GTTACTGCCG GCTGTGGATT CCCGGTTTTG CCGAGCTCGC
AY364318    .G.....G.. .......... .......... .C........ .......... .......... ..........
FeLV-2518   .T.....G.. .......... .......... .......... .......... .......... ..........
FeLV-4314   .......G.. .......... .......... .......... .......... .......... ..........

          3851                                                  FeLV-4314 5' Recomb. Site
FeLV-A      AGCCCCGCTA TACCCTCTCA CTCGACCAGG AACTCTGTTC CAGTGGGGAA CAGAGCAACA ATTGGCCTTC
AY364318    T......... ..T....... .......... .........T .......... ....A..... ..........
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......G.. .......... .......... .......... ..........

          3921
FeLV-A      GAGGACATTA AAAAAGCCCT CTTGAGTTCC CCTGCCCTGG GGTTGCCAGA TATCACCAAA CCCTTTGAAT
AY364318    ..AA...... .......... ......C... .......... .......... .........G ..........
FeLV-2518   ...A...... .......... ......C... .......... .......... .........G ..........
FeLV-4314   ...A...... .......... ......C... .......... .......... .........G ..........

          3991
FeLV-A      TATTTATTGA TGAGAACTCA GGATTTGCAA AGGGGGTGTT AGTCCAAAAA CTGGGACCCT GGAAAAGACC
AY364318    .......... .....G.... ..G.....G. .......... .......... .......... ..........
FeLV-2518   .......... .....G.... ........G. .......... .A........ .......... ..........
FeLV-4314   .G........ .....G.... .....C..G. .......... .......... .......... ..........

          4061
FeLV-A      AGTTGCCTAC CTATCAAAAA AGCTGGATAC AGTGGCATCT GGATGGCCCC CTTGTTTACG CATGGTTGCA
AY364318    .......... .......... .A........ .......... .......... .......... ..........
FeLV-2518   .......... .......... .A........ .........C .......... .......... ..........
FeLV-4314   .......... .......... .A........ .......... .......... .......... ..........

          4131
FeLV-A      GCCATCGCCA TCCTAGTCAA GGATGCAGGG AAGCTAACCC TAGGACAGCC GCTAACTATC CTGACCTCCC
AY364318    .......... .......... .......... ........T. .......... .......G.. ..........
FeLV-2518   .......... .......... .......... .....G.... .......... .......... ..........
FeLV-4314   .......... .......... .......... ........T. .......... .......G.. ..........

          4201
FeLV-A      ACCCAGTTGA GGCACTTGTC CGACAGCCTC CAAATAAATG GCTCTCTAAT GCTAGAATGA CTCATTACCA
AY364318    .......... .......... .......... .......... .......... .......... ..........
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... ..........

          4271
FeLV-A      AGCTATGCTC CTCGATGCAG AGCGAGTCCA TTTCGGGCCG ACAGTCTCCC TTAACCCTGC TACCTTGCTC
AY364318    .......... .......... .......... .........A .......... .......... C........T
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......... .........A .......... .......... C........T

          4341
FeLV-A      CCCCTCCCCA GCGGGGGAAA CCACCACGAC TGTCTCCAGA TTTTAGCCGA GACCCATGGC ACCAGACCCG
AY364318    .......... A..A...... .......... ..C....... .......... .......... ..........
FeLV-2518   .......... .......... .......... .......... .......... .......... ......A...
FeLV-4314   .......... A..A...... .......... ..C....... .......... .......... ..........

          4411
FeLV-A      ACTTAACTGA CCAGCCGTTG CCGGATGCAG ACCTGACCTG GTACACAGAT GGTAGCAGCT TCATCCGTAA
AY364318    .......... .......... .......... .......... ......G... .......... ..........
FeLV-2518   .......... .......... .......... .......... ......G... .......... ..........
FeLV-4314   .......... .......... .......... .......... ......G... .......... ..........

          4481
FeLV-A      TGGCGAGAGA GAGGCCGGAG CCGCAGTAAC AACCGAATCT GAGGTAATCT GGGCTGCTCC CCTCCCACCC
AY364318    C..A...... A......... .......... .......... .......... ........T. ..........
FeLV-2518   C......... A........A .......... .......... .......... ........T. ..........
FeLV-4314   C......... A..-...... .......... .......... .......... ........T. ..........

          4551
FeLV-A      GGAACGTCAG CCCAGCGAGC CGAACTGATT GCCCTGACCC AGGCACTAAA GATGGCAGAA GGTAAGAAGC
AY364318    .......... .......... .......... .......... .......G.. .......A.. ..........
FeLV-2518   .......... .......... .......G.. .......... .......... .......A.. ..........
FeLV-4314   .......... .......... .......... .......... .......G.. .......A.. ..........
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         4621
FeLV-A       TAACTGTCTA TACGGACAGC CGATATGCCT TTGCTACAAC TCATGTACAC GGGGAAATCT ACAGGCGGCG
AY364318     .......... .......... .......... ........G. .......... .......... ..........
FeLV-2518    .......... .......... .......... ........G. .......... .......... ..........
FeLV-4314    .......... .......... .......... ........G. .......... .......... ..........

         4691
FeLV-A       GGGCCTACTA ACTTCAGAAG GAAAAGAAAT TAAAAATAAA AATGAAATCC TCGCCCTACT AGAGGCGTTA
AY364318     ......G... .......... .......... .......... .......... ........T. ..........
FeLV-2518    .......... .......... .......... .......... .......... ........T. ..........
FeLV-4314    ......G... .......... .......... .......... .......... ........T. ..........

         4761
FeLV-A       TTCTTACCCA AAAGACTGAG CATCATCCAT TGCCCGGGAC ACCAAAAAGG TGATAGTCCC CAGGCAAAAG
AY364318     .......... .......... T......... .....T.... .......... .......... .....G....
FeLV-2518    .......... .......... T......... .......... .......... .......... .....G....
FeLV-4314    .......... .......... T......... ..T..A.... .......... .......... .....G....

         4831
FeLV-A       GAAACAGATT AGCTGATGAT ACAGCAAAGA AAGCCGCCAC AGAGACTCAT TCATCACTAA CCGTCTTACC
AY364318     .......... ...C...... .......... .......... .........A .......... ..A.......
FeLV-2518    .......... .......... .......... .......... .......... .......... ..........
FeLV-4314    .......... ...C...... .......... .......... .........A .......... ..A.......

         4901
FeLV-A       CACTGAACTT ATAGAGGGTC CCAAAAGGCC TCCATGGGAA TATGATGACA GTGATTTAGA CCTTGTGCAA
AY364318     .......... .......... .......... .......... .......... .......... .........G
FeLV-2518    .......... .......... .......... .......... .......... .......... .........G
FeLV-4314    .......... .....A.... ......A... .......... ...A...... .......... .........G

         4971
FeLV-A       AAACTCGAAG CTCATTATGA GCCAAAGAGA GGTACCTGGG AGTACCGAGG AAAAACTATC ATGCCTGAAA
AY364318     .....T.... .......... ......A... .......... ......A... G........A ..........
FeLV-2518    .....T.... .......... ......A... .......... .......... .......... ..........
FeLV-4314    .....T.... .......... ......A... .......... ......A... G........A ..........

