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UMI
THE ROLE OF ENDOGENOUS FeLV IN THE INFECTIVITY AND GENOMIC STABILITY OF FeLV SUBGROUP A

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

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* * * * *

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ABSTRACT

Feline leukemia viruses (FeLV) are naturally occurring simple retroviruses associated with certain cancers, and are used as an important model system for studying the carcinogenic mechanisms of retroviruses. Of the three FeLV subgroups A, B and C, FeLV-B viruses are generated \textit{de novo} from recombination between FeLV-A and endogenous FeLV (enFeLV). In a previous study investigating the pathogenicity of two FeLV-A molecular clones, pF6A and pFRA, FRA was found to be more pathogenic and recombinogenic than F6A. The goal of this dissertation was to extend our understanding of the factors contributing to FeLV recombination by evaluating the host cell/virus interactions of pFRA and pF6A. In addition, we investigated the expression of enFeLV RNA in feline tissues and cell lines and its role in the genetic instability of pFRA tagged to GFP gene \textit{in vivo} and \textit{in vitro} as a necessary step toward understanding the recombination process.

FRA and F6A were equally infectious to feline fibroblast (FEA) and feline lymphoid (3201) cell lines. However, FRA was more recombinogenic than F6A in 3201 cells, although no difference in recombination potential between F6A and FRA was observed in FEA cells. These results indicate that the recombinogenic variation between F6A and FRA was cell-dependent. The probable mechanism for the increased recombinogenic potential of FRA in the 3201 cells was traced to the higher quantity of
enFeLV RNA present in the progeny virus of FRA-transfected 3201 cells compared to that of F6A-transfected cells. We tested the possibility that enFeLV RNA expression was enhanced by FRA-LTR compared to F6A-LTR. However, the transfection of 3201 cells with FRA- or F6A-LTR showed no enhancement of enFeLV expression. These results indicate that FRA copackages more enFeLV RNA than F6A thus increasing the probability of exogenous-endogenous recombination. To test the possibility that the pol gene from FRA was responsible for increase recombination, the pol regions were exchanged between pF6A and pFRA. After the transfection of the constructed chimeras into 3201 cells, both chimeras were infectious but no recombinant virus was detected, indicating the presence of another factor (or factors) that may interact with pol to promote recombination.

In preparation for in vivo experimentation to study the spread of the virus from the site of inoculation, the green fluorescent protein transgene (GFP) was inserted 3’ of env gene in the FRA provirus. The pFRA-GFP virus was highly infectious to the feline fibroblast cell line, H927, which continued to mediate strong expression of GFP up to 8 months. By contrast, pFRA-GFP inoculation into cats, and in 3201 cells resulted in unstable FRA-GFP virus with less than 2% of the FRA-GFP infected cells expressing GFP as determined by quantitative real time RT-PCR, expression and immunofluorescence staining assays.

Because F6A and FRA recombinogenicity and FRA-GFP stability were cell-dependent, we hypothesized that the instability of FRA-GFP in 3201 cells was due to the level of enFeLV RNA expression in these cells. As a result, we investigated enFeLV RNA expression in feline lymphoid and fibroblast cells and extended the...
study to various feline tissues. The 3201 cells produced 181 times more enFeLV RNA than fibroblast cells. Lymphoid tissues expressed high level of enFeLV RNA, while the ileum, salivary gland and brain tissues were below the detection limit as determined by real time quantitative RT-PCR.

In conclusion, the data of these studies have shown that the variation in recombination potential between F6A and FRA and the genetic stability of FRA-GFP are cell-dependent and this dependency relies on the level of enFeLV RNA expression. It is probable that the varied expression of enFeLV by feline tissues will affect their performance in mediating FeLV recombination and their susceptibility to infection by subgroup B recombinant FeLV.
Dedicated to my parents and my wife, who encouraged me, my teachers, who enabled me, and my son, who put up with me.
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CHAPTER 1

INTRODUCTION

Feline leukemia virus (FeLV) is a naturally occurring simple retrovirus found in the domestic cat population (46,48,80). Since the isolation of FeLV in 1964 (54), three horizontally transmitted FeLV subgroups; FeLV-A, FeLV-B and FeLV-C have been defined by their unique cell receptor usage in the viral interference assay. Sequence analysis of prototype FeLV subgroup A, B and C viruses show variation in the viral surface glycoprotein (SU) moiety of the envelope gene (52-54,88,89). Another FeLV subgroup, T-cell-tropic FeLV (FeLV-T) with unique interference properties has been recently identified (2). In addition to the exogenous FeLV of feline species, multiple endogenous proviruses are present in the genome of the domestic cat. These endogenous retroviruses play a critical role in the pathogeneses of FeLV (31,46-48,58,75,87,89). The weakly pathogenic FeLV-A is commonly transmitted in nature and highly infectious but rarely leads to disease until new subgroups, B, or C, arise de novo as a result of recombination and/or mutation with endogenous FeLV sequences (enFeLV) (31,46-48,58,75,87,89).

FeLV-B is associated with lymphoma or other myeloproliferative diseases (20,35,58,75,87). FeLV-C is capable of inducing erythroid hypoplasia and immunosuppression (1,32,33,64,74,84,85). Figure 1.1 shows the time frame for the in vivo generation of FeLV-B and -C after the inoculation of FeLV-A molecular clone.
FeLV subgroup B recombinants are detected as early as 8 weeks post inoculation in bone marrow and 12 weeks post inoculation in plasma (81).

FeLV belongs to the gamma retrovirus subfamily of retroviruses. Genetically, FeLV is composed of three genes, \textit{gag}, \textit{pol}, and \textit{env} flanked by long terminal repeats (LTR) (Figure 1.2). Nine proteins are encoded by the FeLV genome, and include the internal structure proteins: p15 (matrix protein, MA), p12 (unknown function), p27 (capsid, CA), and p10 (nucleocapsid, NC); the enzymatic proteins: p14 (protease, PR), p80 (reverse transcriptase, RT), p46 (integrase, IN); and the envelope proteins: gp70 (surface protein, SU), and p15E (transmembrane protein, TM) (43-45,61,89). Although FeLV lacks the discrete accessory and regulatory genes associated with the genetically complex retroviruses, such as human T-cell leukemia virus (HTLV) or bovine leukemia virus (BLV), the U3-LTR region of FeLV-A reportedly exhibits a cellular gene transactivational activity through the generation of an RNA transcript derived from the U3-LTR (38,39).

**FeLV subgroups Receptors:**

As with the other retroviruses, the envelope region of FeLV is divided into two domains, SU and TM (Figure 1.2). Receptor recognition by murine leukemia virus (MLV) and FeLV is determined by the N-terminus of their SU domain (4,6,19,97). The FeLV SU domain consists of several conserved sequences that are interrupted by variable regions A and B (VRA and VRB) (correspond to variable region II and IV) (Figure 1.2) (58). VRA and VRB are highly divergent between MuLV and FeLV, both in length and in sequence.
VRA and VRB are often collectively referred to as the receptor binding domain (RBD). Most of the sequence diversities in VRA and VRB occur in regions that are enclosed by cysteine residues that are conserved in all mammalian type C envelope glycoproteins. As confirmed by X-ray crystallographic study of MLV envelope glycoprotein, these cysteine residues form disulfide-bonded loops, two loops within VRA and one loop in VRB (36). The major determinant for receptor specificity resides in the amino-terminal half of SU, and specifically within the first VRA (VRA1), although additional domains of SU, including VRB and a downstream proline-rich region (PRR), have been implicated as secondary determinants for some SU receptor interactions (4,6-9,19,97).

Interference and tropism studies have documented that FeLV-A, -B, and -C use distinct cellular receptor molecules to infect susceptible cells. The cell tropism of FeLV-A is distinct from those of FeLV-B, and -C. FeLV-B and -C are amphotropic infecting both feline and nonfeline cell lines and recognize membrane-spanning transporter cellular molecules as receptor. By contrast, FeLV-A is ecotropic infecting primarily feline cells and using a distinct but as yet undefined receptor (Table 1.1) (3,55,57,73,83,98,99).

Similar to gibbon ape leukemia virus (GALV), which uses Pit1 as receptor (72), and the amphotropic MuLV, which uses Pit2 as receptor (66,67), FeLV-B uses human, feline and hamster Pit1 and some FeLV-B variants use Pit2 but at lower efficiency (3,19,66,96,99). Pit1 and Pit2 molecules encode a multiple membrane-spanning phosphate transporter molecule (3,55,57,73,99).
The recombinant forms of FeLV-B differ in the amount of envelope SU that is derived from enFeLV, and this may affect whether or not the virus can also use human Pit2 as a receptor (19,99).

It has been shown that the N-terminus portion of VRA of FeLV-B SU is sufficient to confer Pit1 receptor specificity to FeLV-A (19). However, sequences in both VRB and downstream, in the C-terminal half of SU, are secondary determinants for Pit2 receptor specificity because chimeras encoding FeLV-B VRA and VRB infect cells expressing Human Pit2 and Hamster Pit2 more efficiently than chimeras encoding only the VRA of FeLV-B (19). A recent report showed that an arginine at position 73 within VRA of the FeLV-B is necessary for binding and receptor specificity for human Pit2 receptor (96). Interestingly, viruses containing additional FeLV-B sequences in the C-terminus of SU cannot recognize Human Pit2. This result implies that there is a determinant beyond VRB that negatively affects Human Pit2 interactions (19). In contrast, these C-terminal sequences do not play a role in determining feline Pit2 binding and specificity (3).

The newly recognized FeLV subgroup, FeLV-T, evolved from FeLV-A in infected cats but uses the Pit1-enFeLV envelope complex as its receptor (2). FeLV-T is the first example of an oncoretrovirus that uses a two-component receptor complex. The enFeLV envelope protein complex termed FELIX was identified as similar to truncated envelope protein coded for by the CFE-16 enFeLV (2). FeLV-T can repeatedly reinfect T lymphocytes and replicate to a high copy number. In addition, this variant can also superinfect cells that have been infected with the progenitor FeLV-A or FeLV-B (2).
The receptor for FeLV-C has been identified and designated Flvcr by using a human T-lymphocyte cDNA library (98). Flvcr is a glycoprotein and predicted to encode 12 regions that traverse the cell membrane. It is currently speculated that Flvcr functions as an organic anion transporter (83,98). Flvcr is expressed by multiple hematopoietic cells rather than specifically in erythroblasts (98). The FeLV-C determinant for host range has been mapped to the N-terminal portion of VRA (87 to 92 amino acid) (21).

**Endogenous FeLV sequences:**

The cellular DNA of domestic and related small feline species contains polymorphic enFeLV-related sequences (12,16). At least three distinct sets of endogenous retroviral sequences are in the cellular DNA of all domestic cats (102). The first of these is the xenotropic RD-114. There are 15-20 copies of RD-114 retroviral DNA per haploid genome of the feline cell (5,11,13-15,89,94,102). No known disease has been associated with RD-114 (76). Sequence comparisons have suggested that RD-114 was probably derived from cross-species transmissions (zoonoses) of retroviruses from primates to cats (103). A study by Haberman, et al., (42) characterized the *in vitro* infectivity of a limiting dilution derived biological clone with envelope properties of both FeLV and RD-114, however, no definitive molecular study for the proof of the recombinant nature of this biological clone. The second set of endogenous feline retroviruses are replication-defective proviruses related to MAC-1, an endogenous primate retrovirus that can not be induced to replicate in feline cells (18,102). The third family of endogenous feline retroviruses is related to FeLV (enFeLV).
The enFeLV sequences are dispersed throughout the feline genome and expressed as subgenomic transcripts in a tissue-specific manner, but are not inducible as infectious virus particles (12,16,22,69). enFeLV family contains approximately 15 copies per haploid genome arranged as proviruses that generally retain LTR sequences (92,93). However, the enFeLV LTRs are shorter than the LTRs of the exogenous FeLV and can be distinguished from the exogenous FeLV in the unique 3' (U3) region (23,93). In spite of the truncation in the enFeLV U3 region, transcription regulatory studies show the presence of promoter and enhancer function, but variable transcriptional activity due to the influence of negative cis-acting cellular DNA sequences upstream of several loci (17). The enFeLV RNA transcripts retain the primer binding site, packaging signal, leader sequences and open reading frames of pol and env gene (17,58,77). The enFeLVs are known to contribute to the evolution of the exogenous FeLV-B and C through recombination with FeLV-A.

There are two molecular clones derived from enFeLV sequences, CFE-6 and CFE-16 that have been sequenced and molecularly characterized (93). CFE-16 is truncated within the env while the CFE-6 has the entire FeLV-like genome with scattered stop codons in the gag and pol regions (93). The enFeLV RNA transcripts and truncated envelope protein have been detected in tumor cell lines and feline tissues as well as in the feline cell lines, 3201 and MCC cells (65,69-71). The truncated 35 Kda envelope protein produced from CFE-16 was found to occupy the FeLV-B receptor, thus blocking FeLV-B entry into these cells (65). A study by Pandey et al. (1991) showed that cotransfection of the CFE-6 proviral clone with replication-competent FeLV-C/Sarma (pFSC) gave rise to an infectious recombinant virus capable of growing
on human HT1080 cells (a hallmark of FeLV-B), while CFE-16 did not (77). Interestingly, northern blot analysis detected the expression of CFE-16, but not CFE-6, in lymphoma cell lines and in a variety of primary tissues, including lymphoid tissues from healthy specific-pathogen-free cats (65). This result might indicate the presence of negative cis-acting sequences preventing the expression of CFE-6 in feline cells (17).

Recombination and the role of enFeLV in the FeLV pathogenesis:

Natural retrovirus populations are known to have a high degree of genetic diversity as a result of error-prone reverse transcription and recombination generating a pool of heterogenous but closely related genomes, called quasispecies (30,56,63). This diversity in retroviral genes plays critical roles in retroviral carcinogenesis and in the AIDS epidemic. In their life cycle, retroviruses package two copies of viral RNA into each virion (34,59,60). During reverse transcription, both copackaged RNAs can be used as templates to produce a recombinant with a mixture of genetic information (28,29,50,51) as a result of template switching, minus- and plus-strand DNA transfer (40,41). Because these obligatory template-switching events for the completion of virus replication, it has been hypothesized that retroviral reverse transcriptase (RT) has evolutionarily selected to have a low affinity to the template and low processivity (101). The efficiency of recombination depends on the length of the sequence identity between the two copackaged RNAs (105). Intermolecular template switching events during reverse transcriptase between copackaged RNAs can result in homologous and nonhomologous recombination (49,56,100). However, intramolecular template-switching events (within the same template) may result in deletional, insertional, or
duplicational mutations (78,79). The formation of 3’ proto-oncogene-virus junctions of highly oncogenic retroviruses involves such nonhomologous recombination (104).

In addition, a recent report demonstrated that retroviral recombination is temperature dependent and the rate of recombination decreases as temperature is raised from 31 to 43 °C (62).

There is solid evidence to indicate that interactions between exogenous FeLV and enFeLV elements generate recombinant viral quasispecies which represent a variety of chimeric envelope glycoproteins depending on the extent of amino terminal portion replaced by the endogenous envelope sequences (10,58,75,77,81,91). First, FeLV recombinants (FeLV-B) are not present without FeLV-A in naturally infected cats (52,53,88,89). Second, a region of the FeLV-B envelope gene is homologous to murine mink cell focus-forming virus that was acquired via recombination with an endogenous murine virus (35). Third, there is a high degree of homology between the envelope gene of FeLV-B and enFeLV (58,95). Fourth, viruses with properties of FeLV-B were detected following passage of FeLV-A in feline fibroblast cells (FEA), demonstrating that FeLV-B can evolve from FeLV-A (75). Finally, cross over sites between enFeLV and FeLV-A, as well as specific adaptive amino acid mutations, are rapidly selected for replication efficiency and are over represented at later time points after infection (10,25,77,81).

In the FeLV system, the copackaging of the enFeLV RNA and the exogenous viral RNA is one essential requirement for the generation of rFeLV variants via recombination (Figure 1.3). This process begins when FeLV-A infects target cells that
express enFeLV RNA. A fraction of the virion produced from these cells will contain one copy of FeLV-A RNA and one copy of enFeLV RNA.

FeLV-B is thought to be derived through nonhomologous recombination of exogenous FeLV-A sequences with enFeLV sequences during reverse transcription (20,58,75,77,87,90). This recombination occurs in the envelope region, particularly in the SU region. The process of recombination in combination with mutations and in vivo selection produces subgroup B FeLV (10,76). The finding that arginine at position 73 (in variable region A in the env, VRA) is required for the determinant of Pit2 by FeLV-B indicated that recombination and point mutations are needed because this arginine was not found in either the parent FeLV-A nor the endogenous sequences (3). The sites of crossing over between the exogenous FeLV and the enFeLV have been determined and located within 250 base pair in the middle of SU gene (91). These cross over sites were designated A through G, where A has the shortest enFeLV sequences and G has the longest enFeLV sequences starting from the N-terminus of the SU domain of enFeLV. When in vivo-generated FeLV recombinants were examined for the cross over sites within the SU region of the envelope gene, recombinants with relatively greater amounts of enFeLV-derived N-terminal SU substitutions (those with 3' cross over sites of E, F, G, and >G) were generally the predominant species observed at later time point during the course of infection (10,25). This reinforces the idea that recombinants with more endogenously derived SU sequence may have an in vivo selective advantage (76).

Although there is solid evidence for the generation of FeLV-B through recombination of exogenous FeLV with one or more of the estimated 15 enFeLV species, the major enFeLV species that are involved in this recombination have not been
determined. It was shown that the highly transcribed enFeLV (CFE-16) was not the only source of FeLV-B recombinants (65, 77).

However, because of the presence of several polymorphic amino acid positions within the enFeLV-derived domains of FeLV-B which are shared by the exogenous viruses but differ from the known enFeLV sequences, the role of mutations can not be ignored (65).

The origin of FeLV-C is less clear, but may also involve a similar process of recombination with enFeLV sequences in conjunction with mutations (52, 58, 68, 85, 86, 87). In addition, among the envelope variable regions that discriminate FeLV subgroups, variable region VI (downstream of VRB) of CFE-6 near the C-terminus of SU exhibits a considerably homology to FeLV-C than FeLV-B (58, 87).

An obvious advantage for FeLV-A recombination with enFeLV is the formation of recombinant SU (FeLV-B phenotype), which would allow the use of different host cell receptors, thus permitting increased replication and the possibility of superinfection (56, 87). In this respect, the evolution of FeLV subgroups in their natural host and in tissue culture provides a good model to study the role of retrovirus recombination in disease pathogenesis. In fact, FeLV in cats has been widely used as a model to study population dynamics in host-parasite interactions (37).

