Short Communication

Are endogenous feline leukemia viruses really endogenous?

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ABSTRACT

Full length endogenous feline leukemia virus (FeLV) proviruses exist within the genomes of many breeds of domestic cat raising the possibility that they may also exist in a transmissible exogenous form. Such viruses would share receptor usage with the recombinant FeLV-B subgroup, a viral subgroup that arises *in vivo* by recombination between exogenous subgroup A virus (FeLV-A) and endogenous FeLV. Accordingly, all isolates of FeLV-B made to date have contained a "helper" FeLV-A, consistent with their recombinatorial origin. In order to assess whether endogenous viruses are transmitted between cats, we examined primary isolates of FeLV for which the viral subgroup had been determined for the presence of a subgroup B virus that lacked an FeLV-A. Here we describe the identification of two primary field isolates of FeLV (2518 and 4314) that appeared to contain subgroup B virus only by classical interference assays, raising the possibility of between-host transmission of endogenous FeLV. Sequencing of the *env* gene and U3 region of the 3' long terminal repeat (LTR) confirmed that both viral genomes contained endogenous viral *env* genes. However the viral 3' LTRs appeared exogenous in origin with a putative 3' recombination breakpoint residing at the 3' end of the *env* gene. Further, the FeLV-2518 virions also co-packaged a truncated FeLV-A genome containing a defective *env* gene, termed FeLV-2518(A) while no helper subgroup A viral genome was detected in virions of FeLV-4314. The acquisition of an exogenous LTR by the endogenous FeLV in 4314 may have allowed a recombinant FeLV variant to outgrow an exogenous FeLV-A virus that was presumably present during first infection. Given time, a similar evolution may also occur within the 2518 isolate. The data suggest

that endogenous FeLVs may be mobilised by acquisition of exogenous LTRs yielding novel viruses that type biologically as FeLV-B.

Keywords: Feline leukemia virus; endogenous retrovirus; LTRs; RNA co-packaging.

1. INTRODUCTION

Feline Leukemia Virus (FeLV) is a major pathogen of cats that causes a range of diseases, including immunosuppression and both proliferative and non-proliferative haematopoietic disorders. The four subgroups (FeLV-A, -B, -C and –T) are distinguished by their receptor usage as well as their accompanying disease manifestations. Receptor usage is determined by the Receptor-Binding Domain (RBD), located within the surface unit of the envelope glycoprotein (Env) (Bae et al., 1997; Rigby et al., 1992). The prototype FeLV-A subgroup utilises 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) and displays a broader *in vitro* host range (Jarrett et al., 1969; Jarrett et al., 1973). Some FeLV-B isolates are also able to utilise the homologous protein fePit2 (Anderson et al., 2001; Boomer et al., 1997).

Endogenous retroviruses (ERVs) arise after a proviral integration event occurs within a germline cell or during early embryogenesis. The resultant provirus is therefore 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 polymorphisms between individuals. There are numerous FeLV-related endogenous elements (enFeLV) within the domestic cat genome (Benveniste and 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 have intact long terminal repeats (LTRs) (Soe et al., 1983; Soe et al., 1985). As

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), this domain is often analysed to determine the origin of FeLV strains (Tandon et al., 2008). As enFeLV elements are generally mutated and nonfunctional, 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).

FeLV-B arises through recombination events between the *env* genes of FeLV-A and enFeLV transcripts (Neil et al., 1991; Overbaugh et al., 1988; Stewart et al., 1986). Thus FeLV-B is always found alongside FeLV-A, which is generally regarded as the "helper" virus required for FeLV-B transmission (Jarrett and Russell, 1978; Sarma and Log, 1973). 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. The specific enFeLV loci that contribute to FeLV-B formation have not been elucidated.

Recently, full length enFeLV elements with putative functional open reading frames (ORFs) have been characterized (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. We hypothesised that if transcription and packaging of these full-length enFeLV genomes occurred *in vivo,* then they may be transmitted between cats. As the RBD of FeLV-B is encoded by an enFeLV region of the recombinant genome, such infections would present as

subgroup B only by interference. 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 of receptor expression upon the cell surface, preventing re-infection by a virus of the same interference group.