         5041
FeLV-A       AATACGCAAA AGAGTTGATT AGCCATCTGC ATAAGTTAAC ACACCTCAGT GCTAGGAAAA TGAAAACTTT
AY364318     .......... .......... .......... .......... .......... .......... ..........
FeLV-2518    .......... .......... .......... .......... .......... .......... ..........
FeLV-4314    .......... .......... .......... .......... .......... .......... ..........

         5111
FeLV-A       ACTAGAAAGA GAAGAAACTG GGTTTTACCT CCCTAACAGA GACTTACACC TCCGGCAAGT AACAGAGAGC
AY364318     .......... .......... .......... .......... .......... .......... ..........
FeLV-2518    .......... .......... .......... ......A... .......... .......... ..........
FeLV-4314    .......... ..G....... .......... .......... .......... .......... ..........

         5181
FeLV-A       TGCCGGGCAT GTGCTCAAAT CAACGCCGGA AAGATAAAGT TTGGACCTGA TGTAAGGGCC CGAGGCCGCC
AY364318     .......... .......... ......A..G ..A....... .......... .......... .......A..
FeLV-2518    .......... .......... ......A... .......... .......... .......... ..........
FeLV-4314    ....A..... ........G. ......A..G ..A....... .......... .......... .......A..

         5251
FeLV-A       GGCCCGGAAC ACATTGGGAA GTAGACTTCA CTGAAATCAA GCCAGGAATG TATGGATATA AATACCTCTT
AY364318     .........T .......... .......... .......... .......... .......... ..........
FeLV-2518    .......... .......... .......... .......... .......... .......... ..........
FeLV-4314    .........T .......... .......... .......... .......... .......... ..........

         5321
FeLV-A       GGTGTTCATA GATACCTTCT CTGGCTGGGC CGAAGCTTAC CCCGCCAAAC ATGAAACAGC AAAAGTTGTT
AY364318     .......... ..C....... .......... .......... .......... .......... ..........
FeLV-2518    .......... .......... .......... .......... .......... .......... ..........
FeLV-4314    .......... .......... .......... .......... .......... .......... ..........

         5391
FeLV-A       GCCAAGAAAC TCTTAGAAGA AATTTTTCCC CGCTACGGGA TCCCTCAGGT ATTGGGTTCA GATAATGGAC
AY364318     .......... .......... G........T ..T....... .......... .......... ..........
FeLV-2518    .......... .......... ......C... .......... .......... .......... ..........
FeLV-4314    .......... .......... G........T ..T....... .......... .......... ..........

         5461
FeLV-A       CCGCCTTTAT CTCCCAGGTA AGTCAGTCTG TGGCCACCCT ACTGGGGATT AATTGGAAGT TACATTGTGC
AY364318     .......... .......... .......... .......... .......... ........A. ..........
FeLV-2518    .......... .......... .......... .......... .......... .......... ..........
FeLV-4314    .......... .......... .......... .......... .......... ........A. ..........

         5531
FeLV-A       ATACCGACCC CAAAGTTCAG GTCAGGTAGA AAGAATGAAT AGATCAATTA AGGAGACTTT AACTAAATTA
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AY364318    .......... .......... .......... .......... .......... .......... ..........
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... ..........

         5601
FeLV-A      ACGCTAGAAA CTGGCTCTAA GGATTGGGTG CTCCTCCTGC CCCTGGTTTT ATACCGGGTA CGTAACACGC
AY364318    .......... .......... .......... .......... .......... .......... ..........
FeLV-2518   .......... .......... A......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... ..........

         5671
FeLV-A      CAGGCCCCCA CGGGTTAACT CCTTTTGAAA TCCTGTACGG GGCACCCCCA CCTATGGCTC ACTTCTTTGA
AY364318    ....T..... .......... .......... .......... .......... .......... ..........
FeLV-2518   ....T..... T......... .......... ..T....... .......... .......... ..........
FeLV-4314   ....T..... .......... .......... .......... .......... .......... ..........

         5741
FeLV-A      TACTGATATC TCTAGCTTCG CTACCTCCCC CACTATGCAG GCACATTTAC GCGCCCTGCA GCTGGTCCAA
AY364318    .G....C... ...G.T.... .......... ...C...... .......... .......... ..........
FeLV-2518   .G........ .......... .......... .......... .......... .......... ..........
FeLV-4314   .G.G..C... ...G.T.... .......... ...C...... .......... .......... ..........

         5811
FeLV-A      GAAGAGATCC AGAGACCTCT AGCGGCGGCC TACCGAGAAA AACTTGAAAC CCCGGTTGTG CCTCACCCCT
AY364318    .......... .......... ......A... .......... .G..C..... .......... ..........
FeLV-2518   .......... .......... .......... ....A..... .G..C..... .......... ..........
FeLV-4314   .......... .......... ......A... .......... .G..C..... .......... ..........

         5881
FeLV-A      TCAAACCAGG AGACTCCGTC TGGGTTCGGA GACATCAAAC CAAGAACCTC GAGCCACGGT GGAAAGGACC
AY364318    .......... .......... .......... .......... .......... .......... ....G.....
FeLV-2518   .......... .......... .......... .......... ..G....... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... ....G.....

         5951
FeLV-A      ACATATCGTC CTCCTGACCA CCCCCACAGC CTTAAAGGTA GACGGAGTTG CTGCCTGGAT TCACGCCTCT
AY364318    .......... .......... .......G.. ......A... .......... ....T..... C........A
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   .......... .......... .......G.. ......A... .......... ....T..... C........A

         6021
FeLV-A      CACGTGAAAG CTGCAGGACC AACCACCAAT CAAGACCTCT CGGACAGCCC CAGCTCAGAC GATCCATCAA
AY364318    ..T..A..G. .......G.. .......... ..G....C.. .A...GA... .......... ........G.
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   ..T..A..G. .......G.. .......... ..G....C.. .A...GA... .......... ........G.


            Env Start Codon
FeLV-A      GATGGAAAGT CCAACGCACC CAAAACCCTC TAAAGATAAG ACTCTCTCGT GGAACTTAGC GTTTCTGGTG
AY364318    .......G.. .......... .......... .......... ...T...... ..G..C..AT .A........
FeLV-2518   .......... .......... .......... .......... ........A. .......... ..........
FeLV-4314   .......G.. .......... .......... .......... ...T...... ..G..C..AT .A........