The difference in the rate of recombination between enFeLV and FeLV-A has been found to play a key role in FeLV pathogenesis (81). Differential pathogenicity study of two FeLV-A molecular clones, FRA and F6A, revealed that FRA has a more rapid onset of lymphoma and related diseases (81). Molecular analysis of the FeLV provirus in cats demonstrated that pFRA was more recombinogenic than pF6A in vivo.
F6A FeLV-A was molecularly cloned from the small intestine of cat with feline immunodeficiency syndrome and the FRA FeLV-A was molecularly cloned from the thymic tumor tissue of cat which was co-challenged with FeLV-A Rickard plasma preparation and a mixture of in vitro-generated recombinants FeLV (25,75). The comparison of the nucleotide sequence of FRA (8448bp) and F6A (8446bp) revealed an overall 98% homology.

The distinctively different pathogenic properties of two otherwise genetically similar molecular clones of FeLV presented the opportunity to identify factors contributing to the different endogenous/exogenous recombination frequencies of retroviruses. The following section describes the objectives and rationales for the different studies in this dissertation.

Objectives of this dissertation:

First objective (Chapter 2):

The first aim of this dissertation was to answer the following questions: Can the in vivo variation in the recombination rate of FRA and F6A with enFeLV sequences be repeated in a tissue culture system in vitro? Is the variation in the recombination rate of FRA and F6A with enFeLV sequences cell dependent? Can we identify the factors that contribute to the variation in the recombination rate of FRA and F6A with enFeLV sequences?

To address these questions, we have stated the following hypothesis: pFRA has a higher recombination rate than pF6A with the enFeLV env sequences and this variation in recombination rate is based on the level of enFeLV RNA expression. To
approach this hypothesis, the first objective was to design and utilize an *in vitro* tissue culture system to examine the variation in recombination rate of pF6A and pFRA with the enFeLV *env* sequences. We used two different feline cell lines, fibroblast cells (FEA) and lymphoid cells (3201). The importance of using the *in vitro* system is to mimic FeLV infection *in vivo* and to examine the role of the different types of feline cell lines in the infection process independent of multiple variables difficult to analyze *in vivo*. In addition, the *in vitro* system was used to extend our understanding of the factors contributing to FeLV recombination by evaluating the host cell/virus interactions of FeLV-FRA and FeLV-F6A. For this purpose, the F6A- and FRA-FeLV molecular clones were used to transfect the lymphoid and fibroblast cell lines representing the target cells *in vivo*, and the cultures were monitored for virus kinetics and recombinant production as well as the expression of enFeLV RNA.

**Second objective (Chapter 3):**

The second objective of this dissertation was to test the hypothesis that variation in recombination of the FRA and F6A with enFeLV sequences is caused by the variation in the *pol* or the LTR regions between these two viruses.

To determine the role of *pol* region in recombination variation between pF6A and pFRA, 3201 cells were transfected with pF6A, pFRA or the chimeras pF6A/FRA-pol (pF6A with the pFRA *pol* region) or pFRA/F6A-pol (pFRA with the pF6A *pol*). Progeny viruses from these cells were assayed for recombinant FeLV. To study the role of the LTR, stable pZeo.F6A.LTR or pZeo.FRA.LTR transfected 3201 cells were generated and their enFeLV RNA expression was determined and compared with non-transfected 3201 cells using real time quantitative RT-PCR.
Third objective (Chapter 4):

The objective of Chapter 4 was to use GFP tagged FeLV-A to evaluate the genomic stability of FeLV in vitro and in vivo. The following hypothesis was tested: Genomic stability of pFRA-GFP construct is cell dependent relying on the level of enFeLV RNA expression in the infected cells.

Studying the stability of pFRA-GFP could be useful to follow a natural FeLV infection or to design a similar vector with other genes such as drug gene to be used in gene therapy.

Fourth objective (Chapter 5):

The enFeLV sequences have essential roles in the FeLV pathogenesis through recombination and the expression of their envelope proteins, however, the in vivo and the in vitro expression and distribution of enFeLV sequences have not been fully described. In Chapter 4 we have explored the genetic stability of pFRA-GFP in the feline cell lines and in vivo and the possible role of enFeLV expression on that stability.

To complete our studies on FeLV genomic stability and its linkage to enFeLV expression, in Chapter 5 we conducted quantitative measure of enFeLV expression in feline cell lines and in normal feline tissues. The hypothesis to be tested was: Different tissues as well as different feline cell lines may have different level of enFeLV RNA expression which may contribute to the variation in genetic stability of FeLV in different cell types in vitro and in vivo.
To test this hypothesis, total RNA was isolated from three feline cell lines and from different tissues of 6 healthy cats and quantitative real-time RT-PCR was conducted to quantify the expression of enFeLV RNA.
REFERENCES


55. **Johann, S. V., J. J. Gibbons, and B. O'Hara.** 1992. GLVR1, a receptor for gibbon ape leukemia virus, is homologous to a phosphate permease of Neurospora crassa and is expressed at high levels in the brain and thymus. J. Virol. **66:**1635-1640.


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103. van der Kuyl, A. C., J. T. Dekker, and J. Goudsmit. 1999. Discovery of a new endogenous type C retrovirus (FcEV) in cats: evidence for RD-114 being an FcEV(Gag-Pol)/baboon endogenous virus BaEV(Env) recombinant. J. Virol. 73:7994-8002.


### Table 1.1: FeLV cell tropism.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell tropism</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeLV-A</td>
<td>Feline cells (3201 and FEA cell lines)</td>
<td>?</td>
</tr>
<tr>
<td>FeLV-B</td>
<td>Feline, fibroblast cell lines.</td>
<td>Phosphate transporter protein, pit1 and pit2.</td>
</tr>
<tr>
<td></td>
<td>Human fibroblast cell line, HT1080.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D17, dog osteosarcoma cell line.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mink lung fibroblast cell line, MFL.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hamster, baby hamster kidney cell line, BHK.</td>
<td></td>
</tr>
<tr>
<td>FeLV-C</td>
<td>Feline, fibroblast and lymphoid cells.</td>
<td>A member of the major-facilitator superfamily of transporter (organic anion transporter?)</td>
</tr>
<tr>
<td></td>
<td>Human fibroblast cell line, HT1080.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D17, dog osteosarcoma cell line.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mink lung fibroblast cell line, MFL.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Guinea pig, fibroblast cell line (GP104).</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.1: The time frame for the in vivo generation of FeLV-B and -C after the FeLV-A DNA inoculation. This time frame is based on the study by Phipps, et al. 2000 using interference assay.
Figure 1.2: Genetic organization of FeLV genome. The solid line represents the organization of the genome with Rectangles represent precursor proteins. The LTRs are also shown, gray is the U3, black R, and uncolored is U5. The variable regions A (VRA) and B (VRB) and the proline rich region (PRR) of the SU domains are shown.
Figure 13: The diagram depicts the hypothetical generation of FeLV-B and pseudotyped FeLV viruses. Step 1: Infection of a naïve target cells by FeLV-A virus may result in the generation of heterogenous FeLV copackaging FeLV-A and enFeLV genome. Step 2: Sequential infection of naïve cell with the heterogenous FeLV virion may result in recombinant FeLV and/or pseudotyped FeLV. The hypothesized mechanism of the recombination between FeLV-A and enFeLV RNA in heterogenous FeLV virion by RT is shown.
Figure 1.3

Reverse Transcription

FeLV-A

Endogenous

FeLV-B envelope

Recombination as a result of reverse transcriptase

FeLV-A genome

pseudotyping

FeLV-B genome

FeLV-B envelope

FeLV recombinant RNA

Cell

FeLV-A

enFeLV DNA

Step 1

copackaging

Step 2

FeLV-A

FeLV-A

FeLV-A
CHAPTER 2

The Variation in Recombinogenic Activity of Two Feline Leukemia Virus Subgroup A Molecular clones, F6A and FRA Is Cell Dependent

Retroviruses assure their survival by rapidly adapting to host variation through genetic mutation and recombination, and through the use of alternative envelope proteins (pseudotyping). The combination of error-prone reverse transcription and genomic recombination generates a pool of heterogeneous but closely related virions with a high degree of genetic diversity called quasispecies (4,11,15). Pseudotype virion adds to this diversity by offering expanded cell tropism and the capacity to superinfect cells.

In a previous study (18) two closely related molecular clones of FeLV-A, FRA (2) and F6A (16), were shown to have significantly different pathogenesis in young cats. Compared to F6A, FRA caused a more rapid onset of lymphoma or non-regenerative anemia, and had a higher propensity to convert to the FeLV subgroup B genotype presumably through recombination with enFeLV. The distinctively different pathogenic properties of two otherwise genetically similar molecular clones of FeLV presented the opportunity to identify factors contributing to the different endogenous/exogenous recombination frequencies of these viruses. For this purpose, FEA cells (feline embryonic fibroblast) and 3201 cells (feline lymphoid) were transfected with pFRA or pF6A and progeny virus was tested for exogenous and endogenous as well as the recombinant
genotype by RT-PCR and phenotype by growth on HT1080 cells (human fibroblastic). These viruses were also tested for the propensity to form pseudotyped virions that might enhance virus spread in vivo. The results of this study demonstrated that the higher recombinogenic activity of FRA compared to F6A correlates with enhanced packaging efficiency of enFeLV RNA by FRA virion.
MATERIALS AND METHODS

Feline cell transfection and sample collections:

The feline lymphoid cell line 3201 was propagated in a 1:1 (vol/vol) mixture of RPMI 1640 and Leibowitz L-15 medium containing 20% fetal bovine serum (FBS). Feline embryonic fibroblast cells (FEA) was maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS. The medium of 3201 and FEA cells were supplemented with 100 U of penicillin per ml, 100 ug of streptomycin per ml, and 2 mM L-glutamine. The molecular clone FeLV-FRA was derived from a Rickard strain FeLV-induced tumor and molecularly cloned by Chen et. al (2). FeLV-F6A was isolated from the small intestine of a cat infected with a wild type variant of FeLV and molecularly cloned by Overbaugh et. al (16). The 3201 cells (5x10^6 cells) and the FEA cells (1x10^6 cells) were transfected with 25 ug of pFRA or pF6A using electroporation (960uF/250V). Transfection efficiency was determined in parallel using the pEGFP-N1 plasmid (Clontech, Palo Alto, CA) coding for green fluorescent protein. Transfected cells were examined 24 hours after electroporation for GFP expression by fluorescence microscopy. 3201 and FEA cultures were passed weekly at 2x10^6 cell/ml and kept for 28 days at 37°C and 5% CO2 incubation. Tissue culture fluid (TCF) samples were collected and passed through 0.45 uM membrane filters at 7 day intervals for each culture, and tested for the expression of viral antigen (p27) using antigen capture enzyme-linked immunosorbent assay (Petchek FeLV ELISA; Synbiotics, San Diego, Calif).
Optical density (OD) readings were normalized against kit standards using the following formula: relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control.

Inoculation of HT1080 cells:

The human fibrosarcoma cell line HT1080 was cultured in alpha MEM supplemented with 15% FBS, 100 U of penicillin per ml, 100 ug of streptomycin per ml, and 2 mM L-glutamine. The HT1080 cells were inoculated with the TCF filtrate of the transfected FEA and 3201 cells as the indicator cell for detection of recombinant FeLV. Briefly, HT1080 cells were seeded into 24-well plates (1x10^4 cell/ml) the day before infection. On the day of infection, HT1080 cells were treated with polybrene for 30 minutes prior to infection (13). The cells were then washed and incubated for 3 hours with the virus at 200 ul of TCF per well. Fresh medium was added (0.8 ml) and the cells were incubated at 37°C, 5% CO₂ for 24 hours. Thereafter, the medium was replaced and the inoculated HT1080 cells were passed every three or four days for four weeks. Every seven days, supernatants from these cells were collected and tested for the presence of p27 viral antigen using ELISA kit and the OD readings were normalized according to the formula described above.

Virus infectivity:

The 81C focus-forming assay was used to titrate FeLV produced by 3201 cells transfected with FRA or F6A, as previously described (6). The percentage of FeLV-positive FEA or 3201 cells was determined by indirect immunofluorescence staining (18)
using the monoclonal anti-FeLV envelope C11D8 as the primary reagent (CMI, Sacramento, CA). C11D8 has been shown to react with all subgroups of FeLV (9). The positive and negative controls for this assay were FeLV-infected and uninfected FEA or 3201 cells, respectively. Briefly, 2X10^6 of the transfected FEA or 3201 cells were collected at day 7, 14, 21, and 28 post-infection. The cells were washed twice with phosphate buffer saline supplemented with 2 ug/ml bovine serum albumin and 2 ug/ml sodium azide (PBS) and incubated with C11D8 at 37°C for 45 minutes. The cells were then washed 3 times with PBS and the secondary antibody (FITC-labeled anti mouse IgG, Sigma, St. Louis, MO) was added and incubated at 37°C for 45 minutes. The cells were washed 3 times, resuspended in PBS, fixed with 2% paraformaldehyde and analyzed by flow cytometry.

**PCR analysis:**

Primer pairs for the various PCR amplicons are shown in Table 2.1 and their positions in the FeLV genomes are illustrated in Figure 2.1. The genomic DNA isolation and nested PCR were described previously (2). Briefly, genomic DNA was extracted from the inoculated HT1080 cell line at day 28 post-inoculation using a tissue genomic DNA isolation kit (Qiagen, Santa Clarita, CA). The isolated DNA was analyzed by nested PCR. In the first round of amplification, 250 ng of genomic DNA was amplified (30 cycles) using the following primer set: H18, the 5' sense primer, corresponding to F6A sequence 5840 to 5860 at the pol/env junction which is conserved among all exogenous FeLVs; and H20, the 3' antisense primer, complementary to the exogenous U3 sequence in the LTR (F6A 8210 to 8189) (2). To detect the recombinant proviral env
sequences, a 1 ul volume of PCR product from the first round of amplification was used in a second-round amplification for another 30 cycles using the endogenous-specific 5’ sense primer RB56 which is identical to the nucleotide position (210 to 229) indicated in the published CFE-6 sequence (14,20) and the FeLV-A-specific 3’ antisense primer RB17 complementary to the published F6A sequence (7310 to 7330) (5) and FRA sequence (2). To detect the FeLV-A sequences, the FeLV-A-specific 5’ primer RB59 identical to the published sequence of F6A (6259 to 6278) and the 3’ antisense primer RB17 were used in a second-round in amplification with 1 ul of the H20/H18 product as template. The molecular clones F6A and FRA, the biological clone FeLV-IB3 (19), the non-infected HT1080 DNA and ddH20 were used as controls. Nested PCR products were analyzed by electrophoresis on 2% agarose stained with ethidium bromide.

RNA extraction:

At different culture time points, 7, 14, 21 and 28 days post-transfection, total cellular RNA and viral RNA were isolated from a known number of transfected and non-transfected 3201 cells as well as the TCF filtrate from transfected 3201 cells, respectively, using Trizol reagent (Gibco BRL). Twenty-milliliters of the supernatants were ultracentrifuged at 25,000xG for 150 minutes at 4°C then the viral RNA was extracted by Trizol. RNA was treated with 4 U RNase-Free DNase (Ambion, Austin, TX), extracted with phenol-chloroform, precipitated with isopropanol at – 80°C and then washed twice in 75% ethanol. Total RNA was resuspended in DEPC water. The RNA concentration was determined by the absorbance at 260 nm and tested for DNA contamination by 40 cycles RT-PCR in the absence of RT using the upstream primer env-
1 and the downstream primer env-4, which can detect both exogenous and endogenous viral DNA. A known copy number of GFP transcript (12) was added to each supernatant sample to determine the efficiency of viral RNA extraction of each sample.

Quantification of exogenous and endogenous viral RNA expression by RT-PCR:

The exogenous and the endogenous viral RNA copy number were determined by quantitative real-time RT-PCR. The exogenous-specific primers, U3exo sense (2174-2188), accession no. M12500, (22), and the U3exo' antisense (2228-2247), accession no. M12500 (22), were used in this experiment to amplify the exogenous U3 region (10). The RT step was performed with 2 ul of either the extracted viral or cellular RNA at 55°C for one hour using 1 uM U3exo' antisense primer according to the manufacturer's conditions (Gibco BRL). Two microliters of the RT product were added to the PCR reaction in a total volume of 20 ul containing 0.225 uM for each primer and 2 mM Mg and 2 ul of the supplemented reaction mix, FastStart DNA master and Sybr Green I (Roche, Indianapolis, IN). Each cycle consisted of: 95°C denaturation for 5 seconds, 67°C annealing for 10 seconds, and 74°C extension for 10 seconds for 40 cycles in a LightCycler thermal cycler (Roche, Indianapolis, IN). Fluorescence data was acquired at the annealing step and melting curve analysis was performed.

The expression of enFeLV RNA in transfected and control 3201 cultures and the packaged enFeLV RNA were quantified using QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA). The primers used as specific oligonucleotide in the amplification of the enFeLV-U3 region were P20 and P6 (Table 2.1 and Figure 2.1) (3,7,8). Each RT-PCR reaction contained 1 uM of each primer, 0.2 ul QuantiTect RT mix, 10 ul of 2x
QuantiTect SYBR Green RT-PCR master mix, water to a final volume of 20 and 0.5 ug cellular RNA or 2 ul volume of the viral RNA or of the standard RNA transcript. The reaction was run at 50°C for 30 minutes and at 95°C for 15 minutes. The PCR consists of 30 cycles at 94°C for 15 seconds, 57°C for 15 seconds, 72°C for 15 seconds and 74°C for 5 seconds. Fluorescence data was acquired at the annealing step and melting curve analysis was performed.

The RNA samples were adjusted to approximately the same concentration for real-time RT-PCR. The internal control for the cellular RNA samples was based on the copy number of GAPDH RNA gene determined by a separate quantitative real time RT-PCR assays using cat specific GAPDH primers (Table 2.1). The final viral RNA copy number from the real-time PCR assays was adjusted for efficiency of RNA extraction based on the percent recovery of the GFP RNA internal control assayed in parallel real-time RT-PCR assays.

RNA standards for the various real-time RT-PCR assays were in vitro transcribed from DNA cloned into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions (Ambion, Austin, TX). The DNA inserts were derived by RT-PCR of RNA from uninfected 3201 cells for the primer set 7/8 (enFeLV-specific) and 14/15 (GAPDH specific primers) (Table 2.1). The DNA insert for primer set 10/11 (exogenous FeLV-specific) and primer set 16/17 (GFP-specific) were derived by PCR from pF6A and pEGFP-N1, respectively (Table 2.1).
RT-PCR amplification and sequence analysis of env and U3 from the packaged enFeLV RNA and the 3201 cellular RNA:

The U3 and env region of the enFeLV RNA in the virion of the F6A- and FRA-transfected 3201 cells and control 3201 cells were reverse transcribed and amplified using enFeLV specific primers, RB53 and U3.end (Table 2.1 and Figure 2.1). The RT step was performed with 2 ul of either the extracted viral or 3201 cellular RNA at 55°C for one hour using 1 uM U3.end antisense primer in a total volume of 10 ul according to the manufacturer’s conditions (Gibco BRL). Five microliter of each RT reaction was added to 50 ul PCR reaction. The PCR reaction contained 1x PCR buffer, 2 mM MgCl₂, 200 uM dNTPs, 0.4 uM of each primer and 1 U Platinum Tag DNA Polymerase (Gibco BRL). The RT-PCR products were cloned into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions (Ambion, Austin, TX) and utilized in automated DNA sequencing reactions with the forward and reverse primers of pCR2.1 Topo vector.