We examined a group of FeLV field isolates for variants that displayed the FeLV-B phenotype alone. We identified two isolates from the sera of naturally infected cats, and sequenced both the *env* gene and U3 motif of the viral genomes to identify their proviral origin. Although we found evidence for recombination between endogenous and exogenous transcripts, our results indicate that enFeLV may contribute more highly to FeLV transmission and pathogenesis than previously suspected. We predict that these ostensibly-FeLV-B isolates may be transmitted between hosts without the presence of FeLV-A.

2. MATERIALS AND METHODS

2.1. Cell Lines

Mus dunni tail fibroblast (MDTF) cells (ATCC Catalogue CRL-2017) were maintained in low-glucose DMEM, supplemented with 10% Foetal Bovine Serum (FBS), 100U/mL penicillin and 100µg/mL streptomycin. Human embryo kidney (HEK293T) cells (Graham et al., 1977) were maintained in high-glucose DMEM, supplemented with 10% FBS, 100U/mL penicillin and 100µg/mL streptomycin. Feline embryo A

(FEA) cells (Jarrett et al., 1973) were maintained in high-glucose DMEM, supplemented with 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin, 2mM L-Glutamine and 2mM sodium pyruvate. All cells were washed with phosphate-buffered saline and subcultured upon confluency using Trypsin-EDTA. All media and supplements were obtained from InVitrogen Life Technologies Ltd (Paisley, U.K).

MDTF cells stably expressing the human orthologue of the FeLV-C Receptor (hFLVCR1) and the feline thiamine transporter protein (feThTr1) were developed as follows: Viral particles packaging the retroviral receptor expression constructs and pseudotyped with the VSV-G envelope protein (Yee et al., 1994) were produced by transient transfection of HEK293T cells using SuperFect activated dendrimer (QIAgen Ltd, Crawley, U.K.). MDTF cells were transduced with filtered viral particles harvested 72 hours post-transfection. Stably-transduced cells were selected in the presence of 0.8mg/mL Geneticin (InVitrogen) for three weeks. Expression of retroviral receptors was confirmed by immunoblotting (data not shown).

2.2. Receptor Usage Assays

The receptor usage of numerous FeLV isolates from the sera of naturally infected cats was determined using a FeLV receptor interference assay as previously described (Jarrett et al., 1973). These results were then confirmed by transfection of infected FEA cells with a construct containing the β-galactosidase gene and a Murine Leukemia Virus (MLV) packaging signal, pMFG-lacZ (Ohashi et al., 1992).

Pseudotypes were harvested 72 hours post-transfection and used to infect MDTF cells stably expressing the respective FeLV receptors. 48 hours post-infection, MDTF cells were stained for β-galactosidase activity as previously described (Kimpton and Emerman, 1992).

2.3. Immunoblots for Viral Protein Expression

Concentrated virus samples were prepared by ultracentrifugation of cell-free tissue culture fluid from infected cultures. Samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting using the iBlot transfer system (InVitrogen). The primary antibodies for FeLV detection were a 1:500 dilution of mouse monoclonal antibody specific for the capsid (p27) protein (vpg19.1), and a $1:10^5$ dilution of mouse ascites preparation specific against the SU domain of Env (gp70).

For the detection of RD114 virus, goat polyclonal antiserum specific for the RD114 capsid protein was used at a 1:5000 dilution. Secondary antibody was anti-mouse or anti-goat alkaline phosphatase-labelled IgG from horse (Vector Laboratories, Peterborough, U.K). Proteins were visualised using an alkaline phosphatase substrate visualisation kit (Vector Laboratories).