                                        FeLV-2518 5' Recomb. Site
FeLV-A      GGGATCTTAT TCACAATAGA CATAGGAATG GCCAATCCTA GTCCACACCA AATATATAAT GTAACTTGGG
AY364318    ...G...... .A.G.C.... .G.G...... .......... ....G..... .G.G...... .........A
FeLV-2518   .......... .......... ..........  ..........  ....G..... .G.G...... .........A
FeLV-4314   ...G...... .A.G.C.... .G.G...... .......... ....G..... .G.G...... .........A

         6231
FeLV-A      TAATAACCAA TATGCAAACT AACACCCAAG CTAATGCCAC CTCTATGTTA GGAACCTTAA CCGATGCCTA
AY364318    C......... CC.TGT.... GGA..AA.G. .......... ...C.....G ......C.G. .A..C....T
FeLV-2518   C......... CC.TGT.... GGA..AA.G. .......... ...C.....G ......C.G. .A..C....T
FeLV-4314   C......... CC.TGT.... GGA..AACG. .......... ...C.....G ......C.G. .A..C....T

         6301
FeLV-A      CCCTACCCTA CATGTTGACC TATGTGACCT AGTGGGAGAC ACCTGGGAAC CTATAGTCCT AGATCCAACC
AY364318    .......A.G T..T.....T .......TA. .A.A...A.T ..A...A.C. ..TC..AT.A G..A...---
FeLV-2518   .......A.G T..T.....T .......TA. .A.A...A.T ..A...A.C. ..TC..AT.A G..A...---
FeLV-4314   .......A.G T..T.....T .......TA. .A.A.ATA.T ..A...A.C. ..TC..AT.A G..A...---

         6371
FeLV-A      AATGTAAAAC ACGGGGCACG TTACTCCTCC TCAAAGTATG GATGTAAAAC TACAGATAGA AAAAAACAGC
AY364318    ---------- ---------- --------.T. C..GG..... .....G.TCA GC.TATG..G .GGTGG..A.
FeLV-2518   ---------- ---------- --------.T. C..GG..... .....G.TCA GC.TATG..G .GGTGG..A.
FeLV-4314   ---------- ---------- --------.T. C..GG..... .....GGTCA GC.TATG..G .GGTGG..A.

         6441
FeLV-A      A-ACAAACAT ACCCCTTTTA CGTCTGCCCC GGACATGCCC CCTCGCTGGG GCCAAAGGGA ACACATTGTG
AY364318    .G.G.....C ....-..... T.....T..A .........- ---------- ----..CC.G .AG..A....
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FeLV-2518   .G.G.....C ....-..... T.....T..A .........- ---------- ----..CC.G .AG..A....
FeLV-4314   .G.G.....C ....-..... T.....T..A .........- ---------- ----..CC.G .AG..A....

         6511
FeLV-A      GAGGGGCACA AGATGGGTTT TGTGCCGCAT GGGGATGTGA GACCACCGGA GAAGCTTGGT GGAAGCCCTC
AY364318    .G...C.... G........C ..C..T.T.. ....T..C.. .........G ...A.C.AT. ...GA...A.
FeLV-2518   .G...C.... G........C ..C..T.T.. ....T..C.. .........G ...A.C.AT. ...GA...A.
FeLV-4314   .G...C.... G........C ..C..T.T.. ....T..C.. .........G ...A.C.AT. ...GA...A.

         6581
FeLV-A      CTCCTCATGG GACTATATCA CAGTAAAAAG AGGGAGTAGT CAGG------ ---------- ----------
AY364318    .......... .....C.... .........A ....GT..C. ....GAATAT ATCAATGTAG TGGAGGTGGT
FeLV-2518   .......... .....C.... .........A ....GT..C. ....GAATAT ATCAATGTAG TGGAGGTGGT
FeLV-4314   .......... .....C.... .........A ....GT..C. ....GAATAT ATCAATGTAG TGGAGGTGGT

         6651
FeLV-A      ---------- ------ACAA TA--GCTGT- ---------- ---------- -------GAG GGAAAATGCA
AY364318    TGGTGTGGGC CCTGTT..G. ..AA.....T CACTCCTCGA AAACGGGAGC TAGTGAA.G. ..CCGG....
FeLV-2518   TGGTGTGGGC CCTGTT..G. ..AA.....T CACTCCTCGA TAACGGGAGC TAGTGAA.G. ..CCGG....
FeLV-4314   TGGTGTGGGC CCTGTT..G. ..AA.....T CACTCCTCGA TAACGGGAGC TAGTGAA.G. ..CCGG....

         6721
FeLV-A      ACCCCCTGAT TTTGCAGTTC ACCCAGAAGG GAAGACAAGC CTCTTGGGAC GGACCTAAGA TATGGGGATT
AY364318    .....T.... C.....A..T .....A.... ........A. A........T .........T C......GC.
FeLV-2518   .....T.... C.....A..T .....A.... ........A. A........T .........T C......GC.
FeLV-4314   .....T.... C.....A..T .....A.... ........A. A..C.....T .........T C......GC.

         6791
FeLV-A      GCGACTATAC CGTACAGGAT ATGACCCTAT CGCCTTATTC ACGGTGTCCC GGCAGGTGTC AGCCATTACG
AY364318    A......... ...T...... .......... A...C.G... T....A.... ....A..AAT GA........
FeLV-2518   A......... ...T...... .......... A...C.G... T....A.... ....A..AAT GA........
FeLV-4314   A......... ...T...... .......... A...C.G... T....A.... ....A..AAT GA........

         6861
FeLV-A      CCGCCTCAGG CAATGGGACC AAACCTAGTC TTACCTGATC AAAAACCCCC ATCCAGACAA TCCCAAACAG
AY364318    .......... .C........ ...T...... C.G....... .......... ......G... ..T....T..
FeLV-2518   .......... .C........ ...T...... C.G....... .......... ......G... ..T....T..
FeLV-4314   .......... .C........ .......... C.G....... .......... ..T.G.G... ..T....T..

         6931
FeLV-A      GGTCCAAAGT GGCGACCCAG AGGCTCCAAA CGACTGAAAG CGCCCCAAGG ---------- --------TC
AY364318    A....CG... AA.AC.T..C CATTC....G GC.AC.G.G. .A.....G.T ATAACTCTTG TTAATGCC..
FeLV-2518   A....CG... AA.AC.T..C CATTC....G GC.AC.G.G. .A.....G.T ATAACTCTTG TTAATGCC..
FeLV-4314   A....C.... AA.AC.T..C CATTC....G GC.AC.G... .A.....G.T ATAACTCTTG TTAATGCC..

         7001
FeLV-A      TGTTGCCCC- ---------- ----CACCAC CGTGGGTCCC AAACGGATTG GGACCGGAGA TAGGTTAATA
AY364318    CA.......T CTAAGTACCC CTGT....C. ..CAA..... .....T..A. ....A...A. ..........
FeLV-2518   CA.......T CTAAGTACCC CTGT....C. ..CAA..... .....T..A. ....A...A. ..........
FeLV-4314   CA.......T CTAAGTACCC CTGT....C. ...AA..... .....T..A. ....A...A. ..........

         7071
FeLV-A      AATTTAGTAC AAGGGACATA CCTAGCCTTA AATGCCACCG ACCCCAACAA AACTAAAGAC TGTTGGCTCT
AY364318    ........G. .G........ T.....T... ....T...TA .......... .......... ..........
FeLV-2518   ........G. .G........ T.....T... ....T...TA .......... .......... ..........
FeLV-4314   ........G. .G........ T.....T... ....T...TA .......... .......... ..........

         7141
FeLV-A      GCCTGGTTTC TCGACCACCC TATTACGAAG GGATTGCAAT CTTAGGTAAC TACAGCAACC AAACAAACCC
AY364318    .T..A..C.. C.....G... .....T.... .A.....GG. A..G..C..T .......... ....C.....
FeLV-2518   .T..A..C.. C.....G... .....T.... .A.....GG. A..G..C..T .......... ....C.....
FeLV-4314   .T..A..C.. C.....G... .....T.... .A.....GG. A..G..C..T .......... ....C.....