Statistical Analysis:

Statistical analyses of the comparative results were performed using student’s T test or the Mann-Whitney test. P values of <0.05 were considered statistically significant.
RESULTS

FeLV F6A and FRA are equally infectious to lymphoid and fibroblast cells and produce similar virus in cells and tissue culture fluids:

Variations in the rate of retrovirus recombination may be due to the infectious titer of the challenge virus. High titer infectious viruses offer fewer opportunities to copackage heterologous template and to recombine because the available cell population is rapidly infected therefore limiting the extent of subsequent rounds of infection needed to propagate recombinants. In this first study we evaluate the infectivity of the two molecular clones of FeLV subgroup A, F6A and FRA, for viral expression and infection efficiency. Molecular clones of each virus were transfected into 3201 and FEA cells by electroporation. Transfection efficiencies, as determined by parallel transfection with pEGFP, ranged between 9.6 and 11% for the three replicate studies. Cells and TCF filtrate samples were collected weekly for 4 weeks. Virus expression in TCF of the transfected 3201 and FEA cells was measured by FeLV p27 ELISA. The TCF of the transfected 3201 cells was analyzed by viral infectivity titration. In addition, flow cytometric analysis was used to determine the percentage of cells positive for FeLV ENV antigen.

No significant difference of p27 antigen expression in TCF was found between F6A and FRA of 3201 (Figure 2.2A) or FEA cells (Figure 2.2B), although F6A consistently produced higher p27 values than FRA in 3201 cells.

The number of infected 3201 cells, as determined by indirect immunofluorescence staining, was significantly different at day 28 between F6A and FRA (P value= 0.02)
suggesting that FRA spread more rapidly to naïve 3201 cells than F6A (Figure 2.3A). However, when FEA cells were used as target cells for infection, no difference in the number of infected cells was found between F6A and FRA (Figure 2.3B).

Analysis of the exogenous FeLV RNA copy number present in the FRA and F6A infected 3201 cells and in TCF of the infected 3201 cells (progeny virus) were determined by real time RT-PCR for material collected at 7, 14, 21 and 28 days post infection. Cells were counted, the numbers of positive cells were determined by immunofluorescence staining, and the supernatants were analyzed for FeLV p27 antigen by ELISA. To control for the efficiency of the extraction of the virion RNA, a known number of GFP RNA was added to the virions pellets. The quantitative RT-PCR was conducted for both, the exogenous FeLV RNA and the GFP RNA.

From these analyses, no difference in exogenous FeLV RNA copy number was found for either infected cell or TCF for the two molecular clones (P value= 0.68 and 0.944 respectively) (Table 2.2, columns 6 and 8).

**FRA virions contain significantly greater endogenous RNA than F6A:**

Because FRA has an apparent increase in recombinogenic capacity compared to F6A but both viruses were equally infectious in 3201 cells, we next determined if the increase in FeLV recombinants was related to changes in the expression of enFeLV RNA in the FeLV-infected 3201 cells. To do so, total RNA isolated from 7, 14, 21 and 28 day cultures of F6A- or FRA-infected 3201 cells and non-infected 3201 cells was tested for the copy number of enFeLV RNA using real time quantitative RT-PCR. As an internal control for RNA loading, real time quantitative RT-PCR for the GAPDH gene was run in
parallel. The results of 3 replicate assays showed no increase in cellular enFeLV copy number for either FRA or F6A producer cell types compared to uninfected 3201 cells (P value=0.3 and 0.28, respectively) (Table 2.2, column 5).

Next we determined the enFeLV RNA copy number of progeny virus from FRA- and F6A-transfected 3201 cells using real time quantitative RT-PCR. Viral RNA was prepared from the pelleted virions in supernatants of F6A- or FRA-transfected 3201 cells at day 7, 14, 21 and 28 post-transfection. As an internal control for the efficiency of viral RNA extraction, a known copy number of GFP RNA was added to the pelleted virions.

When the ratio of enFeLV RNA to exogenous FeLV RNA were compared for the two FeLV molecular clones, virion from FRA-producer cells were found to have significantly (P value= 0.0043) more endogenous viral RNA than that of the virion from F6A-producer cells (Table 2.2, column 7, 9 and 10).

A truncated enFeLV RNA is expressed into 3201 cells and packaged into the exogenous FeLV virion:

The cross over sites between exogenous FeLV and enFeLV during recombination that produce viable virus have been studied and found to occur in the SU gene (14,20,21). However, this finding does not rule out the possibility that recombination in FeLV genome can occur in the LTR region resulting in recombinant FeLV with enFeLV LTR. If this recombination scenario occurs, the enFeLV quantitative RT-PCR using U3 enFeLV specific primers might also detect the recombinants with enFeLV U3. To study this phenomenon, we conducted enFeLV specific RT-PCR to amplify and sequence the enFeLV env and U3 region from the F6A and FRA virion and control 3201 cells. The
RT-PCR products of the F6A and FRA virion and the control 3201 cells (Figure 2.4) were sequenced (Figure 2.5) and found to be identical to the published sequence of the enFeLV molecular clone, CFE-16 (0.5 Kb) (14,20,21). Neither the full length (1.7 Kb) enFeLV RNA (CFE-6) nor recombinants FeLV with endogenous U3 was detected using this approach (Figure 2.4). These results suggest that virion RNA containing enFeLV U3 are RNAs from enFeLV rather than recombinant containing the enFeLV LTR and exogenous SU region.

FRA has a higher recombination rate than F6A after the transfection into 3201 cells:

To determine if the high packaging frequency of enFeLV RNA into progeny virion from 3201 cells lead to recombination and the production of recombinant virus, we took advantage of the cell tropism of FeLV-A compared to recombinant FeLV. For this purpose, two cell lines with virus restrictive properties, 3201 and HT1080 were used. The 3201 cell line is selectively supportive of infection by FeLV-A but is resistant to infection by FeLV recombinants. By contrast, HT1080 cells selectively support infection by recombinant FeLV (FeLV-B) but are not normally infectable by FeLV-A. TCF of F6A- or FRA-transfected 3201 cells were collected at 7, 14, 21, and 28 days post infection, filtered, and inoculated into naive HT1080 cell cultures. Pretesting of the 3201-derived FRA and F6A inocula by ELISA and virus infectivity assays determined approximately equal virus titers (Tables 2.3 and Figure 2.2A). The supernatants from the inoculated HT1080 cells (HTCF) were analyzed over a period of four weeks by ELISA for the expression of FeLV p27 antigen.
Based on virus p27 production by HT1080 cells, both F6A and FRA from 3201 cells were capable of producing virus infectious to HT1080 cells, however, FRA-transfected 3201 cells produced HT1080-permissive virus as early as day 7 post inoculation (Figure 2.6A), while HT1080-permissive virus was not detected in the F6A-transfected 3201 cells until days 21 post infection (Figure 2.6C). In addition, HT1080-permissive virus from FRA-transfected 3201 cells produced significantly more p27 antigen (Figure 2.6) than HT1080-permissive virus from F6A-transfected 3201 cells (p = <.05 as indicated in Figure 2.6).

**FRA and F6A have the same recombination potential after the transfection into FEA cells:**

An additional study was carried out to determine if the FeLV subgroup A variants could produce recombination FeLV in FEA cells. As was done with the 3201 cells, FEA cells were transfected with the molecular clones of F6A or FRA. The FEA transfected cells were kept for 28 days. At days, 7, 14, 21 and 28, the TCF of the transfected FEA cells were analyzed for FeLV p27 antigen by ELISA, collected, filtered and used to inoculate the HT1080 cells. The HTCF was analyzed for the presence of FeLV p27 antigen by ELISA assay.

The inoculation of the HT1080 cells in this experiment showed no difference in the recombination rate between F6A and FRA (Figure 2.7). In addition, HT1080 permissive virus isolated from FRA-transfected FEA cells produced the same amount of p27 antigen as the HT1080 permissive virus isolated from F6A-transfected FEA cells (Figure 2.7).
HT1080 cells were infected by both recombinant and pseudotype FeLV from the transfected 3201 and FEA cells.

To address the possibility that FeLV pseudotype virus, containing FeLV-A genomes but enFeLV envelopes (FeLV-B phenotype), may be able to infect HT1080 cells producing positive p27 readings, DNA from infected HT1080 cells was analyzed by nested PCR for FeLV-A and FeLV-recombinant (FeLV-B) specific sequences. As controls, the nested PCR products from the molecular clones of F6A and FRA, and the biological clone FeLV-IB3 (subgroup B) were used in parallel. The FeLV-recombinant-specific primer pair, RB56/RB17, produced the expected fragment size of 1.2KB from DNA isolated from HT1080 cells previously inoculated by the TCF from both FRA and F6A transfected 3201 (Figure 2.8A) or FEA cells (Figure 2.9A). However, HT1080 DNA from both inocula also harbored FeLV-A specific sequences, as determined by PCR (Figure 2.8B and 2.9B). We concluded that pseudotyped viruses containing FeLV-A genomes and FeLV-B envelopes mediated the FeLV-A infection into the HT1080 cells because HT1080 cells are not normally infected by FeLV-A.
DISCUSSION

FeLV subgroup A-infected feline cells can give rise to genetic variants as a result of recombination between ecotropic FeLV-A and enFeLV-derived env sequences (14,20,21). In an in vivo study in which cats were infected with two closely related molecular clones of FeLV-A, FRA and F6A, we discovered that FRA was substantially more pathogenic and more prone to produce FeLV subgroup B variants presumably as a result of recombination with enFeLV than F6A (18). The comparison of the nucleotide sequence of FRA (8448bp) and F6A (8446bp) revealed an overall 98% homology (2). This distinct difference in recombination in such closely related viruses provided the opportunity to identify factors that contributed to enhanced recombination frequency of FRA.

The purpose of the current study was to develop an understanding of the role of the host cell-virus interaction that promotes FeLV recombination. The host cell is key to FeLV recombination because it provides the enFeLV template needed for RNA copackaging and subsequent RT template switching leading to recombination. The permissiveness of host cell to virus infection is equally important in regulating the frequency of the second-round infection cycle necessary for recombinants to emerge. Therefore, our first studies were directed at gauging the permissiveness of FEA cells to transfection and infection by the two molecular clones of FeLV. Based on p27 antigen production and the percentage of cells infected, we concluded that FEA cells were equally permissive to the two virus variants. Next, we determined the infection properties of the two molecular clones in 3201 cells. The analysis of p27 antigen production, virus
titer, the percentage of cells infected, and virus RNA copy number showed that 3201 cells were equally permissive to the two virus variants. However, the pF6A transfected 3201 cells were shown to have consistently higher mean virus p27 antigen load than the pFRA throughout the study period. The differential pathogenicity studies in cats infected with the same molecular clones also showed equivalent infectivity and approximately equal virus plasma load (18).

Because FRA was more prone to recombine with enFeLV than F6A in the 3201 cells, we next evaluated the co-packaging of FeLV/enFeLV heterotemplates by 3201 cells infected with each of the two molecular clones. For these studies, LTR specific primers were used to distinguish between exogenous FeLV-A and enFeLV RNA. The virion from FeLV-FRA-infected 3201 cells was found to package more enFeLV RNA than that from F6A infected cells (Table 2.2, column 7, 9 and 10). It has been reported that the U3-LTR region in MuLV and FeLV systems exhibits cellular gene transactivational activity via a specific RNA transcript generated from the U3-LTR region (3,7,8). Because of this transactivational activity of the U3-LTR region, we were interested in testing if the high level of enFeLV RNA in the FRA virion was due to upregulation of enFeLV RNA expression in the FRA-transfected 3201 cells. Unexpectedly, the FRA-transfected 3201 cells were found to have less enFeLV RNA than the control 3201 cells (P value=0.3), but no difference in the enFeLV RNA expression between F6A-transfected 3201 cells and control 3201 cells, (Table 2.2, column 5). This decreased content of enFeLV RNA in the FRA-transfected 3201 cells may relate to increased packaging of enFeLV RNA by FRA virion.
RT-PCR and sequencing analysis of viral and 3201 cellular enFeLV RNA showed that the enFeLV CFE-16 is expressed in the 3201 cells and packaged in the exogenous FeLV virion. These results indicate that CFE-16 is the enFeLV env source for recombinants FeLV, at least in the 3201 cells. However, this observation does not exclude the possibility that the recombination can occur upstream of env gene resulting in recombinants with enFeLV env and 3 LTR but exogenous pol and gag. In addition, the CFE-6 was not detected in either the 3201 cells or in the virion of the F6A and FRA of the transfected 3201 cells. These data are in agreement with other studies showing that CFE-16 was highly transcribed in lymphoid cells (1,14,17). However, because there are several polymorphic amino acid positions within the enFeLV-derived domains of FeLV-B, which are shared by the exogenous viruses but differ from the known enFeLV sequences, the highly transcribed enFeLV CFE-16 may not be the only source of FeLV-B recombinants (1,14,17). While the possibility that the highly transcribed enFeLV proviruses act as intermediates in a multistep recombinational process cannot be ruled out, it is clear that mutations are involved in the genesis of FeLV-B recombinants.

Next we evaluated the output of FeLV recombinant virus by culturing filtered supernatants from the FRA and F6A transfected FEA and 3201 cells onto HT1080 cells which are not permissive to the parent FeLV subgroup A, but are permissive to FeLV recombinants with the FeLV-B phenotype (1,14,17). No difference in the recombination potential of F6A and FRA with enFeLV sequences was found after the transfection of FEA cells. However, we found that FRA-transfected 3201 cells produced significantly higher amounts of HT1080 permissive virus than F6A.
In addition, the FRA-transfected 3201 cells generated HT1080 permissive virus at an earlier time point (day 7) than F6A-transfected 3201 cells (day 21). Why the infection properties and the recombination potential of F6A and FRA are similar in FEA cells but different in 3201 cells probably relates to the level of enFeLV RNA expression in FEA and 3201 cells.

When we examined the viral genotype of the infected HT1080 cells, we discovered that both FeLV-A and FeLV-B genotypes were present. The FeLV-A genotypes were believed to be transmitted to the HT1080 cells through pseudotype virus composed of a FeLV-B envelope and FeLV-A genome.

Our data emphasizes the finding that CFE-16 is the major enFeLV for the generation or recombinants FeLV (1,14,17). In addition, the current study supports the concept that the recombination of retroviruses depends on the ratio of exogenous to endogenous viral RNA. The higher the co-packaging of endogenous viral RNA and exogenous viral RNA the higher likelihood of recombination. The FRA virion of the FRA-transfected 3201 cells package more endogenous viral RNA than the F6A. FRA also has a greater propensity to recombine as shown in this study and as reported in infected cats (18).
REFERENCES


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Table 2.1: Sequences and specificity of primers used for RT and PCR reactions.
Table 2.2: Endogenous and Exogenous FeLV Expression in FRA- and F6A-Transfected 3201 Cells. p27 production was measured by ELISA assay and the optical density (OD) readings were normalized against kit standards using the following formula: relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control. The F6A- or FRA-transfected 3201 cultures were tested for FeLV envelope expression by immunofluorescence staining assay and the percent of positive cells were determined using flow cytometry. The cellular and viral RNA were extracted from uninfected 3201 cultures and the F6A- or FRA-transfected 3201 cultures using Trizol reagent and the copy number of FeLV RNA was determined using real time quantitative RT-PCR. EnFeLV-LTR specific primers and exogenous FeLV-LTR specific primers were used. GAPDH specific quantitative RT-PCR was conducted as an internal control for the cellular enFeLV or exogenous FeLV RNA. A known copy number of GFP RNA was added to the pelleted virion to serve as internal controls for both viral RNA extraction and quantitative RT-PCR of the viral RNA.
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Table 2.2

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Table 2.3: Virus titration for the TCF of F6A- and FRA-transfected 3201 cultures as determined by the 81C focus-forming assay as previously described (6). The average virus titer from three replicates for each study for day 14 and 28 are shown.
Figure 2.1: Map illustrating the position of primers used to distinguish between FeLV-A, recombinant FeLV and enFeLV sequences. The primers used for quantitative PCR of endogenous and exogenous FeLV-RNA and DNA are also shown. Arrows represent the direction of primers and numbers represent the number of the primers in Table 1.
Figure 2.2: ELISA assay of F6A- and FRA-transfected 3201 cells (Figure 2.2A) and FEA cells (Figure 2.2B). p27 production was measured by ELISA assay from 3 replicates at day 7, 14, 21 and 28 post-transfection. Optical density (OD) readings were normalized against kit standards using the following formula: relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control. The standard deviations for each day are also indicated.
Figure 2.2A (3201 cells)

Figure 2.2B (FEA cells)
Figure 2.3: Immunofluorescence staining assay of F6A- and FRA-transfected 3201 cells (Figure 2.3A) and FEA cells (Figure 2.3B). The pattern of FeLV spread in the 3201 culture from 3 replicate studies was determined using immunofluorescence staining assay at day 7, 14, 21 and 28 post-transfection. The monoclonal anti-FeLV envelope C11D8 was used as the primary reagent while FITC-labeled anti mouse IgG was used as the secondary reagent. The percent of positive cells were determined using flow cytometry. The standard deviations for each day are also indicated.
Figure 2.3A (3201 cells)