2.4. Cloning of viral RNA and genomic DNA PCR Products

Viral RNA was extracted from concentrated viral pellets using an RNA preparation kit (QIAmp UltraSense Virus Kit, QIAgen). Following DNase treatment of RNA, cDNA was prepared using oligo-dT primers and a commercial cDNA synthesis kit (Roche Applied Science, Burgess Hill, U.K). Genomic DNA (gDNA) from infected cell cultures was isolated using a commercial kit (DNEasy Blood Kit, QIAgen). A high-fidelity DNA polymerase (Roche) was used to amplify FeLV *env* and LTR transcripts (primer details and PCR conditions available upon request). Viral RNA was included in PCRs to ensure contaminating cellular gDNA was not present. PCR products were separated by electrophoresis and purified from agarose using a commercial kit (QIAquick Gel Extraction Kit, QIAgen). Amplicons were TA-cloned into the pCR2.1 vector (InVitrogen) according to standard procedures. Plasmid purification of positive transformants was conducted with a commercial kit (Plasmid MiniKit, QIAgen).

Both PCR products and plasmid preparations were sequenced using the BigDye Terminator v1.1 Kit and analysed using an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Paisley, U.K) and the computer program DNADynamo (Blue Tractor Software, North Wales, U.K). The sequences described within this study have been deposited with GenBank (accession numbers pending).

3. RESULTS AND DISCUSSION

FEA cells were infected with a panel of 300 FeLV field isolates, and interference assays were used to classify them according to their subgroups. Two isolates,

designated FeLV-2518 and FeLV-4314, displayed the FeLV-B phenotype alone with no evidence of FeLV-A co-infection. This initial result was confirmed using MDTF cells stably transduced to express the various FeLV receptors (feThTr1, hFLVCR1 and fePit1) (data not shown). Although FeLV-B infection is highly associated with FeLV-induced lymphomas (Sheets et al., 1993; Tsatsanis et al., 1994) and leukemias (Jarrett et al., 1978; Tzavaras et al., 1990), the clinical history and disease status of the host is unknown. The disease manifestation of these FeLV isolates is therefore not known.

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 the capsid protein, confirming they were persistently infected (data not shown). A subsequent receptor usage assay confirmed the isolates had retained their FeLV-B phenotype following infection of the HEK293T cells. Immunoblots for detection of gp70 (the surface unit domain of Env) indicated that both isolates produced a full-length envelope glycoprotein (Figure 1). The Env of FeLV-2518 appeared to be of a slightly smaller size than that of FeLV-4314. This may be due to internal deletions within the FeLV-2518 *env* gene or differences in the glycosylation patterns of the proteins.

A PCR was conducted with exogenous FeLV-specific primers, to amplify specifically exogenous *env* transcripts from viral cDNA, prepared from RNA isolated from purified viral particles. Products could not be detected from FeLV-4314 templates; however FeLV-2518 cDNA produced a ~1kB amplicon (Figure 2). This could be

reproduced using cellular gDNA from both infected HEK293T and FEA cells (data not shown), the template of which would be integrated provirus. 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. It also contained a premature termination codon, preventing the final 37 amino acids from being synthesised. This defective exogenous FeLV genome was termed FeLV-2518(A). If this Env protein was expressed it would presumably be secreted; however gp70-specific immunoblots did not indicate it was present in either cell lysates or cell-free supernatant (data not shown). It is possible that this protein, if expressed, may either prevent FeLV-A infection through competitive receptor-binding, or enhance infection with other viruses in a similar manner to that of FeLIX. FeLIX is an endogenously-encoded Env peptide required for FeLV-T infection of T-lymphocytes, allowing the otherwise FeLV-A virus to utilise fePit1 for cell entry (Anderson et al., 2000; Lauring et al., 2001). To date none of our experiments indicate that either of these situations occurs with FeLV-2518(A) Env proteins.

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. Surprisingly, amplicons of the expected size (~500bp) were observed from both FeLV-2518 and -4314 templates (data not shown). Sequence analysis confirmed these were integrated exogenous FeLV U3 motifs. As the sequence of this antisense primer (termed FeLV U3 Rev Kpn1, sequence available upon request) was conserved

between published endogenous and exogenous FeLV sequences, it was then used with a novel sense primer to specifically amplify endogenous FeLV U3 sequences from gDNA. Amplicons were observed in all templates from FEA cells, as expected, but not in those of infected HEK293T cells (data not shown), indicating transmission of an endogenous LTR had not occurred and was therefore not present in either FeLV-2518 or -4314 virions.