         7211
FeLV-A      CCCCCCATCC TGCCTATCTA CTCCGCAACA CAAACTGACC ATATCTGAAG TATCAGGGCA AGGACTGTGC
AY364318    .......... ........CG AC..A..... T........T .....A.... .G..C..... ...TT.....
FeLV-2518   .......... ........CG AC..A..... T........T .....A.... .G..C..... ...TT.....
FeLV-4314   .......... ........CG AC..A..... T........T .....A.... .G..C..... ...TT.....

         7281
FeLV-A      ATAGGGACTG TTCCTAAGAC CCACCAGGCT TTGTGCAATG AGACACAACA GGGACATACA GGGGCGCACT
AY364318    .......... .......... ......A... ........AA ........A. A.......A. ...A.T....
FeLV-2518   .......... .......... ......A... ........AA ........A. A.......A. ...A.T....
FeLV-4314   .......... .......... ......A... ........AA ........A. A.......A. ...A.T....

         7351
FeLV-A      ATCTAGCCGC CCCCAATGGC GCCTATTGGG CCTGTAACAC TGGACTCACC CCATGCATTT CCATGGCGGT
AY364318    .......A.. ...T.GC..T A....C.... .A..C..... C..G..A... ........C. .......A..
FeLV-2518   .......A.. ...T.GC..T A....C.... .A..C..... C..G..A... ........C. .......A..
FeLV-4314   .......A.. ...T.GC..T A....C.... .A..C..... C..G..A... ........C. .......A..
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                FeLV-2518 3’ Recomb. Site
FeLV-A      GCTCAATTGG ACCTCTGATT TTTGTGTCTT AATCGAATTA TGGCCCAGAG TGACTTACCA TCAACCCGAA
AY364318    ......C... .......... ........C. G.....G... .......... .A........ .G.G..T..G
FeLV-2518   ......C... ..T....... ..........  .......... .......... .......... ..........
FeLV-4314   ......C... .......... ......C.C. G.....G... .......... .A........ .G.G..T..G

         7491                                  SU/TM Cleavage Site
FeLV-A      TATGTGTACA CACATTTTGC CAAAGCTGTC AGGTTCCGAA GAGAACCAAT ATCACTAACT GTTGCCCTCA
AY364318    ...A.C..TT .C..C..C.A A..TAAGCC. C.....AAG. ....T..... C..CT.G..A ..........
FeLV-2518   .........T .......... .......... .......... .......... .......... ........T.
FeLV-4314   ...A.C..TT .C..C..C.A A..TAAGCC. C.....AAG. ....T..... C..CT.G..A ..........

         7561
FeLV-A      TGTTGGGAGG ACTCACTGTA GGGGGCATAG CCGCGGGGGT CGGAACAGGG ACTAAAGCCC TCCTTGAAAC
AY364318    ..C....... .A........ ........G. ....C..AA. A......... ...GCG.... .T..C..G..
FeLV-2518   .......... .......... .......... .......... .......... .......... ..........
FeLV-4314   ..C....G.. .A........ ........G. ....C..AA. A......... ...GCG.... .T..C..G..

         7631
FeLV-A      AGCCCAGTTC AGACAACTAC AAATGGCCAT GCACACAGAC ATCCAGGCCC TAGAAGAGTC AATTAGTGCC
AY364318    ......A..T ........G. .......... ......G... ..T......T ....G..... ..........
FeLV-2518   .......... .......... .......... .......... .......... ....G..... ..........
FeLV-4314   ......A..T ........G. .......... ......G... ..T......T ....G..... ..........

            FeLV-4314 3’ Recomb. Site
FeLV-A      TTAGAAAAGT CCCTGACCTC CCTTTCTGAA GTAGTCTTAC AAAACAGACG GGGCCTAGAT ATTCTATTCT
AY364318    ........A. ..T....T.. .......... .......... .......G.. A......... .....G...C
FeLV-2518   .......... .......... .......... ..G....... .......... .......... ..........
FeLV-4314   .......... .......... .......... .......... .......... .......... ..........

         7771
FeLV-A      TACAAGAGGG AGGGCTCTGT GCCGCATTAA AAGAAGAATG TTGCTTCTAT GCGGATCACA CCGGACTCGT
AY364318    ....G..... ......A... ..A..G.... .......... ...T..T... ..A....... .....T.A..
FeLV-2518   .......A.. .......... .......... .......... .......... .......... .......T..
FeLV-4314   .......... .......... .......... .......... .......... ..A....... ..........

         7841
FeLV-A      CCGAGACAAT ATGGCTAAAT TGAGAGAAAG ACTAAAACAG CGGCAACAAC TGTTTGACTC CCAACAGGGA
AY364318    ......T... .......... .A........ .......... ..A....... .A........ ...G......
FeLV-2518   .......... .......... .A........ .......... .......... .......... ..........
FeLV-4314   .......... .......... .AC....... .......... .......... .......... ..........

         7911
FeLV-A      TGGTTTGAAG GATGGTTCAA CAAGTCCCCC TGGTTCACAA CCTTAATTTC CTCCATTATG GGCCCCTTAC
AY364318    .......... .G........ .......... ........T. .......A.. ......C... .........A
FeLV-2518   .......... .......... .......... .....T.... ..C....... .......... ..........
FeLV-4314   .......... .......... A......... .....T.... ..C....... .......... ..........

         7981
FeLV-A      TAATCCTACT CCTAATTCTC CTCTTCGGCC CATGCATCCT TAACAGATTA GTACAATTCG TAAAAGACAG
AY364318    .G.....C.. .......T.A ........A. .T........ ....C.G..G ..G..G..T. .C.....T..
FeLV-2518   .......... .......... .......... .......... ....C..... .......... ..........
FeLV-4314   .......... .......... ..T....... ....GG.... ....C..... .......... ..........

         8051
FeLV-A      AATATCTGTG GTACAAGCCT TAATTTTAAC CCAACAGTAC CAACAGATAA AGCAATACGA TCCGGACCGA
AY364318    ......C..C ..G..GA.T. ..G.GC.... ...G..AC.. ....GCC.GG G.....G... CT.A....A.
FeLV-2518   .......... ........T. .......G.. .......... .......... .......... ........A.
FeLV-4314   .......... .....G..T. .......... .......... ......G... .......... .......A..

               Env Termination Codon, start of 3’U3 Region
FeLV-A      CCATGAT-TT CCAATTAAAT GTATGATTCC ATTTAGTCCC C-AGAAAAAG GGGGGAATGA AAGACCCCCT
AY364318    ..G.ATCACC ...GC..... .......... ......G.T. .T.AG..... ....A..... ........T.
FeLV-2518   .......... .......... ...A...... .......... .....G.... .......... ..........
FeLV-4314   .......C. .......... .......... .......... .......... .......... ..........

         8191
FeLV-A      ACCCCAAAAT TTAGCCAGCT ACTGCAGTGG ---TGCCATT --TCACAAGG CATGGAAAAT TACTCAAGTA
AY364318    -....TTGT. ..GA..CC.. GTCAT.A.AT GCT.AG..A. AG.A..GCCA TT..C..G.C AG.A.C.AG.
FeLV-2518   .......... .....T.... .....T.... .......... .......... T......... ..........
FeLV-4314   .......... .......... .......... .....T...A .......... .......... ..........