Figure 2.3B (FEA cells)
Figure 2.4: RT-PCR detection of the U3 and env region of the enFeLV RNA in the virion of the F6A- and FRA-transfected 3201 cells and control 3201 cells using enFeLV specific primers, RB53 and U3end which produce a 0.49 kb amplicons. The RT step was performed at 55°C for one hour using U3end antisense primer. Five microliter of each RT reaction was added to 50 ul PCR reaction. The PCR reaction contained 1x PCR buffer, 2 mM MgCl2, 200 uM dNTPs, 0.4 uM of each primer and 1 U Platinum Tag DNA Polymerase. The RT-PCR products were cloned into the pCR2.1 Topo vector and utilized in automated DNA sequencing reactions with the forward and reverse primers of pCR2.1 Topo vector. 1; noninfected 3201 cells, 2; viral RNA from the pF6A-transfected 3201 cells, 3; viral RNA from the pFRA-transfected 3201 cells, 4; pFRA, 5; pFGB (FeLV-B molecular clone), L; ladder.
**Figure 2.5:** Comparison of nucleotide sequences of the transcribed enFeLV in 3201 cells, F6A virion of the F6A-transfected 3201 cells and FRA virion of the FRA-transfected 3201 cells with CFE-16 enFeLV (Kumar, D. et al., 1989). The nucleotide sequence begins at the mid of variable region IV and extends into the mid of U3 region of the 3 LTR of CFE-16.
Continued
Figure 2.5
Figure 2.6: Comparative replication of recombinant FeLV from the TCF of F6A- and FRA-transfected 3201 cells at day 7 (A), 14 (B), 21 (C) and 28 (D) post-transfection in HT1080 cells. Shown are average values of 3 replicate studies. The HT1080 cells were inoculated with the TCF filtrate of the transfected 3201 cells as the indicator cell for detection of recombinant FeLV. Every 7 days, supernatants from the inoculated HT1080 cells were collected and tested for the presence of p27 viral antigen using ELISA assay. The OD readings were normalized according to the following formula relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control. The standard deviation for each day is indicated.
Figure 2.7: Comparative replication of recombinant FeLV from the TCF of F6A- and FRA-transfected FEA cells at day 7 (A), 14 (B), 21 (C) and 28 (D) post-transfection in HT1080 cells. Shown are average values of 3 replicate studies. The HT1080 cells were inoculated with the TCF filtrate of the transfected FEA cells as the indicator cell for detection of recombinant FeLV. Every 7 days, supernatants from the inoculated HT1080 cells were collected and tested for the presence of p27 viral antigen using ELISA assay. The OD readings were normalized according to the following formula: relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control. The standard deviations for each day are indicated.
Figure 2.8: Detection of recombinant FeLV and FeLV-A by nested PCR using DNA from HT1080 cells infected with supernatant from FRA or F6A transfected 3201 cells. One ul from the first round PCR, using primers encompassing the entire env gene (H18/H20), was amplified in a second round using recombinants FeLV (RB56/RB17) or FeLV-A (RB59/RB17) specific primers. (A) nested 1.2 kb PCR products using recombinant specific primer. (B) nested 1.09 kb PCR products using FeLV-A specific primer. Lanes: A1, B1) HT1080-FRA; A2,B2) HT1080-F6A. A3,B3) FRA control; A4,B4) F6A control; A5,B5) H2O; A6,B6) uninfected HT1080 cells and A7,B7) the biological clone of FeLV-B, (FeLV-IB3). L; DNA ladder.
**Figure 2.9:** Detection of recombinant FeLV and FeLV-A by nested PCR using DNA from HT1080 cells infected with supernatant from FRA or F6A transfected FEA cells. One ul from the first round PCR, using primers encompassing the entire *env* gene (H18/H20), was amplified in a second round using recombinants FeLV (RB56/RB17) or FeLV-A (RB59/RB17) specific primers. (A) nested 1.26 kb PCR products using recombinant specific primer. (B) nested 1.09 kb PCR products using FeLV-A specific primer. Lanes: A1, B1) HT1080-FRA; A2, B2) HT1080-F6A. A3, B3) the biological clone of FeLV-B (FeLV-IB3); A4, B4) uninfected HT1080 cells; A5, B5) pF6A, A6, B6) pFRA. L: DNA.

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CHAPTER 3

THE ROLE OF POL AND LTR REGIONS IN THE RECOMBINATION VARIATION BETWEEN FeLV SUBGROUP A MOLECULAR CLONES, pF6A AND pFRA

The presence of two genomic RNA molecules in retroviral virions results in a high rate of recombination (11,12). During the process of reverse transcription, a multistep process that is carried out by reverse transcriptase (RT), the viral RNA is converted to double-stranded DNA, which is subsequently integrated into the host genome (10,12). RT is a multifunctional enzyme that possesses RNA-dependent and DNA-dependent polymerase activities and RNase H activity (1,2). To complete the process of reverse transcription, RT must perform two template switches, the first and the second strong-stop template switches (19). It has been proposed that the requirement to perform these two replicative switches confers onto RT the tendency to make additional, nonrequired template switches that result in genetic recombination (11,12). Most DNA polymerases do not switch templates as frequently as RT, suggesting that this process may require some structural or biochemical properties of RT. RT has been mutagenized extensively to identify features important for polymerization, RNase H activity, fidelity and processivity (3,16,31,35,38).
It is thought that RT pauses before switching templates; therefore, mutants with altered pausing and/or processivity may be affected in template switching (3-6,35).

In the FeLV system, the generation of FeLV subgroup B through recombination between the exogenous FeLV (FeLV-A) and the endogenous FeLV (enFeLV) sequences provides a good model to study retroviral recombination in the natural host. In a previous \textit{in vivo} study using two molecular clones of FeLV subgroup A natural isolates, pF6A and pFRA, FRA was more recombinogenic than F6A (32). We have developed an \textit{in vitro} approach to study the recombination frequency between F6A and FRA and found that FRA was also more recombinogenic in that system (Chapter 2).

The distinct difference in recombination in such closely related viruses provided the opportunity to identify factors that contributed to enhanced recombination frequency of FRA. While the mechanism for the difference in recombination rate of F6A and FRA is unknown, several possibilities have been proposed. First, there may be a fundamental difference in the frequency of recombination caused by the genetic variation in the polymerase region. Second, FRA virion contains higher amount of enFeLV RNA than F6A virion (Chapter 2). This property might be due to higher initial enFeLV RNA burden in FRA-infected cells, therefore increasing the probability of copackaging of exogenous and endogenous FeLV RNAs and subsequent recombination. As a result, we predicted that the difference in the enFeLV RNA level might be related to the variation in the LTR between F6A and FRA. Third, the \textit{env} changes may affect the infection rate of the virus such that the higher number of cells infected early leads to a higher copy number of enFeLV RNA copackaged with the exogenous RNA. Fourth, structural features in the RNA templates may promote recombination.
In this study, the first and the second possibilities are addressed. The amino acids differences in the *pol* region between F6A and FRA viruses might be the cause in the delay in the recombinogenic events in F6A versus FRA. The substitutions in the *pol* region may affect recombination through alterations in processivity and affinity of RT to the viral RNA template thereby changing the intermolecular template switching rate, which is the prerequisite for homologous and non-homologous recombination (22,23,25,39,40).

Earlier studies indicted that the FeLV LTR has sequence similarities with MuLV LTRs, in particular with respect to certain nuclear protein-binding sites (24,28,36,37). In addition, the U3 region of the FeLV LTR has been shown to have a role in disease pathogenesis similar to that of MuLV LTR U3 region (15,20,29,36,37). It has been shown that the MuLV and FeLV LTRs have transactivation properties related to the U3 region of their LTRs (8,9,17,18). In addition, we have shown that the FRA-transfected 3201 cells produced virion with a higher enFeLV RNA content than the F6A-transfected 3201 cells (Chapter 2). As a result, we predicted that the FRA LTR might have higher potential than F6A LTR to enhance the enFeLV RNA expression resulting in the increase of enFeLV RNA packaging.

To approach these two possibilities, we formed the following hypothesis: The variation in recombination of the FRA and F6A with enFeLV sequences is caused by the variation in the *pol* or the LTR regions between these two viruses.

To identify the viral genetics region responsible for the observed difference, chimeric proviruses with the *pol* region exchanged between FRA and F6A were constructed. Using these chimeras in our *in vitro* system (Chapter 2) may lead us to a
better understanding of the role of pol region in retrovirus recombination. If the pol gene was responsible, we expect to see a higher rate of recombination with the F6A/FRA pol than the FRA/F6A pol constructs. In addition, plasmid vectors with F6A or FRA LTRs have been constructed and used to examine the enhancement of enFeLV RNA expression in the feline lymphoid cells. We expected FRA-LTR to enhance the enFeLV RNA expression in a rate higher than that of F6A-LTR.
MATERIALS AND METHODS

Plasmids:

The FeLV-A molecular clones used in this study include pF6A, pFRA and the chimeras pFRA/F6A-pol and pF6A/FRA-pol. The pF6A has been molecularly cloned by Overbaugh et al (1988) (30). The pFRA plasmid and the pFRA/F6A-pol and pF6A/FRA-pol chimeras were provided by Roy-Burman, P (University of South California, School of Medicine). The pFRA/F6A-pol is the pFRA with the pF6A pol region. The pF6A/FRA-pol is the pF6A with the pFRA pol region. Figure 3.1 has illustration for the construction of the pFRA/F6A-pol and pF6A/FRA-pol chimeras.

The plasmid pZeo.F6A.LTR and pZeo.FRA.LTR were constructed by PCR cloning the LTR of pF6A or pFRA into the BamHI and HindIII sites of the plasmid pZeoSV2 (Invitrogen, Carlsbad, CA). The pF6A and pFRA LTR fragments were generated by PCR using FeLV-A LTR specific primers linked to HindIII (sense primer, 5’ TgAAAgACCCCCTACCCCAAA ‘3) and BamHI (antisense primer, 5’ TgAAAagACCCCTgAACTAggT ‘3) restriction enzyme sites. The PCR products and the pZeoSV2 vector were digested with BamHI and HindIII for one hour at 37°C. The pF6A or pFRA LTRs were ligated to pZeoSV2 using T4 ligase (Invitrogen) at 16°C for 8 hours.

pEGFP-N1 plasmid (Clontech, Palo Alto, CA) coding for green fluorescent protein was used to determine the transfection efficiency for each transfection assay.
Cell lines:

The feline lymphoid cell line 3201 was propagated in a 1:1 (vol/vol) mixture of RPMI 1640 and Leibowitz L-15 medium containing 20% fetal bovine serum (FBS) supplemented with 100 U of penicillin per ml, 100 ug of streptomycin per ml, and 2 mM L-glutamine. The human fibrosarcoma cell line HT1080 was cultured in alpha MEM supplemented with 15% FBS supplemented with 100 U of penicillin per ml, 100 ug of streptomycin per ml, and 2 mM L-glutamine.

3201 cells transfection and sample collection:

To study the effects of switching the pol regions between pFRA and pF6A, the 3201 cells (5x10^6 cells) were transfected with 25 ug of pFRA, pF6A, pFRA/F6A-pol or pF6A/FRA-pol using electroporation (960uF/250V). Transfection efficiency was determined in parallel using the pEGFP-N1 plasmid. The transfected cells were examined 24 hours after electroporation for GFP expression by fluorescence microscopy. 3201 cells were passed weekly at 2x10^5 cell/ml and kept for 28 days at 37°C and 5% CO₂ incubation. Tissue culture fluid (TCF) samples were collected and passed through 0.45 uM membrane filters at 7 day intervals for each culture, and tested for the expression of viral antigen (p27) using antigen capture enzyme-linked immunosorbent assay (Petchek FeLV ELISA; Synbiotics, San Diego, Calif). Optical density (OD) readings were normalized against kit standards using the following formula: relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control.
3201 cell lines containing stably integrated FeLV LTRs were generated by transfecting pZeo.F6A.LTR or pZeo.FRA.LTR by electroporation. These plasmids contain a Zeocin resistance gene expression cassette. BamHI-digested and self ligated pZeoSv2 was used as a negative vector control in the transfection assays. One day after transfection, cells were split at a low density in the presence of 0.5mg/ml Zeocin (Invitrogen). Zeocin-resistant cell colonies were pooled and passaged in the presence of Zeocin.

**Inoculation of HT1080 cells:**

The HT1080 cells were inoculated with the TCF filtrate of the FeLV-A molecular clones (pF6A, pFRA, pF6A/FRA-pol and pFRA/F6A-pol) transfected 3201 cells as the indicator cell for detection of recombinant FeLV. Briefly, HT1080 cells were seeded into 24-well plates (1x10^4 cell/ml) the day before infection. On the day of infection, HT1080 cells were treated with polybrene for 30 minutes prior to infection (26). The cells were then washed and incubated for 3 hours with the virus at 200 ul of TCF per well. Fresh medium was added (0.8 ml) and the cells were incubated at 37°C, 5% CO_2 for 24 hours. Thereafter, the medium was replaced and the inoculated HT1080 cells were passed every three or four days for four weeks. Every seven days, supernatants from these cells were collected and tested for the presence of p27 viral antigen using ELISA kit and the OD readings were normalized according to the formula described above.
**Virus infectivity:**

The 81C focus-forming assay was used to titrate FeLV produced at day 28 by 3201 cells transfected with FeLV-A molecular clones as previously described (14). The percentage of FeLV-positive 3201 cells was determined by indirect immunofluorescence staining (32) using the monoclonal anti-FeLV envelope C11D8 as the primary reagent (CMI, Sacramento, CA). C11D8 has been shown to react with all subgroups of FeLV (21). The positive and negative controls for this assay were FeLV-infected and uninfected 3201 cells, respectively. Briefly, 2X10⁶ of the transfected 3201 cells were collected at day 7, 14, 21, and 28 post-infection. The cells were washed twice with phosphate buffer saline supplemented with 2 ug/ml bovine serum albumin and 2 ug/ml sodium azide (PBS) and incubated with C11D8 at 37°C for 45 minutes. The cells were then washed 3 times with PBS and the secondary antibody (FITC-labeled anti mouse IgG, Sigma, St. Louis, MO) was added and incubated at 37°C for 45 minutes. The cells were washed 3 times, resuspended in PBS, fixed with 2% paraformaldehyde and analyzed by flow cytometry.

**PCR analysis of the inoculated HT1080 cells genomic DNA:**

Primer pairs for the various PCR amplicons are listed in (Chapter 2, Table 2.1). The genomic DNA was extracted from the HT1080 cell line at day 28 post-inoculation using a tissue genomic DNA isolation kit (Qiagen, Santa Clarita, CA). The isolated DNA was analyzed by nested PCR (7). In the first round of amplification, 250 ng of genomic DNA was amplified (30 cycles) using the following primer set: H18, the 5' sense primer, corresponding to F6A sequence 5840 to 5860 at the pol/env junction
which is conserved among all exogenous FeLVs; and H20, the 3’ antisense primer, complementary to the exogenous U3 sequence in the LTR (F6A 8210 to 8189) (30). To detect the recombinant proviral env sequences, a 1-ul volume of PCR product from the first round of amplification was used in a second-round amplification for 40 cycles using the endogenous-specific 5’ sense primer RB56 which is identical to the nucleotide position (210 to 229) indicated in the published CFE-6 sequence (27,34) and the FeLV-A-specific 3’ antisense primer RB17 complementary to the published F6A sequence (7310 to 7330) (13)and FRA sequence (7). To detect the FeLV-A sequences, the FeLV-A-specific 5’ primer RB59 identical to the published sequence of F6A (6259 to 6278) and the 3’ antisense primer RB17 were used in a second-round in amplification with 1 ul of the H20/H18 product as template. The molecular clones F6A and FRA, the biological clone FeLV-IB3 (33) the non-infected HT1080 DNA and ddH20 were used as controls. Nested PCR products were analyzed by electrophoresis on 2% agarose stained with ethidium bromide.

**RNA extraction:**

Total cellular RNA was extracted from a known number of stably pZeo.F6A.LTR or pZeo.FRA.LTR transfected 3201 cells and negative control nontransfected 3201 cells. Total cellular RNA and viral RNA were also isolated from a 1x10⁷ of FeLV-A molecular clones transfected (day 28 post-transfection) and non-transfected 3201 cells as well as the TCF filtrate from transfected 3201 cells, respectively. All the RNA extraction was done using Trizol reagent (Gibco BRL). For the preparations of viral RNA, 20 ml of the supernatants were ultracentrifuged at
25,000g for 150 minutes at 4°C to pellet virions then the viral RNA was extracted by Trizol. RNA was treated with 4 U RNase-Free DNase (Ambion, Austin, TX), extracted with phenol-chloroform, precipitated with isopropanol at - 80°C and then washed twice in 75% ethanol. Total RNA was resuspended in DEPC water. The RNA concentration was determined by the absorbance at 260 nm and tested for DNA contamination by 40 cycles RT-PCR in the absence of RT using the upstream primer env-1 and the downstream primer env-4 (Chapter 2, Table 2.1), which can detect both exogenous and endogenous viral DNA.

**Quantification of endogenous viral and cellular RNA by Real Time RT-PCR:**

The expression of enFeLV RNA in noninfected 3201, pZeo.F6A.LTR or pZeo.FRA.LTR transfected 3201 cells and chimers transfected 3201 cells and the viral enFeLV RNA in the supernatants of the chimers transfected 3201 cells were quantified using QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA). The primers used to specifically amplify the enFeLV-U3 region were P20 and P6 (Chapter 2, Table 2.1) (18). Each RT-PCR reaction contained 1 uM of each primer, 0.2 ul QuantiTect RT mix, 10 ul of 2x QuantiTect SYBR Green RT-PCR master mix, water to a final volume of 20 and 0.5 ug cellular RNA or 2 ul volume of the viral RNA or of the standard RNA transcript. The reaction was run at 50°C for 30 minutes and at 95°C for 15 minutes. The PCR consists of 30 cycles at, 94°C for 15 seconds, 57°C for 15 seconds, 72°C for 15 seconds and 74°C for 5 seconds. Fluorescence data was acquired at the annealing step and melting curve analysis was performed.
The RNA samples were adjusted to approximately the same concentration for real-time RT-PCR. The internal control for the cellular RNA samples was based on the copy number of GAPDH RNA gene determined by a separate quantitative real time RT-PCR assays using cat specific GAPDH primers (Chapter 2, Table 2.1).

RNA standards for the various real-time RT-PCR assays were \textit{in vitro} transcribed from DNA cloned into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions (Ambion, Austin, TX) as described in Chapter 2.

\textbf{Statistical Analysis:}

Statistical analyses of the comparative results were performed using student’s T test. P values of <0.05 were set as statistically significant.
RESULTS

Infectivity properties of FeLV pF6A, pFRA and the chimeras, pF6A/FRA-pol and pFRA/F6A-pol in feline lymphoid cell line:

In initial studies parental proviruses, pF6A and pFRA and the chimeras, pF6A/FRA-pol and pFRA/F6A-pol (Figure 3.1) were compared for their p27 expression and infectivity in 3201 cells. Molecular clones of each virus were transfected into 3201 cells by electroporation. Transfection efficiencies, as determined by simultaneous transfection with pEGFP, ranged between 10 and 11% for the three replicate studies. Cells and filtered tissue culture fluid (TCF) samples were collected weekly for 4 weeks. The p27 expression was measured by p27 ELISA and the percent of positive cells was determined using immunofluorescence staining assay. The infectious virus titer was determined using the 81C focus-forming assay (14).

From 3 triplicate studies, no difference in p27 antigen expression in TCF of the transfected 3201 cells was found between wild type and the chimera viruses (Figure 3.2). The number of infected cells was also found to be similar, although the chimeric viruses had relatively higher number of positive cells than the corresponding wild type viruses (Figure 3.3).