Although there appeared to be exogenous LTRs present in both virus isolates, the lack of a full length exogenous *env* transcript in either case led us to predict an endogenous virus was contributing to the replication and transmission potential of both isolates, despite the fact that endogenous U3 sequences could not be detected in infected HEK293T cells. To ensure the replication-competent endogenous feline retrovirus RD-114 was not contributing to transmission of these isolates, we performed both immunoblots against the capsid protein and a PCR to detect RD-114 *env* transcripts. We did not detect either RD-114 transcripts or proteins (data not shown). We then conducted a PCR upon gDNA from infected HEK293T cells, using an enFeLV-env-specific sense primer and the conserved U3 antisense primer. Amplicons of the expected size, 2.2kB, were observed from both FeLV-2518 and -4314 templates (see Figure 3). Cloning and sequence analysis of these revealed both products to be recombinant *env* sequences with an exogenous FeLV LTR. This correlates with the seemingly-conflicting results produced in other PCR assays. We surmise that these ostensibly-endogenous FeLV genomes are producing the full-length Env glycoproteins observed in Figure 1. It is not known which of the viral genomes found within FeLV-2518 virions contributes the remaining viral proteins.

The 3' recombination breakpoint of FeLV-4314 is approximately 200bp downstream of the SU/TM cleavage site (Figure 4). It consists 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 are 12 randomly-distributed mutations, encoding 7 amino acid substitutions, which distinguish this from the full-length enFeLV genome AY364318 (Roca et al., 2004). This indicates that although the contributing enFeLV locus is not definitively known it is likely to be a recently-endogenised retrovirus. We hypothesise 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.

Comparatively, the 3' recombination breakpoint of the FeLV-2518 genome is an 18 nucleotide stretch encoding the WTSDFC motif, ~200bp upstream of the SU/TM cleavage site (Figure 4). Within the endogenously-encoded region, there is only one nucleotide difference (causing a K to I substitution) when compared to the AY364318 genome. Sequence comparison of FeLV-2518 and FeLV-2518(A) revealed homologous stretches both within the 3' region of *env* and across the U3 region. 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). As this *env* is assumed to be defective and not expressed, this genetic drift may have occurred since the recombination event that formed the

functional FeLV-2518 viral genome, as there is no longer selective pressure upon the exogenous genome to retain functionality.

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 coinfection of FeLV-A. This is also the first identification of a 3' recombination breakpoint being identified within the TM domain of a FeLV-B isolate, as the majority of recombination sites span a central 250bp region of SU (Sheets et al., 1992). The 5' recombination breakpoints of both FeLV-2518 and -4314 are yet to be identified; however we hypothesise the majority of the viral genomes are of endogenous origin and have arisen from a recently-endogenised FeLV element. Evidence for this comes from the fact that both these viral genomes, as well as the full-length enFeLV genomes (Roca et al., 2004) contain the C11D8 epitope (MGPNL, located in the central region of the SU domain (Elder et al., 1987; Nunberg et al., 1984)) which is not found in defective enFeLV elements such as CFE-6 (McDougall et al., 1994; Sheets et al., 1993). This also provides evidence that, although mostly-endogenously encoded, these Env glycoproteins may evoke an immune response in their hosts.

Prior to the discovery of putatively conserved enFeLV genomes, some 5' recombination points in FeLV-B genomes were known to be located within the *pol* gene indicating it must be functional in some enFeLV loci (Overbaugh et al., 1988;

Pandey et al., 1991). The few enFeLV-encoded *gag* genes studied were found to be highly mutated and thus assumed to be defective; however both the packaging signal and leader sequences of these are intact (Berry et al., 1988). It is therefore feasible that expression and packaging of defective RNA transcripts alongside exogenous genomes may occur even in host cats lacking the full-length enFeLV loci. Although the co-packaging of two distinct FeLV genomes within one virion has not been observed directly, co-packaging of both FeLV and MLV RNAs resulting in novel recombinant genomes has been observed *in vitro* (Pandey et al., 1991; Yin and Hu, 1997). There is also the notable case of AKR mice, which develop leukemia 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 et al., 1989).