         8261
FeLV-A      TGTTCCCATG AGATACAAGG AAGTTAGAGG CTA------A AACAGGATAT CTGTGGTTAA GCACCTGGGC
AY364318    A....AGGG. TCT..TCCTA .GTCC.CC.T T..GCTGCC. .......... .......C.G C....C..C.
FeLV-2518   .......... .....T.... .......... .A........ .......... .......... ..........
FeLV-4314   .......... .....T.... .......... .G........ .......... .......... ..........

         8331
FeLV-A      C--------- ---CCGGCTT GAGGCCAAGA ACAGTTAAAC CCCGGATATA GCTGAAACAG C----AGAAG
AY364318    .TAAGATAGC CAC.T...CC T.A.ATGG.. .TG.A-..GT A.T.AC.CC. C.C..T.G.C .CTAG...T.
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FeLV-2518   ......... ......... .........G ......... ......... ......... .........
FeLV-4314   ......... ......... ......... ......... ......... ......... .........

         8401
FeLV-A      TTTCAAGGCC GCTACCAGCA GTCTCCAGGC TCCCC----- -AGTTGACC- ---------- ----------
AY364318    AGC.T..T.A ..C...CATG T.T.T.CCC. ..ATTCTGGG A.A.C.C..T CAGAAAAGAA AAGAAAAAGA
FeLV-2518   ......... ...G...... ......... ......... ......... ......... .........
FeLV-4314   ......... A..G.....T ......... ......... ......... ......... .........

         8471
FeLV-A      ---------- --AGGGTTCG ACCTTCCGCC TCATTTAAAC TAACCAAT-- --CCCC---- --ACGCCTCT
AY364318    AAAAAAAAAA AA.AAAAAAA .AAAC.A... ........CT GG......AA GA....GTAA CT.T..T...
FeLV-2518   ......... ....A..... ......... ......... ......... ......... .........
FeLV-4314   ......... ....A..... ......... ......... ......... ......... .........

         8541
FeLV-A      CGCTTCTGTG CGCGCGCTTT C--------- -TGCTATAAA ACGAGCCATC AGCCCC-CAA CGGGCGCGCA
AY364318    .........A AC.......C TGCCACTCCA ACC....... .A.TCT.CC. .....AA... GA........
FeLV-2518   ......... ......... ......... ......... .......--- ------.--- ----------
FeLV-4314   ......... ......... ......... ......... ......... .........-- ----------

         8611
FeLV-A      AGTCTTTGCT GAGACTTGAC CGCCCCGGGT ACCCGTGTAC GAATAAACCT CTTGCTGATT GCATCTGACT
AY364318    ......... ......... ......... ......... ......... .......T.. .........
FeLV-2518   ---------- ---------- ---------- ---------- ---------- ---------- ----------
FeLV-4314   ---------- ---------- ---------- ---------- ---------- ---------- ----------

         8681
FeLV-A      CGTGGTCTCG GTGTTCTGTG GGCGCGGGGT CTCATCGCCG AGGAAGACCT AGTTCAGGGG TCTTTCA---
AY364318    ......... ......C... ...A..... ......... ......... ....AG.... .......AGT
FeLV-2518   ---------- ---------- ---------- ---------- ---------- ---------- -------...
FeLV-4314   ---------- ---------- ---------- ---------- ---------- ---------- -------...
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(B) Gag-pol polyprotein alignment

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                  1
FeLV-A (Rickard)  MSGASSGTAI GAHLFGVSP- --EYRVLIGD EGAGPSKSLS EVSFSVWYRS RAARLVIFCL
enFeLV AY364318   ......... ..R...I.SV LG........ ......R.P. ......... .......L..
FeLV-4314         .....M.... ......... ......... ........P. ........Q. .........
FeLV-2518         .....M.... ......... ......... ......R... ........Q. .........

                 61
FeLV-A (Rickard)  VASFLVPCLT FLIAETVMGQ TITTPLSLTL DHWSEVRARA HNQGVEVRKK KWITLCEAEW
enFeLV AY364318   ......... .....A.... .V........ N.....Q... R......... .........
FeLV-4314         ......... ......... ......... ......... ......... .........
FeLV-2518         ......... ......... ......... ......... ......... .........

                121
FeLV-A (Rickard)  VMMNVGWPRE GTFSLDNISQ VEKKIFAPGP YGHPDQVPYI TTWRSLATDP PSWVRPFLPP
enFeLV AY364318   ......... ...TI..... ..ER...... ......I... ......... .P........
FeLV-4314         ......... ......S... ......... ......... ......... .........
FeLV-2518         ......... ......... ......... ......... ......... .........

                181
FeLV-A (Rickard)  PK-PPTPLPQ PLSPQPSAPL TSSLYPVLPK SDPPKPPVLP PDPSSPLIDL LTEEPPPYPG
enFeLV AY364318   ..H.R.DP.E .........P I......... P....A.... .N........ .........
FeLV-4314         ......... ......... ......... PG........ ......... .........
FeLV-2518         ......... ......... ......... ......... ......... .........

                241
FeLV-A (Rickard)  GHGP-PPSGP RTPTASPIAS RLRERRENPA EESQALPLRE GPNNRPQYWP FSASDLYNWK
enFeLV AY364318   ....T..... ......... ......... .K........ ......... .........
FeLV-4314         ......... ......... ......... ......... ......... .........
FeLV-2518         ......... ......... ......... ......... ......... .........

                301
FeLV-A (Rickard)  SHNPPFSQDP VALTNLIESI LVTHQPTWDD CQQLLQALLT GEERQRVLLE ARKQVPGEDG
enFeLV AY364318   L......... ......... ......... .......... A......... .........
FeLV-4314         ......... ......... ......... ......... ......... .........
FeLV-2518         ......... ......... ......... ......... ......... .........

                361
FeLV-A (Rickard)  RPTQLPNVID ETFPLTRPNW DFATPAGREH LRLYRQLLLA GLRGAARRPT NLAQVKQVVQ
enFeLV AY364318   ........V. .A........ ......... ......... ......... .........
FeLV-4314         .......... .A........ ......... ......... ......... .........
FeLV-2518         ......... ......... ......... ......... ......... .........P

                421
FeLV-A (Rickard)  GKEETPAAFL ERLKEAYRMY TPYDPEDPGQ AASVILSFIY QSSPDIRNKL QRLEGLQGFT
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enFeLV AY364318   .......S.. .......... .......... .......... .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........

                481
FeLV-A (Rickard)  LSDLLKEAEK IYNKRETPEE REERLWQRQE ERDKKRHKEM TKVLATVVAQ NRDKDREESK
enFeLV AY364318   .......... .......... .......... .......... .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........

                541
FeLV-A (Rickard)  LGDQRKIPLG KDQCAYCKEK GHWVRDCPKR PRKKPANSTL LNLGDZESQG QDPPPEPRIT
enFeLV AY364318   .......... .......... .......... ......D... ...E...... ..........
FeLV-4314         .......... .......... .......... .K........ .......... ..........
FeLV-2518         .......... .........R .......... .......... .......... ..........

                601
FeLV-A (Rickard)  LKIGGQPVTF LVDTGAQHSV LTRPDGPLSD RTALVQGATG SKNYRWTTDR RVQLATGKVT
enFeLV AY364318   ..V....... .......... .......... .S........ .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........