When the infectious virus titer on 81C cells was compared between the wild type and the chimeras, significant differences were found (Figure 3.4). The FRA/F6A-pol chimera virus had a significantly lower titer than the wild type FRA (p value=0.0006). The titer of the F6A/FRA-pol chimera virus was not significantly different from the wild type F6A (p value=0.4).
Recombination potential of the FeLV-A molecular clones with the enFeLV in the transfected 3201 cells:

An additional study was carried out to determine if the switch of the pol region between pF6A and pFRA affects the recombination rate of pF6A and pFRA. 3201 cells were transfected with the molecular clones of F6A, FRA, F6A/FRA-pol or FRA/F6A-pol. The 3201 transfected cells were kept for 28 days. At days, 7, 14, 21 and 28, the TCF of the transfected 3201 cells were analyzed for FeLV p27 antigen by ELISA and infectious virus titer by the 81C focus-forming assay, collected, filtered and used to inoculate the HT1080 cells. The inoculation of the HT1080 cells was used as a determinant for the production of HT1080 permissive virus (recombinants or pseudotype, FeLV-A packaged into FeLV-B envelope). The HT1080 tissue culture fluids (HTCF) were analyzed for the presence of FeLV p27 antigen by ELISA assay.

The inoculation of the HT1080 cells in this experiment showed that the FRA virus had a higher recombination rate than the F6A virus, which was constant with the results of chapter 2. However, the results of three triplicate studies showed no detectable virus in the HT1080 cells inoculated with TCF from the 3201 cells transfected with either chimera clone. These results suggest that the recombination potential of F6A and FRA were lost after the switching of the pol region (Figure 3.5 and 3.6).

The genotype of the HT1080 permissive viruses:

The TCF collected from the transfected 3201 cells was used to inoculate HT1080 cells. HT1080 cells are known to be infected only by FeLV-recombinants (FeLV-B) and are used as an indicator cell for the FeLV recombinants. However, we
have shown that HT1080 cells could also be infected by pseudotype virus (FeLV-A genome packaged into FeLV-B envelope). Using this approach, the HT1080 cells inoculated with TCF of the 3201 cells transfected with either chimera clone were found not to produce infectious recombinant virus. Because of this, we wanted to use a more sensitive assay to screen for recombinant virus in the HT1080 cells inoculated with TCF of the 3201 cells transfected with F6A/FRA-pol or FRA/F6A-pol chimera. For this purpose, nested PCR analysis was conducted after the extraction of the DNA of the TCF inoculated HT1080 cells. In the first round, the envelope gene of the FeLV was amplified using the H18 and H20 primers. In the second round, recombinant specific primer (RB56) and FeLV-A specific primer (RB59) were used as upstream primers in combination with the downstream RB17 primer. The proper negative and positive controls were used (Figure 3.7 and 3.8).

Using the recombinants specific primer RB56, the expected fragment size of 1.257KB was detected in the DNA isolated from HT1080 cells inoculated by the TCF from the pF6A or pFRA transfected 3201 cells but not from the chimera transfected 3201 cells (Figure 3.7). However, when the FeLV-A specific primers were used, the HT1080 cells inoculated by TCF from the F6A, FRA, or chimeras-transfected 3201 cells were found to harbor FeLV-A env (1.09KB) sequences presumably as a result of pseudotyping into the transfected 3201 cells (Figure 3.8).
Expression and packaging of enFeLV RNA in the chimeras transfected 3201 cultures:

Because of the recombination potential loss of the chimera clones with enFeLV RNA, we wanted to determine if this loss was related to changes in the expression or packaging of enFeLV RNA. To study this, we performed quantitative real time RT-PCR for the enFeLV RNA in the chimera transfected 3201 cells and in the produced virions. Total RNA was isolated from 28 day cultures of chimera transfected 3201 cells and non-infected 3201 cells and tested for the copy number of enFeLV RNA using real time quantitative RT-PCR. As an internal control for RNA loading, real time quantitative RT-PCR for the GAPDH gene was run in parallel. The results of this study showed no changes in cellular enFeLV copy number for both F6A/FRA-pol and FRA/F6A-pol producer cell types compared to uninfected 3201 cells. In addition, both chimeras were found to package enFeLV RNA in their virion (Table 3.1).

The expression activation of lymphoid cell enFeLV RNA by FeLV-A LTR:

To assess possible enhancement of enFeLV RNA expression by FeLV-A LTR, 3201 cell lines containing stable integrated FeLV LTR were generated by transfection with pZeo.F6A.LTR or pZeo.FRA.LTR. Total cellular RNA was extracted from the transfected 3201 cells and nontransfected 3201 cells and the quantity of enFeLV RNA was determined using real time quantitative RT-PCR.

Based in duplicate studies, no differences in the enFeLV RNA expression were found between F6A-LTR or FRA-LTR transfected and control 3201 cells (Table 3.2). These results indicate that the FeLV-A LTR does not enhance the enFeLV RNA expression.
DISCUSSION

We have previously demonstrated that FeLV subgroup A, pFRA, is more recombinogenic than the pF6A with enFeLV sequences (Chapter 2) (32). The comparison of the nucleotide sequence of pF6A and pFRA revealed 98% homology. The highest variations in nucleotide sequence between pF6A and pFRA were found in pol and env regions. In addition, when the virions generated from the pF6A- or pFRA-transfected 3201 cells were examined for their enFeLV RNA contents, FRA-virion was found to have higher enFeLV RNA content than F6A-virion (Chapter 2). Furthermore, it has been shown that the expression of exogenous FeLV-LTR can induce transcription of collagenase IV gene in murine fibroblasts as well as in feline fibroblasts (17). Based on these observations we hypothesized that the variation in recombination of the FRA and F6A with enFeLV sequences was caused by the variation in the LTR or the pol regions between these two viruses.

To study the possible upregulation of enFeLV RNA by the FeLV subgroup A LTR, stable pZeo.F6A.LTR or pZeo.FRA.LTR transfected 3201 cells were generated and their enFeLV RNA expression was determined and compared with non-transfected 3201 cells. No differences were found in the expression of enFeLV RNA between pZeo.F6A.LTR and pZeo.FRA.LTR transfected 3201 cells and control 3201 cells. This result indicates that the FeLV-A LTR has no upregulation effects in the expression of enFeLV RNA in feline lymphoid cells. This result is in agreement with the study by Ghosh, S. et al., (2000) (18) where F6A LTR had no effects in the upregulation of enFeLV RNA in the fibroblast cells.

To determine the role of pol region in recombination variation between pF6A
and pFRA, we have transfected 3201 cells with pF6A, pFRA, pF6A/FRA-pol or pFRA/F6A-pol. The 3201 cells are known to be susceptible to FeLV subgroup A but resistance to FeLV recombinant. Therefore, the recombinantation potential of the wild types and the chimeras were determined by the inoculation of the HT1080 cells with the TCF of the transfected 3201 cells. When the 3201 cells were transfected with pF6A, pFRA, pF6A/FRA-pol or pFRA/F6A-pol, no significant differences were found between pF6A and pF6A/FRA-pol and between pFRA and pFRA/F6A-pol in the p27 antigen production and the number of infected 3201 cells as examined by p27 ELISA and immunofluorescence staining assays, respectively (Figure 3.2 and 3.3). However, significant differences were found when virus preparations were titrated on 81C cells (Figure 3.4). While the titer of the FRA/F6A-pol chimera virus was significantly lower than the wild type FRA, both F6A and F6A/FRA-pol had a high infectious virus titer. The results suggest that substituting the pol gene in the chimera virus affected their permissiveness on the 81C cell but not 3201 cells.

In contrast to wild types F6A and FRA, when the chimera viruses were tested for their potential to generate infectious HT1080 permissive virus (recombinants or pseudotype) neither F6A/FRA-pol nor FRA/F6A-pol generated infectious HT1080 permissive virus. However, nested PCR analysis showed that both chimeras were able to pseudotype with the enFeLV envelope protein and generated pseudotype viruses. We do not know why these chimera constructs do not recombine with enFeLV RNA. Because enFeLV RNA expression and packaging in the exogenous FeLV virion are required for the generation of recombinants FeLV, we predicted that the switch of pol region between pF6A and pFRA caused a reduction in the packaging of enFeLV RNA.

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resulting in no generation of recombinants FeLV. However, the quantitative RT-PCR analysis showed that both pF6A/FRA-pol and pFRA/F6A-pol transfected 3201 cells express enFeLV RNA similar to the control 3201 cells. In addition, the FeLV virion generated from pF6A/FRA-pol and pFRA/F6A-pol transfected 3201 cells was found to package enFeLV RNA. For the pFRA/F6A-pol construct, the lack of recombinant FeLV production might be due to the reduction of infectious virus titer. However, the pF6A/FRA-pol was found to have higher infectious virus titer than the pF6A, but no recombinants FeLV generated after the transfection of 3201 cells. These results indicated that the F6A/FRA-pol is more stable than the parent virus, pF6A.

The switch of \textit{pol} region between pF6A and pFRA might have caused some changes in the structural or biochemical properties of RT resulting in a change in the processivity or fidelity of RT. The secondary structure of the viral RNA genome might also have been affected due to this switch. Any one of these changes might have effects in the recombination mechanism.
REFERENCES


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35. **Snyder, C. S. and M. J. Roth.** 2000. Comparison of second-strand transfer requirements and RNase H cleavages catalyzed by human immunodeficiency virus type 1 reverse transcriptase (RT) and E478Q RT. *J. Virol.* **74:**9668-9679.


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**Table 3.1:** enFeLV RNA expression in control uninfected 3201 cells, chimera transfected 3201 cells and the virion of the chimera transfected 3201 cultures. The enFeLV RNA copy number was determined by real time quantitative RT-PCR using enFeLV-LTR specific primers (P6 and P20). As an internal control for the cellular enFeLV RNA, GAPDH specific real time quantitative RT-PCR was conducted in parallel and the enFeLV RNA copy number was adjusted based on the copy number of GAPDH RNA. Each RT-PCR reaction contained 1 uM of each primer, 0.2 ul QuantiTect RT mix, 10 ul of 2x QuantiTect SYBR Green RT-PCR master mix, water to a final volume of 20 and 0.5 ug cellular RNA or 2 ul volume of the viral RNA or of the standard RNA transcript. The reaction was run at 50°C for 30 minutes and at 95°C for 15 minutes. The PCR consists of 30 cycles at, 94°C for 15 seconds, 57°C for 15 seconds, 72°C for 15 seconds and 74°C for 5 seconds. Fluorescence data was acquired at the annealing step and melting curve analysis was performed.
Table 3.2: enFeLV RNA expression in control 3201 cells and in F6A-LTR or FRA-LTR transfected 3201 cells. The enFeLV RNA copy number was determined by real-time quantitative RT-PCR using enFeLV-LTR specific primers (P6 and P20). The RNA samples were adjusted to approximately the same concentration for real-time RT-PCR. The internal control for the cellular RNA samples was based on the copy number of GAPDH RNA gene determined by a separate quantitative real time RT-PCR assays using GAPDH specific primers. Each RT-PCR reaction contained 1 uM of each primer, 0.2 ul QuantiTect RT mix, 10 ul of 2x QuantiTect SYBR Green RT-PCR master mix, water to a final volume of 20 and 0.5 ug cellular RNA or 2 ul volume of the viral RNA or of the standard RNA transcript. The reaction was run at 50°C for 30 minutes and at 95°C for 15 minutes. The PCR consists of 30 cycles at, 94°C for 15 seconds, 57°C for 15 seconds, 72°C for 15 seconds and 74°C for 5 seconds. Fluorescence data was acquired at the annealing step and melting curve analysis was performed.
Figure 3.1: Strategy used to exchange pol region between pF6A and pFRA molecular clones using restriction enzymes.

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Figure 3.2: ELISA assays from 3 replicate studies of pF6A-, pFRA-, pF6A/FRA-pol- and pFRA/F6A-pol-transfected 3201 cells. p27 was determined by commercial enzyme-linked immunosorbent assay at day 7, 14, 21 and 28 post-transfection as described in Materials and Methods. Optical density (OD) readings were normalized against kit standards using the following formula: relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control. The standard deviations for each day are also indicated.
Figure 3.3: Immunofluorescence staining assays from 3 replicate studies of pF6A-, pFRA-, pF6A/FRA-pol- and pFRA/F6A-pol-transfected 3201 cells. The pattern of FeLV spread in the 3201 culture was determined using immunofluorescence staining assay at day 7, 14, 21 and 28 post-transfection. The monoclonal anti-FeLV envelope C11D8 was used as the primary reagent while FITC-labeled anti mouse IgG was used as the secondary reagent. The percent of positive cells were determined using flow cytometry. The standard deviations for each culture are also indicated.
Figure 3.4: Virus titration assays for the TCF from 3 replicate studies of pF6A-, pFRA-, pF6A/FRA-pol- and pFRA/F6A-pol-transfected 3201 cultures as determined by the 81C focus-forming assay (14). The average virus titer from three replicates for each study for day 28 are shown. The standard deviations for each culture are also indicated.
Figure 3.5: Comparative replication from 3 replicate studies of recombinant FeLV from the TCF of pFRA- and pFRA/F6A-pol-transfected 3201 cells at day 7, 14, 21, and 28 post-transfection in HT1080 cells. The HT1080 cells were inoculated with 200 ul TCF filtrate of the transfected 3201 cells as the indicator cell for detection of recombinant FeLV. Every 7 days, supernatants from the inoculated HT1080 cells were collected and tested for the presence of p27 viral antigen using ELISA assay. The OD readings were normalized according to the following formula relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control. The standard deviations for each day are indicated.
Figure 3.6: Comparative replication from 3 replicate studies of recombinant FeLV from the TCF of pF6A- and pF6A/FRA-pol-transfected 3201 cells at day 7, 14, 21, and 28 post-transfection in HT1080 cells. The HT1080 cells were inoculated with 200 ul TCF filtrate of the transfected 3201 cells as the indicator cell for detection of recombinant FeLV. Every 7 days, supernatants from the inoculated HT1080 cells were collected and tested for the presence of p27 viral antigen using ELISA assay. The OD readings were normalized according to the following formula relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control. The standard deviations for each day are indicated.
Figure 3.7: Detection of recombinant FeLV env sequences by nested PCR using DNA from HT1080 cells infected with supernatant from pF6A-, pFRA-, pF6A/FRA-pol- or pFRA/F6A-pol-transfected 3201 cells. One ul from the first round PCR, using primers encompassing the entire env gene (H18/H20), was amplified in second round using recombinants FeLV (RB56/RB17) specific primers. Lane: 1) HT1080-FRA; 2) HT1080-F6A; 3) HT1080-FRA/F6A-pol; 4) HT1080-F6A/FRA-pol, 5); ddH2O 6) pFRA; 7) uninfected HT1080 cells and 8) the biological clone of FeLV-B, (FeLV-IB3). L: DNA ladder.
Figure 3.8: Detection of FeLV-A env sequences by nested PCR using DNA from HT1080 cells infected with supernatant from pF6A-, pFRA-, pF6A/FRA-pol- or pFRA/F6A-pol-transfected 3201 cells. One ul from the first round PCR, using primers encompassing the entire env gene (H18/H20), was amplified in second round using FeLV-A (RB59/RB17) specific primers. Lane: 1) HT1080-FRA; 2) HT1080-F6A; 3) HT1080-FRA/F6A-pol; 4) HT1080-F6A/FRA-pol, 5); ddH2O 6) uninfected HT1080 cells; 7) the biological clone of FeLV-B, (FeLV-IB3); 8) pFRA and L: DNA ladder.
CHAPTER 4

IN VITRO AND IN VIVO GENOMIC STABILITY OF FELINE LEUKEMIA VIRUS SUBGROUP A TAGGED WITH GREEN FLUORESCENT PROTEIN

Replicating retrovirus populations are characterized by a high degree of genetic change (7). This genetic diversity is the product of base misincorporations (2,10,27), rearrangements (6,36), and both homologous (5,14-16,32) and nonhomologous (24,36,37,39) recombination events in the viral genome. The genetic variability of retroviruses gives the ability to adapt quickly to changes in selective pressures.

A number of replication-competent retroviral vectors have been constructed by the insertion of heterologous sequences into full-length viral genomes. Such vectors were derived from several retrovirus species, including Rous sarcoma virus (1,21), murine leukemia virus (8,20,26,33,34), spleen necrosis virus (9) human foamy virus (30) and human immunodeficiency virus (17,19,23,35). In studies in which the structure of these lengthened viruses was examined, the inserted sequences were usually partially or completely lost from the virus population within few passages through cultures of susceptible cells (9,20,26,30,33).

The other retrovirus vector of interest is feline leukemia virus (FeLV). FeLV is a simple retrovirus which is categorized into three subgroups (A, B, and C) by viral
interference assays that identify genetic sequence variation in the viral surface glycoprotein (SU) moiety of the envelope (env) gene (28,29). The in vitro biological properties of FeLV-A tagged with green fluorescent protein gene (pFRA-GFP) have been studied in feline fibroblast cells, H927 cell line (3). After the transfection of H927 cells, the FRA-GFP was found to be replication competent, genetically stable, and exhibited in vitro biological properties similar to those of the parental virus molecular clone (pFRA). Almost 100% of the pFRA-GFP-transfected H927 cells continuously express GFP for at least 8 months of passages in culture, suggesting that the GFP transgene persisted in the proviral genome of FRA-GFP. In addition, recombinant-competent FeLV (exhibiting FeLV-B envelope properties) expressing GFP was detected after the inoculation of human fibroblast cells, HT1080, with the supernatant of the pFRA-GFP-transfected H927 cultures. HT1080 cells are known to express FeLV-recombinants receptor but not FeLV-A receptor. However, PCR analysis of the DNA of the inoculated HT1080 cells showed the presence of recombinant FeLV with GFP transgene and recombinant FeLV with no GFP transgene. This in vitro study demonstrated that a foreign gene can be incorporated into the FeLV provirus and can be efficiently expressed in cells without disrupting functions critical for virus replication.

Because the results of Chapter 2 demonstrated that the cell type has a role in recombination potential of FeLV, the current Chapter was designed to test the genomic stability of pFRA-GFP in vitro and in vivo. Because of varied cell types that can be infected in vivo we predicted that pFRA-GFP will be less stable in vivo than in the fibroblast culture. To test this hypothesis, the objective of this Chapter was to study the
genomic stability of the FRA-GFP in feline lymphoid cells (3201 cell line) and in cats to compare it with the genetic stability of pFRA-GFP in H927 cells. The assays used were direct detection of the GFP expression in 3201 cells and in tissues and quantitative RT-PCR analysis for FeLV and GFP expression. Stability of pFRA-GFP could be useful as a means to follow the natural spread of FeLV infection or to design a similar vector with other genes such as drug gene to be used in gene therapy.
MATERIALS AND METHODS

FRA-GFP construct:

The FRA-GFP plasmid was provided to us from Dr. Pradip Roy-Burman, University of South California School of Medicine, Los Angeles, California. The generation of the pFRA-GFP construct (Figure 4.1) was described previously (3). Briefly, a 550-bp internal ribosomal entry site (IRES) sequence of encephalomyocarditis virus attached to a polylinker (MCS) was fused to the env gene by overlap extension PCR. The Emerald-green (emG) GFP gene was then cloned in the MCS of pFRA-IRES (in the env-3’ UTR boundary), resulting in pFRA-GFP. The insertion of the IRES-GFP cassette results in 1.3 Kb increase in the FRA provirus.