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 and 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; Levy et al., 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. These include cases of FeLV-related multicentric lymphoma (Athas et al., 1995; Chandhasin et al., 2004) and acute myeloid leukemia (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. The U3 domain of exogenous gammaretroviral LTRs is also known to activate the AP-1 pathway (Abujamra et al., 2003; Ghosh and Faller, 1999; Weng et al., 1995), which is involved in cancer progression. Endogenous LTRs do not exert this effect when expressed (Ghosh et al., 2000), therefore the acquisition of exogenous LTRs for a mostly-endogenous FeLV genome would almost certainly alter its pathogenic potential.

4. CONCLUSIONS

Although we did not identify any instances of enFeLV transmission, which would have displayed a FeLV-B phenotype by interference and receptor usage assays, we did identify two FeLV field isolates (FeLV-2518 and -4314) that presented as FeLV-B alone, without FeLV-A co-infection. These viral genomes are recombinants and it is suspected that the majority is of endogenous origin. We hypothesise that in the case of FeLV-4314, the exogenous U3 region allowed the recombinant virus to achieve higher rates of transcription due to its 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. It is not known which *gag-pol* genes (either endogenous or exogenous) contribute to the viral replication of this isolate. Future work will concentrate on cloning and sequencing the entire viral genome of both isolates, including the truncated FeLV-2518(A) genome, to identify the 5' recombination points. Additionally, studies are underway to determine whether the

two genomes within FeLV-2518 co-package and dimerise within virions or are packaged separately. It may also be of interest to determine whether the viruses are able to utilise the FeLV-B receptor homologue fePit2, as it has been observed that the degree of viral genome originating from enFeLV correlates to the ability of isolates to utilise fePit2 (Boomer et al., 1997; Sugai et al., 2001).

To the best of our knowledge, this is 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.

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Conflict of Interest Statement

All authors declare that there is no conflict of interest.

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Figure 1: Immunoblot against the gp70 (SU) domain of Env. Samples were concentrated viral particles, prepared as described in text.

Figure 2: PCR products from viral cDNA. Primers specifically amplified the *env* genes of exogenous FeLV transcripts.