                661
FeLV-A (Rickard)  HSFLYVPECP YPLLGRDLLT KLKAQIHFTG EGANVVGPKG LPLQVLTLQL EEEYRLFEPE
enFeLV AY364318   .......... .......... .......... ........M. .......... ..........
FeLV-4314         .......... .......... .......... ........R. .......... ..........
FeLV-2518         .......... .......... .......... ........R. .......... ..........

                721
FeLV-A (Rickard)  STQKQEMDIW LKNFPQAWAE TGGMGTAHCQ APVLIQLKAT ATPISIRQYP MPHEAYQGIK
enFeLV AY364318   .EL..G..S. .......... ...I.M.... ..I....... .......... ..........
FeLV-4314         ........T. .......... .....M.... .......... .......... ..........
FeLV-2518         .......... .......... .....M.... ..I....... .......... ..........

                781
FeLV-A (Rickard)  PHIRRMLDQG ILKPCQSPWN TPLLPVKKPG TEDYRPVQDL REVNKRVEDI HPTVPNPYNL
enFeLV AY364318   .......... .......... .......... .G........ .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........

                841
FeLV-A (Rickard)  LSTLPPSHPW YTVLDLKDAF FCLRLHSESQ LLFAFEWRDP EIGLSGQLTW TRLPQGFKNS
enFeLV AY364318   .......... .......... ......P... .......K.. .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... .......... ......P... .......... .......... ..........

                901
FeLV-A (Rickard)  PTLFDEALHS DLADFRVRYP ALVLLQYVDD LLLAAATRTE CLEGTKALLE TLGNKGYRAS
enFeLV AY364318   .......... .......... .......... .......K.. .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .A........ .......... .......... .......... .......... ..........

                961
FeLV-A (Rickard)  AKKAQICLQE VTYLGYSLKD GQRWLTKARK EAILSIPVPK NSRQVREFLG TAGYCRLWIP
enFeLV AY364318   .........K ........E. .......... .......... .P........ ..........
FeLV-4314         .......... .......... .......... .......... .P........ ..........
FeLV-2518         .......... .......... .......... .......... .P........ ..........

               1021                        FeLV-4314 5' Recomb. Site
FeLV-A (Rickard)  GFAELAAPLY PLTRPGTLFQ WGTEQQLAFE DIKKALLSSP ALGLPDITKP FELFIDENSG
enFeLV AY364318   .......... .......... .......... N......... .......... .......S..
FeLV-4314         .......... .......... ....[.....]. N......... .......... .......S..
FeLV-2518         .......... .......... .......... N......... .......... .......S..

               1081
FeLV-A (Rickard)  FAKGVLVQKL GPWKRPVAYL SKKLDTVASG WPPCLRMVAA IAILVKDAGK LTLGQPLTIL
enFeLV AY364318   .......... .......... .......... .......... .......... ........V.
FeLV-4314         .......... .......... .......... .......... .......... ........V.
FeLV-2518         ......I... .......... .......... .......... .......... ..........

               1141
FeLV-A (Rickard)  TSHPVEALVR QPPNKWLSNA RMTHYQAMLL DAERVHFGPT VSLNPATLLP LPSGGNHHDC
enFeLV AY364318   .......... .......... .......... .......... .......... ..NE......
FeLV-4314         .......... .......... .......... .......... .......... ..NE......
FeLV-2518         .......... .......... .......... .......... .......... ..........

               1201
FeLV-A (Rickard)  LQILAETHGT RPDLTDQPLP DADLTWYTDG SSFIRNGERE AGAAVTTESE VIWAAPLPPG
enFeLV AY364318   .......... .......... .......... .........K .......... .....S....
FeLV-4314         .......... .......... .......... .........K .......... .....S....
FeLV-2518         .......... .T........ .......... .........K ..T....... .....S....
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                1261
FeLV-A (Rickard)  TSAQRAELIA LTQALKMAEG KKLTVYTDSR YAFATTHVHG EIYRRRGLLT SEGKEIKNKN
enFeLV AY364318   .......... ........K. .......... .....A.... .......... ..........
FeLV-4314         .......... ........K. .......... .....A.... .......... ..........
FeLV-2518         ........V. ........K. .......... .....A.... .......... ..........

                1321
FeLV-A (Rickard)  EILALLEALF LPKRLSIIHC PGHQKGDSPQ AKGNRLADDT AKKAATETHS SLTVLPTELI
enFeLV AY364318   .......... .......... .......... .......... ........Q. ...I......
FeLV-4314         .......... .......... .......... .......... ........Q. ...I......
FeLV-2518         .......... .......... .......... .......... .......... ..........

                1381
FeLV-A (Rickard)  EGPKRPPWEY DDSDLDLVQK LEAHYEPKRG TWEYRGKTIM PEKYAKELIS HLHKLTHLSA
enFeLV AY364318   .......... .......... .......... ....Q..... .......... ..........
FeLV-4314         ....K..... N......... .......... ....Q..... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........

                1441
FeLV-A (Rickard)  RKMKTLLERE ETGFYLPNRD LHLRQVTESC RACAQINAGK IKFGPDVRAR GRRPGTHWEV
enFeLV AY364318   .......... .......... .......... .......... .......... .H...I....
FeLV-4314         .......... .......... .......... Q....V.... .......... .H...I....
FeLV-2518         .......... .......K.. .......... .......... .......... ..........

                1501
FeLV-A (Rickard)  DFTEIKPGMY GYKYLLVFID TFSGWAEAYP AKHETAKVVA KKLLEEIFPR YGIPQVLGSD
enFeLV AY364318   .......... .......... .......... .......... .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........

                1561
FeLV-A (Rickard)  NGPAFISQVS QSVATLLGIN WKLHCAYRPQ SSGQVERMNR SIKETLTKLT LETGSKDWVL
enFeLV AY364318   .......... .......... .......... .......... .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........

                1621
FeLV-A (Rickard)  LLPLVLYRVR NTPGPHGLTP FEILYGAPPP MAHFFDTDIS SFATSPTMQA HLRALQLVQE
enFeLV AY364318   .......... .......... .......... ......A... G......... ..........
FeLV-4314         .......... .......... .......... ......A... G......... ..........
FeLV-2518         .......... .......... .......... ......A... .......... ..........

                1681
FeLV-A (Rickard)  EIQRPLAAAY REKLETPVVP HPFKPGDSVW VRRHQTKNLE PRWKGPHIVL LTTPTALKVD
enFeLV AY364318   .......... .......... .......... .......... .......... ..........
FeLV-4314         .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... Q......... .......... ......R... .......... ..........

                1741
FeLV-A (Rickard)  GVAAWIHASH VKAAGPTTNQ DLSDSPSSDD PSRWKVQRTQ NPLKIRLSRG TZ
enFeLV AY364318   .......... .......... .P..D..... .......... .......... ..
FeLV-4314         .......... .......... .P..D..... .......... .......... ..
FeLV-2518         .......... .......... .......... .......... ........H. ..
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(C) Env protein precursor alignment

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                  FeLV-2518 5′ Recomb. Site          Fusion motif
FeLV-A (Rickard)  MESPTHPKPS KDKTLSWNLA FLVGILFTID IGMANPSPHQ IYNVTWVITN MQTNTQANAT
FeLV-4314         ..G....... ....F..D.M I...V.LRL. V.......... V.....T... LV.G.T....
enFeLV AY364318   ..G....... ....F..D.M I...V.LRL. V.......... V.....T... LV.G.K....
FeLV-2518         ...........  .......... .......... .......... V.....T... LV.G.K....
FeLV-2518(A)      .......... .......... .......... ........P. M......... V.........