Transfection of 3201 cultures:

The feline lymphoid cell line (3201 cells) were transfected with 25ug pFRA-GFP or 25 ug pFRA DNA using electroporation (960uF/250V). Samples, supernatants and cells, were collected weekly for 4 weeks to test for viral load (p27 ELISA), FeLV envelope expression and the GFP expression using indirect immunofluorescent assay (IFA). In addition, the H927 cells were transfected with 25 ug pFRA-GFP to control for the infectivity of pFRA-GFP.

Virus infectivity in 3201 cultures:

The p27 ELISA (Petcheck FeLV ELISA, Synbiotics, San Diego CA) was used to test the viral load in the cultures of the transfected 3201 cells. The percentage of GFP
positive 3201 cells and FeLV-positive 3201 cells was determined by flow cytometry using IFA (25). To determine the number of FeLV positive cells, monoclonal anti-FeLV envelope C11D8 were used as the primary reagent (CMI, Sacramento, CA). C11D8 has been shown to react with all subgroups of FeLV (11). The positive and negative controls for FeLV envelope expression were known FeLV-infected and uninfected 3201 cells, respectively. The positive control for GFP expression was H927-GFP cells (100% positive for GFP). Briefly, $2 \times 10^6$ of the transfected 3201 or H927 cells were collected at day 7, 14, 21, and 28 post-transfection. The cells were washed twice with phosphate buffer saline supplemented with 2 ug/ml bovine serum albumin and 2 ug/ml sodium azide (PBS) and incubated with C11D8 at 37°C for 45 minutes. The cells were then washed 3 times with PBS and the secondary antibody (PE-labeled anti mouse IgG, Birmingham, AL) was added and incubated at 37°C for 45 minutes. The cells were washed 3 times, resuspended in PBS, fixed with 2% paraformaldehyde and analyzed by flow cytometry for GFP expression and for the FeLV envelope.

**Kitten inoculation:**

Two groups of SPF cats were used in two different studies. The first study consisted of three SPF 24 to 48 hour old kittens. Two of these kittens (AGP4-1 and AGP4-2) were inoculated intradermally with 50ug of pFRA-GFP plasmid DNA combined with a cationic lipid compound (DOTAP; Roche Diagnostic Crop., Indianapolis, Ind.) in a final volume of 0.5 ml of HEPES-buffered saline (4,38). Third kitten (AGP4-3) was inoculated with the supernatant of pFRA-GFP transfected H927
cells (100% positive for GFP expression). The kittens remained with their own queens until the end of the study, 9 weeks, where they were sacrificed for necropsy.

The second study consisted of six SPF 24 to 48 hour old kittens. Three SPF kittens (IQA1-1, IQA1-2, and IQA1-3) were inoculated intradermally with 175μg of pFRA-GFP plasmid DNA combined with DOTAP in a final volume of 0.5 ml of HEPES-buffered saline (4,38). The other three kittens (AXQ1-3, AXQ1-4, and AXQ1-5) were inoculated with $1\times10^7$ pFRA-GFP-transfected H927 cells (100% positive for GFP expression) intraperitoneal. The kittens remained with their own queens until weaning at 10 weeks of age. Precaution was taken to avoid contamination between the two groups in which each group was separated in an individual cage. All the kittens were sacrificed at week 12.

In both studies, all works was performed in accordance with University Laboratory Animal Care and Use Committee and by Department of Health Education and Welfare publication No. NIH 74-23, Guide for the Care and Use of Laboratory Animals.

Sample collection and processing:

Weekly blood samples were collected from the inoculated cats. Blood smears and the separation of plasma samples were done at the time of bleeding. Complete necropsies were performed at the conclusion of the study (9 weeks for the first study and 12 weeks for the second study) to collect tissue samples for histopathologic examination as well as the examination of frank GFP direct fluorescence in the tissues.
Tissue samples were collected in 10% neutral buffered formalin, 100% methanol, or in OCT medium (Sakura Finetek, Torrance, Calif) as well as snap frozen in liquid nitrogen.

**FeLV Viremia:**

FeLV viremia was measured by antigen capture enzyme linked immuno-sorbent assay (ELISA) for the detection of P27 capsid protein in plasma. Optical density (OD) readings were normalized against kit standards using the following formula: relative OD = OD of unknown - OD of negative control/OD of positive control - OD of negative control.

**Recovery of GFP gene in the plasma by inoculation of H927 culture:**

H927 cells were inoculated by 0.2 ml plasma of the FRA-GFP inoculated cats. The plasmas used to inoculate the H927 cultures were collected at week 5 post-inoculation for the first study and at week 5 and 12 post-inoculation for the second study. The inoculated cells were incubated for 21 days at 37°C. The cultures were then tested by p27 ELISA and by direct fluorescent microscope to test for the GFP expression.

**Indirect immunofluorescence assay for FeLV:**

For the detection of FeLV group-specific antigen (GSA), methanol-fixed blood smears were stained by indirect immunofluorescence assay using goat anti FeLV antibody as described previously (12).
**GFP expression analysis in tissues and blood smears:**

Examination of the frank GFP direct fluorescence was carried out on blood smears and the collected tissues. The blood smears were fixed (absolute methanol or 10% Formalin for 10 minutes) and then counterstained for 2 minutes in 0.02% Evans blue. Non-fixed blood smears were also tested for the GFP direct fluorescence.

To test GFP direct fluorescence in tissues, tissues collected at necropsy was embedded in OCT compound and frozen in liquid nitrogen. Frozen sections were cut (10 μM), adhered to charged slides, briefly air dried, and fixed in 10% formalin for 10 minutes. The fixed tissues were washed in phosphate buffered saline (PBS) and counterstained in 0.02% Evans blue for 2 minutes. The counterstained tissues were then washed three times in PBS and checked under the fluorescent microscope.

**Quantification of the expression of exogenous FeLV RNA and the GFP RNA:**

Viral RNA was extracted from 100 ul plasma at week 5 post-inoculation using TRI Reagent-LS (MRC, Cincinnati, OH). The exogenous viral RNA and the GFP RNA copy number were determined by quantitative real-time RT-PCR. The exogenous-specific primers, U3exo sense (2174-2188), accession no M12500 (31) and the U3exo’ antisense (2228-2247), accession no. M12500 (31) were used in this experiment to amplify the exogenous U3 region (13). For the amplification of GFP RNA, GFP specific primers were used (Chapter 2, Table 2.1) (18).

The RT step was performed with 2 ul of the extracted viral RNA at 55°C for one hour using 1 uM U3exo’ antisense primer according to the manufacturer’s conditions.
Two microliters of the RT product were added to the PCR reaction in a total volume of 20 ul containing 0.225 uM for each primer and 2 mM Mg and 2 ul of the supplemented reaction mix, FastStart DNA master and Sybr Green I (Roche, Indianapolis, IN). Each cycle consisted of: 95°C denaturation for 5 seconds, 67°C annealing for 10 seconds, and 74°C extension for 10 seconds for 40 cycles in a LightCycler thermal cycler (Roche, Indianapolis, IN). Fluorescence data was acquired at the annealing step and melting curve analysis was performed.

The copy number of the GFP RNA was determined using QuantiTect SYBR Green RT-PCR kit according to the manufacturer’s conditions (Qiagen, Valencia, CA). Briefly, 2 ul of the plasma extracted RNA was added to 20 ul total volume reaction containing 1 uM of upstream and downstream GFP primers, 10 ul 2x QuantiTect SYBR Green RT-PCR Master Mix and 0.2 ul QuantiTect RT Mix. The RT cycle was at 50°C for 20 minutes and followed by 15 minutes at 95°C denaturation and DNA polymerase activation step. Each cycle consisted of: 94°C denaturation for 5 seconds, 60°C annealing for 5 seconds, and 72°C extension for 5 seconds for 30 cycles in a LightCycler thermal cycler (Roche, Indianapolis, IN). Fluorescence data was acquired at the annealing step and melting curve analysis was performed.

RNA standards for the real-time RT-PCR assays were *in vitro* transcribed from DNA cloned into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions (Ambion, Austin, TX). The DNA insert for the FeLV primers and GFP primer were derived by PCR from pF6A and pEGFP-N1, respectively.
Quantifying the copy number of exogenous FeLV and the GFP DNA in the genomic DNA of the inoculated cats:

Genomic DNA was isolated from the frozen mesenteric lymph node, salivary gland and bone marrow of the FRA-GFP inoculated cats using genomic DNA purification kit (Gentra, Minneapolis, Minnesota).

The exogenous provirus FeLV and the GFP DNA copy number were determined by quantitative real-time PCR using FastStart DNA master and Sybr Green I kit (Roche, Indianapolis, IN). The exogenous-specific primers, U3exo sense and the U3exo’ antisense (see above) were used in this experiment to amplify the exogenous U3 region (13). For the amplification of GFP RNA, GFP specific primers were used (18).

To determine the copy number of exogenous FeLV provirus, two 2 ul of the genomic DNA (300 ng) were added to the PCR reaction in a total volume of 20 ul containing 0.3 uM for each primer and 2 mM Mg and 2 ul of the supplemented reaction mix. Each cycle consisted of: 95°C denaturation for 5 seconds, 67°C annealing for 10 seconds, and 74°C extension for 10 seconds for 40 cycles in a LightCycler thermal cycler. Fluorescence data was acquired at the annealing step and melting curve analysis was performed.

The copy number of the GFP DNA was determined using 2 ul of the genomic DNA (300 ng) added to 20 ul PCR reaction containing 0.3 uM for each GFP primer and 4 mM Mg and 2 ul of the supplemented reaction mix. Each cycle consisted of: 95°C denaturation for 0 seconds, 60°C annealing for 0 seconds, 72°C extension for 5 seconds,
and 80°C for 5 seconds for 30 cycles in a LightCycler thermal cycler. Fluorescence data was acquired at the annealing step and melting curve analysis was performed.

DNA standards for the real-time PCR assays were DNA cloned into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The DNA insert for the exogenous FeLV primers and the GFP primers were derived by PCR from pF6A and pEGFP-N1, respectively.

**Detection of FeLV recombinants in the tissues of FRA-GFP inoculated kittens:**

Genomic DNA was isolated from the frozen mesenteric lymph node, salivary gland and bone marrow of the FRA-GFP inoculated cats as described above. The isolated DNA was analyzed by nested PCR to detect recombinant FeLV sequences. In the first round of amplification, 250 ng of genomic DNA was amplified (40 cycles) using the following primer set: H18, the 5' sense primer, corresponding to F6A sequence 5840 to 5860 at the pol/env junction which is conserved among all exogenous FeLVs; and H20, the 3' antisense primer, complementary to the exogenous U3 sequence in the LTR (F6A 8210 to 8189) (Chapter 2, Table 2.1) (22). The first primer pairs (H18 and H20) were chosen to amplify the entire env-GFP sequences. To detect the recombinant proviral env sequences, a 1 ul volume of PCR product from the first round of amplification was used in a second-round amplification for 35 cycles using the endogenous-specific 5' sense primer RB53 (Chapter 2, Table 2.1) and the H20 antisense primer. The H20 antisenes primer is located downstream of the GFP insert. So, recombinant sequences with or without GFP sequences will be detected using this
technique. The molecular clones pFRA and pFRA-GFP were used as negative control while the molecular clone pFGB (FeLV-B) was used as the positive control. Nested PCR products were analyzed by electrophoresis on 2% agarose stained with ethidium bromide.

**Statistical Analysis:**

Statistical analyses of the comparative results were performed using student’s T test. P values of <0.05 were set as statistically significant.
RESULTS

Replication properties of pFRA-GFP in vitro:

Feline 3201 lymphoid cultures transfected with pFRA-GFP or pFRA plasmid were evaluated for p27 antigen expression by p27 ELISA. In addition, the pFRA-GFP- or pFRA-transfected 3201 cultures were evaluated for the envelope expression by IFA assay and for the expression of GFP using flow cytometry. As can be seen from figures 4.2 and 4.3, these two assays revealed no difference in replication properties between pFRA-GFP and pFRA (Figure 4.2 and 4.3). From a maximum of 75% of 3201 staining positive for FeLV envelope, less than 2% co-express GFP (Figure 4.3). As a control, the transfection of H927 cells resulted in almost 100% of the cells expressing GFP at 21 day post-transfection.

In vivo infectivity of pFRA-GFP:

To determine in vivo infectivity, 50ug of pFRA-GFP plasmid DNA or the supernatants of pFRA-GFP-transfected H927 cells were inoculated intradermally and intraperitoneally, respectively, into three neonate cats using protocols described previously (4,38). FeLV antigenemia, a presumptive test for viremia, was measured by p27 ELISA in serial plasma samples from the inoculated cats. Only the cat which was inoculated with the supernatants of pFRA-GFP-transfected H927 cells became viremic while the other two remained nonviremic through the 9-week study (Table 4.1).

In a second study, 175ug of pFRA-GFP plasmid DNA and the pFRA-GFP-transfected H927 cells were inoculated intradermally or intraperitoneally, respectively,
into six neonate cats (three each) using the same protocols described of the first study. In this study, all the cats, which were transfected with 175ug of pFRA-GFP DNA developed FeLV viremia 3 to 5 weeks post-transfection, but only two of the cats inoculated with the pFRA-GFP transfected H927 developed viremia at this time point. The other cat remained nonviremic through the 12-week study (Table 4.1).

To detect the FeLV GSA in blood smears, IFA was conducted using anti-GSA antibodies as the primary antibody and anti-goat FITC-conjugated as the secondary antibody (12). In both studies, strong correlation was detected between ELISA and the IFA in the blood samples. All the viremic cats (in both study) were found to be positive for the GSA between 3 to 5 weeks (Table 4.1).

**GFP expression in blood smears and tissues:**

Number of tissues was collected and processed in different methods in order to test the expression of GFP. The tissue samples were collected in 10% neutral buffered formalin, 100% methanol, or in OCT medium as well as snap frozen in liquid nitrogen. The GFP was found only in those tissues which were embedded in OCT compound, fixed in 10% formalin and counterstained in 0.02% Evans blue for 2 minutes. The detection of GFP direct fluorescence in the frozen tissues provides solid evidence for the delivery and the expression of GFP gene in the infected tissues (Table 4.2).

While no GFP was detected in the first study, two cats from the second study, IQA1-1 and IQA1-3, were found to have the GFP in the OCT-embedded tissues. These two cats were transfected with 175ug pFRA-GFP DNA.
In addition, all the inoculated cats of the second study, except AXQ1-3, were found to have the spleen positive for GFP expression (Table 4.2 and Figure 4.4A-E).

In an attempt to recover the FRA-GFP virus expressing GFP, H927 cells were inoculated with plasma collected at week 5 of the first study and week 5 and 12 of the second study from the FRA-GFP inoculated cats. In the first study, only the H927 cells inoculated with the plasma of the cats inoculated with the supernatant of the pFRA-GFP transfected H927 cells was found to be positive by ELISA, but no GFP expression was detected in the inoculated cells. In the second study, all the plasma inoculated H927 cultures were positive by p27 ELISA, but only H927 cultures that were inoculated with IQA1-1 and IQA1-3 plasma were found to express GFP (Table 4.2). This experiment showed that the infectious FRA-GFP was only recovered from cats that expressed GFP in their tissues.

Genetic stability of pFRA-GFP upon inoculation of Kittens:

When the kittens were inoculated with the pFRA-GFP plasmid or the pFRA-GFP-transfected H927, blood samples were collected weekly until the end of the study (12 weeks). To test the stability of the FRA-GFP virus, viral RNA was extracted from the plasma of week 5 for the group of the second study and quantitative real time RT-PCR was conducted for both, exogenous FeLV RNA and GFP RNA. From 0.0024% to 2.33% of pFRA-GFP virion was found to have the GFP insert (Table 4.3). To test if this significant reduction in the number of FeLV virion having the intact FRA-GFP RNA is due to deletion of GFP insert or to inhibition of the FRA-GFP provirus expression, we
conducted quantitative real time PCR for the exogenous FeLV provirus against the GFP DNA in the genome of the mesenteric lymph node, salivary gland and bone marrow of the FRA-GFP inoculated cats of the second study. The result of the quantitative real time PCR clearly showed that a high number of the FRA-GFP provirus have lost the GFP gene (Table 4.3).

To test for the generation of recombinants FeLV with or without GFP insert, we conducted nested PCR analysis using primer pairs flanking the area of recombination and the GFP insert. All the tested tissues, mesenteric lymph node, bone marrow and salivary gland had recombinant FeLV sequences present (Figure 4.5). However, the fragment sizes of all of the detected recombinant FeLV sequences were found to be larger than the FeLV-B molecular clone fragment (1.8 Kb) and less than the expected size for recombinant having intact GFP insert (3 Kb) which may indicate that the recombinant FeLV in the FRA-GFP inoculated kittens have truncated GFP gene.
DISCUSSION

We have conducted in vivo and in vitro studies using replication-competent FeLV-A containing IRES-GFP construct (pFRA-GFP) at the env-3’ UTR boundary to examine virus stability. Use of the IRES-GFP insert allowed us to track virus spread by flow cytometry in vitro using 3201 and H927 cells, with loss of fluorescence serving as a reliable surrogate marker for genetic instability. In addition, direct checking of GFP expression in tissues of the FRA-GFP inoculated cats was used to determine the genetic stability of FRA-GFP in vivo. Because of a variety of obstacles that may interfere with the detection of GFP expression in the tissues, RT-PCR as well as PCR analysis for the genomic RNA and DNA of selected tissues was also used to test the stability of FRA-GFP genome. In a previous study, the use of the same pFRA-GFP construct had successfully shown the stability of the FRA-GFP insert over multiple serial passages in feline fibroblast cells for at least 8 months (3).

In the current study, the in vivo infectivity of the pFRA-GFP is similar to the wild type, pFRA, in that the detection of viremia beginning between 4 and 5 weeks post-transfection was similar to that in studies involving the parent pFRA (Table 4.1) (4,25). In addition, strong correlation between p27 ELISA and IF assay was found in the six inoculated cats of the second study (Table 4.1). However, the infectivity of pFRA-GFP is dependent on the amount of the plasmid DNA in the inoculums. Unlike the study by Phipps, A. et al (2000) (25), when we used 50ug of pFRA-GFP DNA, none of the inoculated cats became viremic. When we increased the amount of pFRA-GFP DNA, 175ug, all of the inoculated cats became viremic.
The viral kinetics in vitro was also found to be similar between the wild type pFRA and the pFRA-GFP as can be seen from the p27 ELISA and IF assays (Figure 4.1 and 4.2). As with the in vitro study by Chang, et al (2001) (3), our study showed that pFRA-GFP has the in vivo and the in vitro properties of the wild type pFRA. However, the results of our in vivo and in vitro study are different from pFRA-GFP transfected H927 cells study by Chang, et al. (2001) (3). In their study as well as in the current study, almost 100% of the pFRA-GFP-transfected H927 cells were found to continuously express GFP for at least 8 months of passages in culture, suggesting that the GFP transgene persisted in the proviral genome of FRA-GFP. In contrast, FRA-GFP transfected 3201 cells showed a maximum of 75% positive staining for FeLV envelope on 3201 cells but less than 2% co-expression of GFP, (Figure 4.3) indicating genetic disruption for the IRES-GFP insert in the cellular genome.