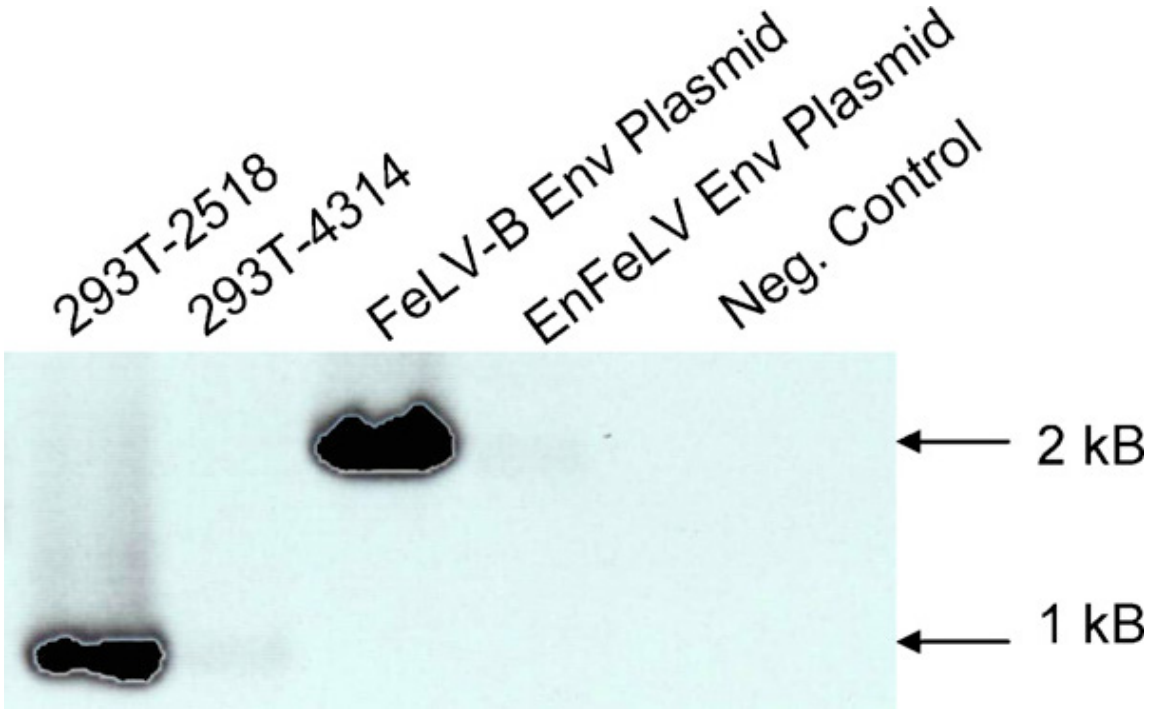

Figure 3: PCR products from gDNA templates. Sense primer was specific to the 5' region of endogenous FeLV *env*, antisense primer was based upon a portion of the U3 motif conserved between enFeLV and exogenous FeLV.

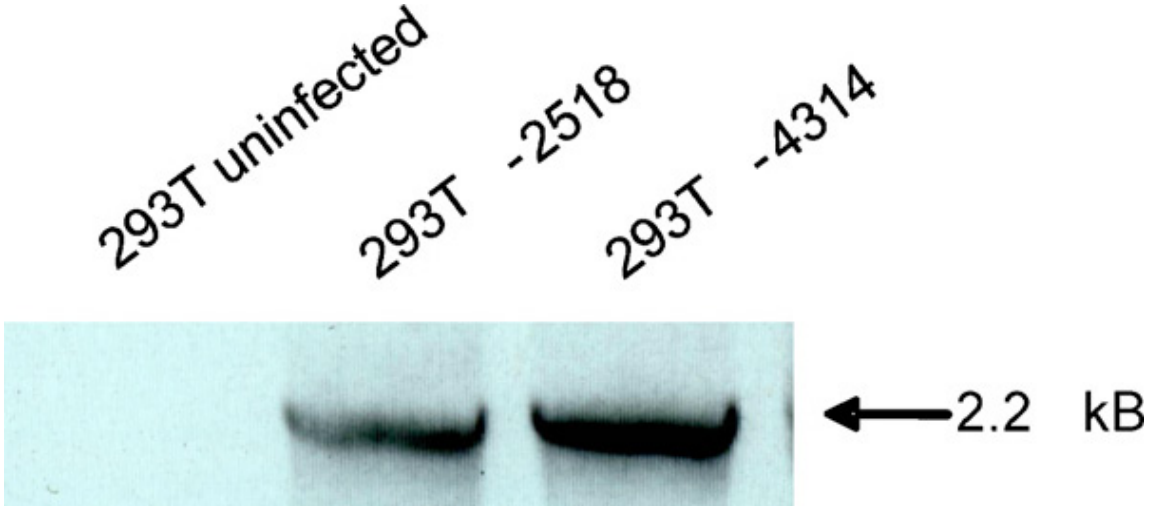

Figure 4: (a) Partial Env protein alignment of FeLV-2518, -4314 and prototype FeLV-A (Glasgow-1) sequences. Boxed regions indicate predicted 3' recombination breakpoints for the isolates described in this study. Vertical line denotes the SU/TM cleavage site. (b) Schematic of the *env* and LTR genomic regions described within this study.

(A)

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FeLV-2518 CISMAVLNWTSDFCVLIELWPRVTYHQPEYVYSHFAKAVRFRREPISLTVALML
FeLV-4314 CISMAVLNWTSDFCALIELWPRVTYHEPEYIYSHFENKPRFKRDPISLTVALML
FeLV-A    CISMAVLNWTSDFCVLIELWPRVTYHQPEYVYTHFAKAVRFRREPISLTVALML 456
          **************.***********:***:*:** :  **.*.**********

          GGLTVGGIAAGVGTGTKALLETAQFRQLQMAMHTDIQALEESISALEKSLTSLS
          GGITVGGMAAGIGTGTAALLETAQFRQLQMAMHTDIQALEESISALEKSLTSLS
          GGLTVGGIAAGVGTGTKALLETAQFRQLQMAMHTDIQALEESISALEKSLTSLS 510
          **:****:***:**** ************************************
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(B)