                   61
FeLV-A (Rickard)  SMLGTLTDAY PTLHVDLCDL VGDTWEPIVL DPTNVKHGAR YSSSKYGCKT TDRKKQQQTY
FeLV-4314         .........F ..MYF....I IDN..N.SDQ E.F------- ---PG...GQ PM.RW..RNT
enFeLV AY364318   .........F ..MYF....I I.N..N.SDQ E.F------- ---PG...DQ PM.RW..RNT
FeLV-2518         .........F ..MYF....I I.N..N.SDQ E.F------- ---PG...DQ PM.RW..RNT
FeLV-2518(A)      .......... .......... .......... N......... ......R... ..........

                  121
FeLV-A (Rickard)  PFYVCPGHAP SLGPKGTHCG GAQDGFCAAW GCETTGEAWW KPSSSWDYIT VKRGSSQDNS
FeLV-4314         .........- ----NRKQ.. .P......V. .......TY. R.T....... ..K.VT.GIY
enFeLV AY364318   .........- ----NRKQ.. .P......V. .......TY. R.T....... ..K.VT.GIY
FeLV-2518         .........- ----NRKQ.. .P......V. .......TY. R.T....... ..K.VT.GIY
FeLV-2518(A)      .......... .......... .....Y.... .......... ..T....... ..........

                   181
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FeLV-A (Rickard)  ---------- C--------- -----EGKCN PLILQFTQKG RQASWDGPKI WGLRLYRTGY
FeLV-4314         QCSGGGWCGP .YDKAVHSSI TGASEG.R.. .......... ..T......S .......S..
enFeLV AY364318   QCSGGGWCGP .YDKAVHSSK TGASEG.R.. .......... ..T......S .......S..
FeLV-2518         QCSGGGWCGP .YDKAVHSSI TGASEG.R.. .......... ..T......S .......S..
FeLV-2518(A)      .......... .......... .......R.. ..V....... .......... ..........

                241
FeLV-A (Rickard)  DPIALFTVSR QVSAITPPQA MGPNLVLPDQ KPPSRQSQTG SKVATQRLQT TESAP-----
FeLV-4314         ......S... ..MT...... .......... ...FG...IE .Q.TPHHS.G NG.T.GITLV
enFeLV AY364318   ......S... ..MT...... .......... ........IE .R.TPHHS.G NGGT.GITLV
FeLV-2518         ......S... ..MT...... .......... ........IE .R.TPHHS.G NGGT.GITLV
FeLV-2518(A)      .......... ...T...... .......... .....L...R .......P.. N.........

                301
FeLV-A (Rickard)  -RSVAP---- -TTVGPKRIG TGDRLINLVQ GTYLALNATD PNKTKDCWLC LVSRPPYYEG
FeLV-4314         NA.I..LSTP V.P.S..... ..N....... .......V.N .......... ..........
enFeLV AY364318   NA.I..LSTP V.PAS..... ..N....... .......V.N .......... ..........
FeLV-2518         NA.I..LSTP V.PAS..... ..N....... .......V.N .......... ..........
FeLV-2518(A)      .......... ...I...... .......... XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX

                361
FeLV-A (Rickard)  IAILGNYSNQ TNPPPSCLST PQHKLTISEV SGQGLCIGTV PKTHQALCNE TQQGHTGAHY
FeLV-4314         ..V....... .........D .......... .......... ........KK ..K..K.T..
enFeLV AY364318   ..V....... .........D .......... .......... ........KK ..K..K.T..
FeLV-2518         ..V....... .........D .......... .......... ........KK ..K..K.T..
FeLV-2518(A)      XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX

                421                  FeLV-2518 3′ Recomb. Site     SU/TM Cleavage Site
FeLV-A (Rickard)  LAAPNGAYWA CNTGLTPCIS MAVLNWTSDF CVLIELWPRV TYHQPEYVYT HFAKAVRFRR
FeLV-4314         ....S.T... .......... .......... .A........ ...E...I.S ..ENKP..K.
enFeLV AY364318   ....S.T... .......... .......... .......... ...E...I.S ..ENKP..K.
FeLV-2518         ....S.T... .......... .......... .......... .........S ..........
FeLV-2518(A)      XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX

                481                                          FeLV-4314 3′ Recomb. Site
FeLV-A (Rickard)  EPISLTVALM LGGLTVGGIA AGVGTGTKAL LETAQFRQLQ MAMHTDIQAL EESISALEKS
FeLV-4314         D......... ...I....M. ..I....A.. .......... .......... ..........
enFeLV AY364318   D......... ...I....M. ..I....A.. .......... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........
FeLV-2518(A)      XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX

                541
FeLV-A (Rickard)  LTSLSEVVLQ NRRGLDILFL QEGGLCAALK EE-------C CFYADHTGLV RDNMAKLRER
FeLV-4314         .......... .......... .......... .......... .......... ..........
enFeLV AY364318   .......... .......... .......... .......... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........
FeLV-2518(A)      XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX

                601
FeLV-A (Rickard)  LKQRQQLFDS QQGWFEGWFN KSPWFTTLIS SIMGPLLILL LILLFGPCIL NRLVQFVKDR
FeLV-4314         .......... .........K .......... .......... .......WV. ..........
enFeLV AY364318   .......... .......... .......... ......M... .......... ..........
FeLV-2518         .......... .......... .......... .......... .......... ..........
FeLV-2518(A)      XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXX... .......--- ----------