When the tissues of the inoculated cats of the second study were tested for GFP expression, only two out of six cats were shown to have the GFP expressed in the collected tissues. However, 5 out of the six cats expressed GFP in the spleen tissues, which might be due to trapping blood-born antigen. To determine if the lack of GFP expression in the inoculated cats was due to the disruption of GFP insert or to the inhibition of the GFP gene expression, quantitative RT-PCR and PCR analysis were conducted for the tissues of the cats of the second study. Using the quantitative RT-PCR, from 0.0024 to 2.33% of plasma virion was found to have the GFP insert (Table 4.3). However, the percent of proviral DNA having the GFP insert was found to vary between the tested tissues, mesenteric lymph node, bone marrow and salivary gland.
The highest percent of proviral DNA with GFP insert was found to be in the salivary gland and the lowest was found to be in the mesenteric lymph node (Table 4.3). In addition, the recombinants FeLV sequences were detected in the mesenteric lymph node, bone marrow and salivary gland tissues. However, neither of the tested tissues was found to have recombinant sequences with intact GFP gene.

Our results showed that the biological patterns of FRA-GFP *in vivo* and *in vitro* in 3201 cells are the same indicating that lymphoid cell line (but not fibroblast cell line) mimics *in vivo* environment in the FeLV infection. In addition, the data of the current study imply that genetic stability of FeLV is cell dependent.

The outcomes of the current study lead us to the following questions. First, why is pFRA-GFP more stable in fibroblast cells than in lymphoid cells. Second, why is pFRA-GFP more stable in salivary gland than in lymph nodes. The results of next chapter showed that 3201 lymphoid cells express more enFeLV RNA than the fibroblast cells. In addition, we have shown that mesenteric lymph nodes express high level of enFeLV RNA while no detectable expression of enFeLV RNA is found in salivary gland tissues. Based on the results of Chapter 5, we predict that the level of enFeLV RNA expression in the infected cell plays a critical role in the stability of FRA-GFP. Then, what is the role of the level of enFeLV RNA expression in the genetic stability of FeLV-A? When the results of the current Chapter are combined with the results described in Chapter 2 and 5, it is likely that the role of enFeLV in FeLV-A genomic instability is related to its recombination with FeLV-A sequences.
REFERENCES


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Table 4.1: Summary of the results of viremia (p27 ELISA) and IFA of FRA-GFP inoculated cats. FeLV viremia was measured by p27 ELISA for the detection of capsid protein in plasma. Optical density (OD) readings were normalized against kit standards using the following formula: relative OD = OD of unknown − OD of negative control/OD of positive control − OD of negative control. The IFA assay was conducted for the detection of FeLV group-specific antigen (GSA) using methanol-fixed blood smears stained by goat anti FeLV antibody as described previously (12).
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<th>Assay</th>
<th>Time (weeks post-inoculation)</th>
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</tbody>
</table>

Table 4.1
Table 4.2: GFP expression in the FRA-GFP inoculated cats. Tissues collected at necropsy was embedded in OCT compound and frozen in liquid nitrogen. Frozen sections were cut (10 μM), adhered to charged slides, briefly air dried, and fixed in 10% formalin for 10 minutes. The fixed tissues were washed in phosphate buffered saline (PBS) and counterstained in 0.02% Evans blue for 2 minutes. The counterstained tissues were then washed three times in PBS and checked under the fluorescent microscope. For the detection of GFP expression in the plasma-inoculated H927 cells, the cells were washed 2 times in PBS and directly checked under the fluorescent microscope.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Challenge</th>
<th>Bone marrow</th>
<th>Mesenteric lymph node</th>
<th>Peritoneal lymph node</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Salivary gland</th>
<th>H927 cell line wk 5</th>
<th>H927 cell line wk 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGP4-1</td>
<td>50ug pFRA-GFP DNA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>AGP4-2</td>
<td>H927-FRA-GFP supernatant</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>H927</td>
<td>wk 5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>H927</td>
<td>wk 12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
</tbody>
</table>

- (negative) no GFP was detected or + (positive for GFP), number of + represents the degree of GFP expression in the tissues. * H927 culture was inoculated with the plasma (plasma collected at week 5 and plasma collected at week 12) of the FRA-GFP inoculated cats.

Table 4.2
Table 4.3: Summary of the results of quantitative real time RT-PCR and PCR for the FRA-GFP inoculated cats. Viral RNA was extracted from the plasma of week 5 for the group of the second study and quantitative real time RT-PCR was conducted for both, exogenous FeLV RNA and GFP RNA using FeLV-A LTR specific primers and GFP specific primers, respectively. Genomic DNA was extracted from mesenteric lymph node, bone marrow and salivary gland to conduct quantitative real time PCR for the exogenous FeLV provirus and the GFP DNA in the genome of the FRA-GFP inoculated cats of the second study using FeLV-A LTR specific primers and GFP specific primers, respectively. To determine the % of GFP RNA to FeLV RNA or the % of GFP DNA to FeLV provirus, the following formula was used:

\[
\% \text{ Of GFP to FeLV} = \frac{\text{GFP copy number}}{\text{FeLV copy number}} \times 100.
\]
<table>
<thead>
<tr>
<th>Animal</th>
<th>Challenge</th>
<th>RNA</th>
<th>Plasma</th>
<th>Mesenteric lymph node</th>
<th>Bone marrow</th>
<th>Salivary gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQA1-1</td>
<td></td>
<td>GFP copy/ml</td>
<td>5.65x10⁵</td>
<td>7.18x10⁴</td>
<td>1.91x10⁴</td>
<td>1.77x10⁴</td>
</tr>
<tr>
<td></td>
<td>FeLV copy/ml</td>
<td>9.45x10⁷</td>
<td>9.00x10⁵</td>
<td>1.26x10⁶</td>
<td>5.92x10⁵</td>
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<tr>
<td></td>
<td>%GFP to FeLV</td>
<td>0.6%</td>
<td>0.8%</td>
<td>1.5%</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>IQA1-2</td>
<td>175ug pFRA-GFP DNA</td>
<td>GFP copy/ml</td>
<td>2.80x10⁴</td>
<td>1.22x10³</td>
<td>1.48x10³</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>FeLV copy/ml</td>
<td>2.80x10⁷</td>
<td>1.87x10⁶</td>
<td>8.80x10⁵</td>
<td>nd</td>
<td></td>
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<tr>
<td></td>
<td>%GFP to FeLV</td>
<td>1%</td>
<td>0.065%</td>
<td>0.168%</td>
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<tr>
<td>IQA1-3</td>
<td></td>
<td>GFP copy/ml</td>
<td>3.40x10⁴</td>
<td>5.50x10³</td>
<td>3.52x10⁴</td>
<td>4.19x10³</td>
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<tr>
<td></td>
<td>FeLV copy/ml</td>
<td>5.00x10⁷</td>
<td>1.00x10⁶</td>
<td>1.24x10⁶</td>
<td>3.40x10⁵</td>
<td></td>
</tr>
<tr>
<td></td>
<td>%GFP to FeLV</td>
<td>0.068%</td>
<td>0.55%</td>
<td>2.83%</td>
<td>1.23%</td>
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<tr>
<td>AXQ1-3</td>
<td>pFRA-GFP-transfected</td>
<td>GFP copy/ml</td>
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<td>3.40x10⁵</td>
<td>1.50x10⁵</td>
<td>1.07x10³</td>
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<td>FeLV copy/ml</td>
<td>3.78x10⁷</td>
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<td>9.57x10²</td>
<td>5.26x10³</td>
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<tr>
<td></td>
<td>%GFP to FeLV</td>
<td>0.34%</td>
<td>6%</td>
<td>15.6%</td>
<td>20.48%</td>
<td></td>
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<tr>
<td>AXQ1-4</td>
<td>H927 cells</td>
<td>GFP copy/ml</td>
<td>1.40x10⁴</td>
<td>2.63x10²</td>
<td>1.99x10³</td>
<td>1.52x10³</td>
</tr>
<tr>
<td></td>
<td>FeLV copy/ml</td>
<td>6.00x10⁴</td>
<td>6.54x10⁴</td>
<td>9.79x10³</td>
<td>5.45x10³</td>
<td></td>
</tr>
<tr>
<td></td>
<td>%GFP to FeLV</td>
<td>2.33%</td>
<td>4.03%</td>
<td>20.39%</td>
<td>27.8%</td>
<td></td>
</tr>
<tr>
<td>AXQ1-5</td>
<td>(1x10⁷ total cells/cat)</td>
<td>GFP copy/ml</td>
<td>1.78x10⁴</td>
<td>9.36x10²</td>
<td>1.35x10³</td>
<td>1.59x10³</td>
</tr>
<tr>
<td></td>
<td>FeLV copy/ml</td>
<td>7.50x10⁸</td>
<td>1.80x10⁶</td>
<td>7.43x10⁵</td>
<td>2.04x10⁵</td>
<td></td>
</tr>
<tr>
<td></td>
<td>%GFP to FeLV</td>
<td>0.0024%</td>
<td>0.0515%</td>
<td>0.182%</td>
<td>0.78%</td>
<td></td>
</tr>
</tbody>
</table>

nd. Not done

Table 4.3
Figure 4.1: Construction of pFRA-GFP provirus as described by Chang, et al., 2001 (3). Step 1: Schematic representation of the overlap extension PCR for fusing the env fragment with the IRES-MCS fragment. The two primers used, env-L and IRES-R2, for overlapping extension PCR are indicated. Step 2: Conversion from pFRA to pFRA-IRES using Nar I restriction enzyme and subsequent legation. Step 3: Conversion from pFRA-IRES to pFRA-GFP using restriction enzymes, AatII and NdeI and subsequent legation.
Figure 4.1

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Figure 4.2: ELISA assay of pFRA-GFP and pFRA-transfected 3201 cells. P27 production was measured by ELISA assay at day 7, 14, 21 and 28 post-transfection. Optical density (OD) readings were normalized against kit standards using the following formula: relative OD = OD of unknown – OD of negative control/ OD of positive control – OD of negative control. The standard deviations for each day are also indicated.
Figure 4.3: Immunofluorescence staining assay of pFRA-GFP- and pFRA-transfected 3201 cells. The H927 cells transfected with pFRA-GFP are also included as positive control. The patterns of FRA-GFP and FRA spread in the 3201 culture were determined using immunofluorescence staining assay at day 7, 14, 21 and 28 post-transfection. The monoclonal anti-FeLV envelope C11D8 was used as the primary reagent while PE-labeled anti mouse IgG was used as the secondary reagent. 3201-FRA-GFP C11D8 positive: the percent of FeLV-envelope positive cells in the pFRA-GFP transfected 3201 cultures; 3201-FRA C11D8 positive: the percent of FeLV-envelope positive cells in the pFRA transfected 3201 culture; 3201-FRA-GFP GFP positive: the percent of cells dual positive to GFP expression and FeLV-envelope in the pFRA-GFP transfected 3201 culture. H927-FRA-GFP C11D8 positive: the percent of cells positive to FeLV-envelope in the pFRA-GFP transfected H927 culture. H927-FRA-GFP GFP positive: the percent of cells dual positive to GFP expression and FeLV-envelope in the pFRA-GFP transfected H927 culture.

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Figure 4.3

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**Figure 4.4:** GFP expression in the tissues of pFRA-GFP inoculated cats. The tissues were fixed with 10% formalin and counterstained in 0.02% Evans blue and checked under the fluorescent microscope. A: Salivary gland. B: Mesenteric lymph node. C: Bone marrow. D: Spleen. E: Thymus.
Figure 4.4 A: Salivary gland

Figure 4.4 B: Mesenteric lymph node
Figure 4.4 C: Bone marrow
Figure 4.4 D: Spleen

pFRA-GFP

Negative control

Figure 4.4 E: Thymus
Figure 4.5: Detection of recombinant FeLV sequences. The genomic DNA samples were isolated from, mesenteric lymph node (Lane 1), bone marrow (Lane 2) and salivary gland (Lane 3) of FRA-GFP inoculated kittens using genomic DNA purification kit (Gentra, Minneapolis, Minnesota) and amplified by nested PCR, first with primer pair H18 and H20 for 40 cycles and second with primer pairs RB53 and H20 in for 35 cycles. The FeLV-B molecular clone, pFGB, (Lane 4) was used as positive control with an expected size of the amplified fragment of 1.8 Kb. The FeLV-A molecular clones, pFRA (Lane 5) and pFRA-GFP (Lane 6) were used as negative controls. Lane 7 is ddH2O, L is ladder.
CHAPTER 5

QUANTIFICATION OF ENDOGENOUS FELV RNA IN VIVO AND IN VITRO

In addition to the exogenous FeLVs of feline species, multiple endogenous FeLV (enFeLV) proviruses are present in the genomes of domestic cats and related small feline species (1). The family members of enFeLV sequences are polymorphic and present at approximately 15 copies per cell. Many (if not all) of enFeLVs contain extensive deletions and none have yielded infectious virus as a result of mutations in the \textit{gag} gene (10,20,21). Although enFeLV elements are mostly defective genomes, they play at least three vital roles in the pathogenesis of the exogenous FeLV. First, enFeLV sequences recombine \textit{de novo} with the exogenous FeLV-A \textit{env} sequences giving rise to a new subgroup known as FeLV-B (4,5,10,11,16-18,20,22). Second, expression of the enFeLV \textit{env} may lead to pseudotyping through which the FeLV-A genome can be delivered to cells normally resistant to infection by FeLV-A. Third, because enFeLV \textit{env} sequences are closely related to the \textit{env} of FeLV-B, expression of enFeLV \textit{env} may lead to the blockage of FeLV-B receptor (5,6). These three roles are restricted to the expression of the enFeLV \textit{env} region.

In a previous study, we have shown that coinoculation of FeLV-A with FeLV-B caused inhibition of FeLV-A (pFRA) infection (13). This apparent interference was
time and titer dependent. Even though none of the FeLV-B inoculated cats became viremic, FeLV-B was capable of establishing focal FeLV infections primarily of the salivary gland and in some cats in the bladder epithelium. Because enFeLV and FeLV-B use the same cellular receptor (6), we predicted that the FeLV-B localized infection of salivary gland and bladder epitheliums was permitted because of the lack of enFeLV RNA expression in these tissues (12).

Relevant to the vital role of enFeLV in the FeLV pathogenesis, the in vivo (as well as in vitro) expression of enFeLV sequences has not been fully described. Earlier studies revealed low-level expression of enFeLV in placenta, fetal lymphoid tissues, and some FeLV-negative lymphomas (2,6-9). Among different feline cell lines that have been tested for the enFeLV RNA expression, only the lymphoid cell lines were found to express enFeLV RNA (2,6,7).

The objective of this study was to test and quantify the enFeLV RNA expression in different tissues in vivo and in different cell lines in vitro using quantitative real time RT-PCR. The hypothesis being tested is: Different tissues as well as different feline cell lines may have different level of enFeLV RNA expression which may contribute to the variation in genetic stability of FeLV in different cell type in vivo and in vitro.
MATERIALS AND METHODS

Animals:

Six SPF control cats were used in this study to test the expression of enFeLV RNA in their tissues. The tissues of choices were: tonsil, spleen, thymus, lymph node, bone marrow, peripheral blood mononuclear cells (PBMC), bladder, intestine, salivary gland and brain.

Cell lines:

Three FeLV-negative cell lines were tested for the enFeLV RNA expression. These cell lines were, 3201 cells, which are FeLV-negative lymphoid tumor cell line (19), and feline fibroblast cell lines represented by feline embryonic fibroblast cell line (FEA) and another feline embryonic fibroblast cell line (H927 cells) (14). The RNA was extracted from 5 independent patches of 3201 cell line, 5 independent patches of FEA cell line and one patch of H927 cell line.

RNA extraction:

Total RNA isolation was performed from 10 selected tissues and from three different cell lines. The RNA was extracted from the homogenized tissues or the feline cell lines using Purescript RNA isolation kit (Gentra, Minneapolis, MN). RNAs were treated with RNase-Free DNase I (Ambion, Austin, TX), extracted with phenol-chloroform, precipitated with isopropanol at −80°C and then washed twice in 75% ethanol. Total RNA was resuspended in DEPC water. The RNA concentration was determined by the absorbance at 260 nm and tested for DNA contamination by RT-PCR in the absence of RT using endogenous specific primers.
Quantitative Real-Time RT-PCR:

To quantify the expression of enFeLV RNA, the quantitative real time RT-PCR was performed into two separate steps, RT and real time PCR using the upstream primer RB53 which is specific for enFeLV sequences (614 to 629), 5’ ACAACgggAgCTAgTg 3’ (5,18) and the downstream primer env-4 (Chapter 2, Table 2.1). RT step was performed using 0.6 ug RNA and 2 ul volume of standard RNA transcript (2x10⁷ to 20 copy). The RNA was subjected to first-strand cDNA synthesis using reverse transcriptase (Gibco BRL) according to the manufacturer’s instructions for 1 hour at 55°C in a 10-ul reaction volume containing 1 uM of the downstream primer, env-4. After completion of first strand synthesis, two microliter of the RT reaction was added to the real time PCR reaction in a total volume of 20 ul containing 0.225 uM for each primer, 2 mM Mg and 2 ul of the supplemented reaction mix (Roche). The reaction was run at 95°C for 8 minutes and at 60°C annealing temperature for 10 seconds, 74°C extension for 10 seconds, 95°C denaturing for 5 seconds for 40 cycles.

The RNA samples were adjusted to approximately the same concentration for real-time RT-PCR. The loading control for the RNA samples was based on the copy number of GAPDH RNA gene determined by a parallel quantitative real time RT-PCR assays. The GAPDH gene primer sequences are 5’ gCCgTggAATTTgCCgT 3’ as the sense primer and 5’ gCCATCAATgACCCCTTCAT 3’ as the antisense primer.
Construction of the standard RNA transcript:

To construct the standard RNA transcripts for the quantification of enFeLV and GAPDH gene, RT-PCRs were conducted with 0.5 ug 3201 total RNA using env-1 and env-4 premiers (Chapter 2, Table 2.1) or GAPDH RNA specific primers. The RT-PCR products were cloned into pCR2.1 Topo vector according to the manufacture’s instructions (Invitrogen) and used as the template for PCR using M13 forward and M14 reverse primers. The PCR products of M13F and M13R primers have the T7 promoter downstream of the cloned fragment so that these products can be used for \textit{in vitro} transcription assay (Ambion, Austin, TX). The \textit{in vitro} transcription products generated by T7 polymerase were used as the template for the standard RNA transcript for the quantitative RT-PCR.
RESULTS

Lymphoid cell line expresses more enFeLV RNA than fibroblast cell lines:

To test the enFeLV RNA expression in 3201 cells and fibroblast cells (FEA and H927 cell lines), genomic RNA was extracted from 5 different cultures of either 3201 cells or the fibroblast cells. The quantitative RT-PCR analyses were conducted using enFeLV env specific primers, RB53 and env-4 (Chapter 2, Table 1). As an internal control, quantitative real time RT-PCR for GAPDH gene was conducted in parallel and the results of the enFeLV RNA copy number was adjusted based on the copy number of GAPDH RNA. The results of the quantitative RT-PCR showed that fibroblast cell lines (FEA and H927) express enFeLV RNA but at a low level. The enFeLV RNA expression by 3201 cells was found to be 181 times more than its expression in the fibroblast cells (Table 5.1).

Endogenous FeLV RNA is highly expressed in lymphoid tissues:

Six SPF control cats were used in this study to test the expression of enFeLV RNA in their tissues. The tissues of choices were: tonsil, spleen, thymus, lymph node, bone marrow, peripheral blood mononuclear cells (PBMC), bladder, intestine, salivary gland and brain. The genomic RNA was extracted from these tissues using Purescript RNA isolation kit. Quantitative RT-PCR analysis of enFeLV RNA expression revealed high expression for the enFeLV RNA in lymphoid organs, thymus, bone marrow, lymph nodes, spleen, tonsils, and PBMC.
High expression of enFeLV RNA was also found in bladder. Expression of enFeLV RNA was below the limit of detection, less than 30 copies/1ug RNA, in salivary gland, brain and ileum (Table 5.2).
DISCUSSION

Endogenous FeLV RNA expression was previously detected in placenta, fetal hemopoietic tissues, and some malignant lymphomas of FeLV-negative cats using in situ hybridization and liquid hybridization (2,7). In addition, enFeLV RNA was detected in feline lymphoid organs and other feline tissues as well as feline lymphoid cell lines using northern blot analysis (6). Neither of the previous studies has detected enFeLV RNA expression in fibroblast cell lines or has quantified the variation in the expression of enFeLV RNA in different tissues. The purpose of the current study was to quantify the enFeLV RNA expression in different feline tissues as well as lymphoid and fibroblast cell lines using quantitative real time RT-PCR. Because of the importance of the env expression of enFeLV in the pathogenesis of FeLV infection, the endogenous specific RT-PCR primers were developed to amplify the env region of enFeLV RNA. Using GAPDH specific primers as an internal control for RNA loading and enFeLV specific primers to amplify the enFeLV env sequences, the data of this study showed that endogenous proviral transcripts are abundantly expressed in a variety of normal feline tissues of lymphoid organs including thymus, spleen, tonsil, lymph nodes, bone marrow and PBMC. In addition, high expression of enFeLV sequences was found in the bladder. However, no expression of enFeLV RNA was detected in the brain, ileum and salivary gland. Although other studies have not detected enFeLV RNA expression in lymph nodes and spleen (7), the results of the current study were in agreement with the northern blot analysis study by McDougall, (6) where the thymus, spleen, lymph nodes and bone marrow were found to express enFeLV RNA.
It has been reported that feline fibroblast cell lines (FEA and H927) do not express enFeLV RNA sequences (2,6,7). In FeLV, the generation of recombinants and pseudotypes requires the expression of enFeLV RNA. In Chapter 2 of this dissertation, we showed that the transfection of FEA cultures with FeLV-A resulted in the generation of FeLV-recombinants and pseudotypes FeLV suggesting that enFeLV provirus is expressed in FEA culture. Furthermore, the transfection of H927 cell line with pFRA (FeLV-A) led to the generation of recombinant FeLV (Chapter 4) (3). To study the expression of enFeLV RNA in FEA and H927 cells, we have conducted quantitative real time RT-PCR and compared the FEA and H927 cells with the lymphoid cell line, 3201 cells. The quantitative real time RT-PCR analysis showed that FEA and H927 cells had low level expression of enFeLV RNA compare to the 3201 cells (181 fold difference, Table 5.1). Furthermore, we predict that the resistance of the 3201 cultures to FeLV-B infection is due to the high expression of enFeLV RNA but not to the absence of the FeLV-B receptor. In contrast, the susceptibility of the fibroblast cells to FeLV-B infection might be due to the low expression of enFeLV RNA.

An interesting observation was found when the \textit{env} region (instead of LTR region, Chapters 2 and 3) was used to quantify the level of enFeLV RNA expression in 3201 cells. The use of \textit{env} specific primers showed 100 times ($10^4$) lower enFeLV RNA expression than the use of LTR specific primers ($10^6$). This observation suggests the presence of larger number of enFeLV species with LTRs but with truncation in the SU region. This type of enFeLV should not have a role in the generation of competent FeLV recombinants since the SU region of the enFeLV is the main participant in the
The level of enFeLV RNA expression may contribute to the genetic instability of FeLV genome. The transfection of the feline fibroblast cell line with pFRA-GFP (FeLV-A tagged with green fluorescent protein) resulted in stable expression of GFP for long-term propagation in culture, almost 100% of cells are FeLV-GFP positive (3). On the other hand, the FRA-GFP in the 3201 cultures was unstable (<2% of the cells are FeLV-GFP positive) (Chapter 4, Figure 4.3). In addition, when the cats were inoculated with FRA-GFP, the virus was found to be more stable in the salivary gland tissues than in the mesenteric lymph node tissues as was determined by quantitative real time PCR (Chapter 4, Table 4.3). The cause of genetic instability of FeLV in 3201 cell line and the mesenteric lymph node tissues is likely due to the high expression of enFeLV. The high expression of enFeLV RNA in 3201 cultures or in the mesenteric lymph node tissues leads to the recombination between the FeLV-GFP and the enFeLV which may result in the deletion of the GFP gene.

In a recent study, we determined that FeLV-B had limited replication in vivo and no viremia was developed in the inoculated cats, however, FeLV-B was capable of establishing focal FeLV infections primarily of the salivary gland in neonatal cats inoculated with the cell-free virus (13). Based on the pattern of FeLV antigen distribution in the FeLV-B infected cats (Table 5.3) and on the data of the current study, the localized infection of FeLV-B in the salivary gland is permitted probably due to the lack of enFeLV RNA expression.
Likewise, the resistance of lymphoid tissues (lymph nodes, spleen, thymus, tonsil and bone marrow) to FeLV-B infection would be due to the high level of expression of enFeLV in these tissues and not to the absence of FeLV-B receptor.

The susceptibility of the bladder epithelium to FeLV-B in some cats (13) and our finding that the bladder tissues express high level of enFeLV RNA show that enFeLV-mediated resistance to FeLV-B is not ubiquitous. The finding that the enFeLV (CFE-16) truncated envelope protein (35-KDa protein) confer resistant to FeLV-B infection *in vitro* and the susceptibility of feline Q201 helper T-cell line (does not express the 35-Kda protein) to FeLV-B (6) support this idea. Alternatively, the 35-Kda envelope protein might be expressed at low level while the FeLV-B receptor is highly expressed in the bladder tissues. Another possibility is that the expression of enFeLV RNA by bladder tissue may vary within cat population. Because the data of the current study showed that the brain tissues do not express enFeLV RNA, the resistance of the brain tissues to FeLV-B (13) might be due to the lack of the expression of FeLV-B receptor. However, since the 35-Kda enFeLV envelope protein appears to be a soluble mediator of resistance to FeLV-B infection (6), it is possible that this protein confers resistance in nonexpressing cells *in vivo* in selected tissues.

In conclusion, the *in vivo* as well as the *in vitro* resistance of cells expressing enFeLV to FeLV-B demonstrates the potential for endogenous retroviruses to interfere with exogenous retrovirus infection that may provide another approach to vaccine development based on endogenous retrovirus system.
REFERENCES


Table 5.1: Summary of the results of quantitative real time RT-PCR of enFeLV RNA expression in feline cell lines. The genomic RNA was extracted from 5 independent cultures of 3201 cells or 5 independent fibroblast cultures, FEA and H927 cells. The quantitative real time RT-PCR was performed into two separate steps, RT and real time PCR using the upstream primer RB53 which is specific for enFeLV sequences and the downstream primer env-4. RT step was performed using 0.6 ug RNA or 2 ul volume of standard RNA transcript (2x10^7 to 20 copy). The RNA was subjected to first-strand cDNA synthesis using reverse transcriptase (Gibco BRL) for 1 hour at 55°C in a 10-ul reaction volume containing 1 uM of the downstream primer, env-4. After completion of first strand synthesis, two microliter of the RT reaction was added to the real time PCR reaction in a total volume of 20 ul containing 0.225 uM for each primer, 2 mM Mg and 2 ul of the supplemented reaction mix (Roche). The reaction was run at 95°C for 8 minutes and at 60°C annealing temperature for 10 seconds, 74°C extension for 10 seconds, 95°C denaturing for 5 seconds for 40 cycles. The RNA samples were adjusted to approximately the same concentration for real-time RT-PCR. The internal control for the RNA samples was based on the copy number of GAPDH gene determined by a parallel quantitative real time RT-PCR. The RT-PCR assays were repeated five times with five independent samples.
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<th>H927 cell line</th>
</tr>
</thead>
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<td></td>
</tr>
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<td>$1.2 \times 10^2$</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>$2.84 \times 10^2$</td>
<td></td>
</tr>
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<td>$2.64 \times 10^2$</td>
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</tr>
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<td>$1.8 \times 10^2$</td>
<td>$1.8 \times 10^2$</td>
</tr>
<tr>
<td>Average</td>
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<td>$2.5 \times 10^2$</td>
<td>$1.8 \times 10^2$</td>
</tr>
</tbody>
</table>

Table 5.1
Table 5.2: Summary of results of quantitative real time RT-PCR of enFeLV RNA expression in feline tissues. Genomic RNA was extracted from six SPF control cats using Purescript RNA isolation kit. From each animal, the RNA was extracted from tonsil, spleen, thymus, lymph node, bone marrow, peripheral blood mononuclear cells (PBMC), bladder, intestine, salivary gland and brain. The quantitative real time RT-PCR was performed into two separate steps, RT and real time PCR using the upstream primer RB53 which is specific for enFeLV sequences and the downstream primer env-4. RT step was performed using 0.6 ug RNA or 2 ul volume of standard RNA transcript (2x10^7 to 20 copy). The RNA was subjected to first-strand cDNA synthesis using reverse transcriptase (Gibco BRL) for 1 hour at 55°C in a 10-ul reaction volume containing 1 uM of the downstream primer, env-4. After completion of first strand synthesis, two microliter of the RT reaction was added to the real time PCR reaction in a total volume of 20 ul containing 0.225 uM for each primer, 2 mM Mg and 2 ul of the supplemented reaction mix (Roche). The reaction was run at 95°C for 8 minutes and at 60°C annealing temperature for 10 seconds, 74°C extension for 10 seconds, 95°C denaturing for 5 seconds for 40 cycles. The RNA samples were adjusted to approximately the same concentration for real-time RT-PCR. The internal control for the RNA samples was based on the copy number of GAPDH gene determined by a parallel quantitative real time RT-PCR. The detection limit was determined based on the standard RNA transcript.
RNA copy Number/ug total RNA

<table>
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<tr>
<th>Animal</th>
<th>SG</th>
<th>Tonsil</th>
<th>Spleen</th>
<th>Brain</th>
<th>Thymus</th>
<th>Bladder</th>
<th>PBMC</th>
<th>MLN</th>
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<th>BM</th>
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</tbody>
</table>

enFeLV RNA copy number/ug total RNA. SG, salivary gland; PBMC, peripheral blood mononuclear cell. MLN, mesenteric lymph node. BM, bone marrow. nd, not done.

Table 5.2: Summary of results of quantitative real time RT-PCR of enFeLV RNA expression in feline tissues.
<table>
<thead>
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<tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>

+, positive for FeLV antigen. - negative for FeLV antigen. nd= not done because tissue was not available for examination.

**Table 5.3: FeLV antigen distribution in tissues from FeLV-1B3 infected neonatal cat.** The tissues samples were fixed in 100% methanol and stained with ant-FeLV Gag specific antigen (13)
PERSPECTIVES AND FUTURE DIRECTIONS

The purpose of this research project was to extend our understanding of the factors contributing to FeLV recombination by evaluating the host cell/virus interactions of two closely related molecular clones of FeLV-A, pFRA and pF6A. In addition, we investigated enFeLV RNA expression in feline tissues and cell lines and its role in the genetic stability of a pFRA vector containing green fluorescent protein transgene (GFP) in vivo and in vitro as a means to better understanding the dynamics of the recombination process. These studies have shed light on the contribution of enFeLV RNA expression to the recombination variation between F6A and FRA, and the stability and infectivity of FeLV-A in vivo and in vitro. Our findings revealed the pivotal role that the copackaging of endogenous with exogenous FeLV RNA plays in the emergence of novel pathogenic retroviral agents. Furthermore, elucidating factors, such as RT variation, which impact recombinogenic potential are important for the development of retroviral based gene therapy vectors, retroviral based vaccines, and the field of xenotransplantation.

Historically, the study of genetically simple retroviruses such as feline leukemia virus laid the foundation upon which modern cancer genetics and retrovirus research are based. While no genetically simple retrovirus has been identified as an etiological agent for human disease, continued FeLV research may lead to the future
discovery of novel proto-oncogenes, novel tumor suppressor genes, modified-live retroviral vaccines and retroviral gene therapy vectors, which are important for the advancement of human medicine.

As with most research studies, the work described in this dissertation poses many new questions and, in this case, provide technical and theoretical avenues for the pursuit of answers. As future directions, based on the current understanding of the FeLV system, the following three major studies may be considered:

**First:** Identify factors that are involved in the recombination variation between F6A and FRA with enFeLV. This will be done through considering the following experiments:

1. **Switching env region:**

   The spread of the FeLV-A into the infected culture correlate with the amount of the packaged enFeLV RNA, the higher the number of infected cells the higher the amount of the packaged enFeLV RNA. In our studies, we showed that the FRA-transfected 3201 cultures had higher number of positive cells at day 21 and day 28 post-transfection than the F6A-transfected 3201 cells. The spread efficiency of the FeLV into the infected culture is related to the envelope properties of the virus. The env regions of F6A and FRA have a number of amino acid differences scattered in the SU domain. Can these variations in the env region affect the recombination rate of pF6A and pFRA with enFeLV in the transfected 3201 cultures through changing the infectivity kinetics of the virus? To answer this question, the objective of this study will be to investigate the role of env region in the recombination variation between pF6A and pFRA proviruses by switching the env gene between pF6A and pFRA.
using restriction enzymes and subsequent ligation. The resulting chimeras will be used to transfect 3201 cells and the rate of recombination will be determined by inoculating HT1080 cells with the supernatants of the transfected 3201 cells. The enFeLV RNA packaging will be determined in the chimera virions using quantitative real time RT-PCR. If the variation in the env region is the cause for the delay of recombinant generation in the F6A-transfected 3201 cells, we expect F6A/FRA-env (F6A with FRA env gene) virion to contain more enFeLV RNA and to be more recombinogenic than FRA/F6A-env (FRA with F6A env gene).

If the env gene was found to be the cause for the recombination variation between F6A and FRA, the remainder of the study will be to narrow down the variation to specific amino acid residues within the responsible region. For this purpose, site directed substitution mutants will be constructed to determine the amino acids responsible for the differential effect.

2- Substitution mutations in the pol region:

In Chapter 3, the pol region between pF6A and pFRA was switched and the resulted chimeras, pF6A/FRA-pol and pFRA/F6A-pol, were used to study the role of the genetic differences in pol region between pF6A and pFRA in the recombination with enFeLV sequences. Unlike the parent viruses, the transfection of 3201 cells with these chimeras did not lead to the generation of recombinant FeLV indicating that the switch of pol region between pF6A and pFRA resulted in the loss of the recombination potential with enFeLV sequences. This finding brings the opportunity to determine the amino acids that are critical in recombination mechanism. As a result, the objective of this experiment will be to conduct substitution mutational
analysis in the pol region between pF6A and pFRA to identify the critical amino acids that lead to the loss of recombination with enFeLV. The 3201 cells will be transfected with the mutants FeLV and the supernatant of the transfected 3201 cells will be used to inoculate HT1080 cells as a determinants for recombinant generation.

Second: In preparation for in vivo experiment to study the spread of the virus from the site of inoculation, the GFP gene was inserted 3' of env gene in the FRA provirus. pFRA-GFP inoculation in vivo and in 3201 cells resulted in unstable FRA-GFP virus with less than 2% of the FRA-GFP expressing GFP as was determined by quantitative RT-PCR, GFP expression and immunofluorescent staining assays.

In another study, pF6A/FRA-pol (F6A with FRA pol) inoculation in 3201 cells resulted in highly infectious virus as was determined by 81 C focus forming assay, p27 ELISA assay, and immunofluorescence envelope-staining assay. However, no recombinant virus was detected in the pF6A/FRA-pol-transfected 3201 cells, as determined by the inoculation of HT1080 cells and nested PCR, indicating that pF6A/FRA-pol is more stable than the pF6A and pFRA. As a result, the objective of this study will be to generate stable FeLV-GFP vector by inserting the GFP gene 3' of the env gene of the pF6A/FRA-pol. We expect this vector to be more stable than pFRA-GFP. The stability of pF6A/FRA-pol-GFP vector will be evaluated by inoculating 3201 and H927 cells. If the vector was found to be stable, as will be determined by direct checking of GFP expression, quantitative RT-PCR and sequencing studies, it will be used to study the spread of the virus from the site of inoculation in vivo.


green fluorescent protein reporter exhibits in vitro biological properties similar to those of the parental FeLV-A. J. Virol. 75:8837-8841.


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68. **Hofmann-Lehmann, R., J. B. Huder, S. Gruber, F. Boretti, B. Sigrist, and H. Lutz.** 2001. Feline leukaemia provirus load during the course of


77. **Johann, S. V., J. J. Gibbons, and B. O'Hara.** 1992. GLVR1, a receptor for gibbon ape leukemia virus, is homologous to a phosphate permease of Neurospora crassa and is expressed at high levels in the brain and thymus. J. Virol. 66:1635-1640.


129. **Snyder, C. S. and M. J. Roth.** 2000. Comparison of second-strand transfer requirements and RNase H cleavages catalyzed by human immunodeficiency virus type 1 reverse transcriptase (RT) and E478Q RT. *J. Virol.* 74:9668-9679.


148. van der Kuyl, A. C., J. T. Dekker, and J. Goudsmit. 1999. Discovery of a new endogenous type C retrovirus (FcEV) in cats: evidence for RD-114 being an FcEV(Gag-Pol)/baboon endogenous virus BaEV(Env) recombinant. J. Virol. 73:7994-8002.