                661
FeLV-A (Rickard)  ISVVQALILT QQYQQIKQYD PDRP----Z
FeLV-4314         .......... .....V.... .........
enFeLV AY364318   .....T.V.. ..H.RLG.C. S.Q.YHPS.
FeLV-2518         .......... .......... ..Q......
FeLV-2518(A)      ---------- ---------- ----.....
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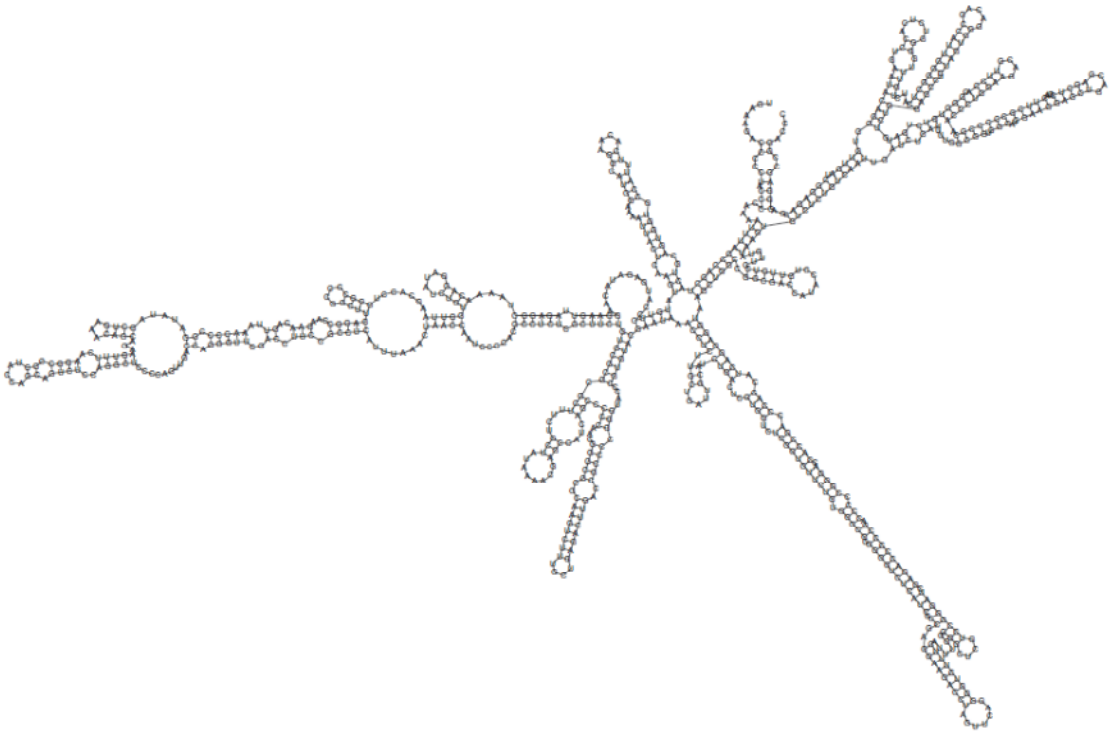
8.5 Predicted secondary structures within FeLV *env* RNA

FeLV-A *env* RNA

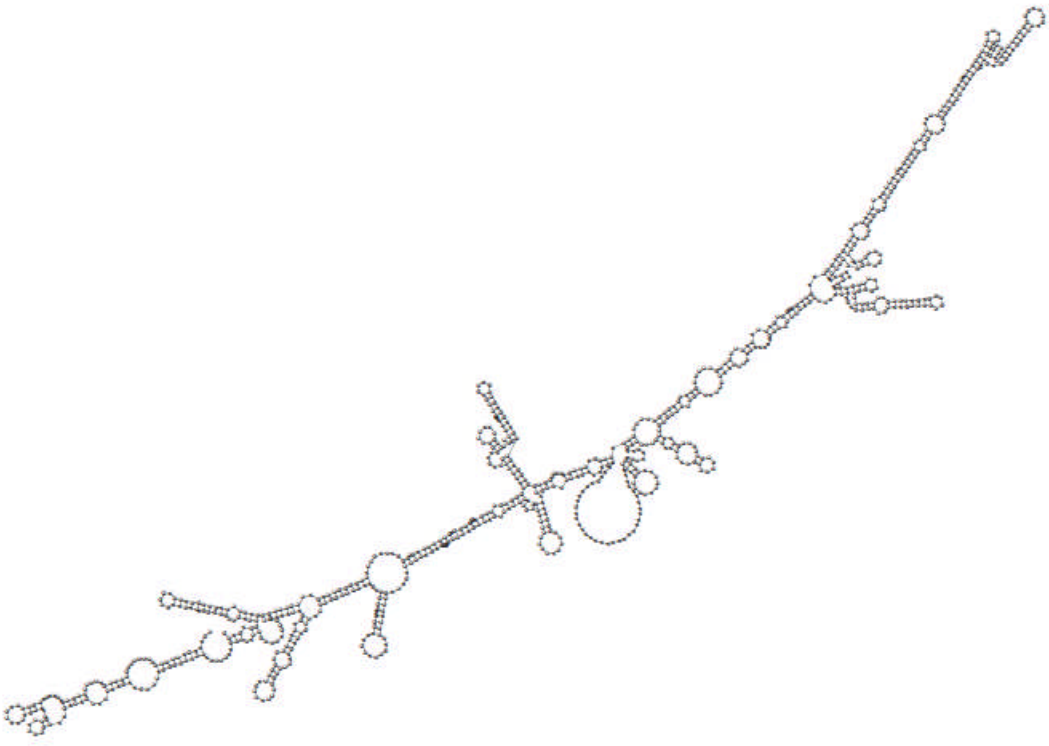
enFeLV *env* RNA

8.6 Predicted secondary structures within FeLV leader sequences

FeLV-A Monomeric RNA

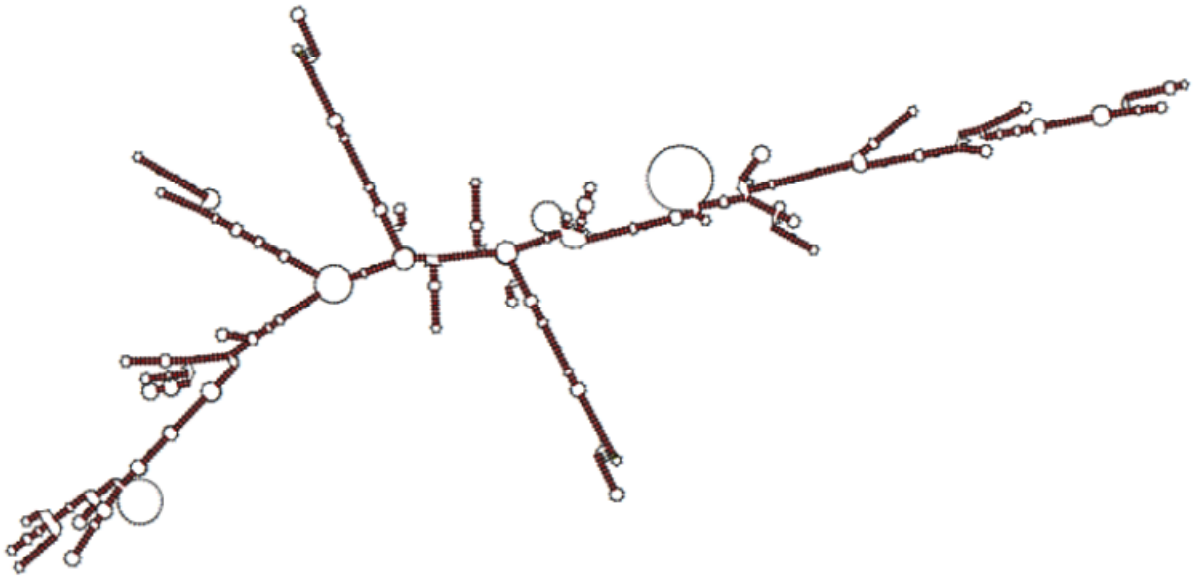


enFeLV Monomeric RNA



FeLV-A Homodimeric RNA

enFeLV Homodimeric RNA



FeLV-A/enFeLV Heterodimeric RNA

8.7 Publications arising from this work

Stewart H, Jarrett O, Hosie M, Willett B. (2013). Complete genome sequences of two feline leukaemia virus subgroup B isolates with novel recombination sites. *Genome Announcements* (in press).

Stewart H, Adema K, McMonagle E, Hosie M, Willett B. (2012). Identification of novel subgroup A variants with enhanced receptor binding and replicative capacity in primary isolates of anaemogenic strains of feline leukaemia virus. *Retrovirology* 9(48).

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