MOLECULAR DETECTION AND ANALYSIS OF FELINE LEUKEMIA VIRUS (FeLV) LONG TERMINAL REPEAT (LTR) SEQUENCES IN NEOPLASTIC AND NON-NEOPLASTIC FeLV-INDUCED DISEASES OF DOMESTIC CATS

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MOLECULAR DETECTION AND ANALYSIS OF FELINE LEUKEMIA VIRUS (FeLV) LONG TERMINAL REPEAT SEQUENCES IN NEOPLASTIC AND NON-NEOPLASTIC FeLV-INDUCED DISEASES OF DOMESTIC CATS

The feline leukemia virus (FeLV) causes degenerative and proliferative hemolymphatic diseases in domestic cats. Some cats with clinicopathologic evidence of FeLV infection have no detectable FeLV antigen within tissues. To test the hypothesis that FeLV provirus is present but not expressing antigen in some cats, a region of the FeLV long terminal repeat (LTR) was detected using the polymerase chain reaction (PCR) and compared to FeLV antigen detection using immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (ELISA), in tissues from cats with suspected FeLV-related disease.

Using formalin-fixed, paraffin-embedded lymphosarcomas (LSAs) from 70 cats, FeLV DNA was detected significantly more often than antigen in tumors from cats $\geq$7yr. Conclusions were that latent or defective FeLV may be more prevalent in LSAs from older cats.

Using peripheral blood samples from 68 cats with varying evidence of FeLV-related disease, there was no difference in the prevalence of FeLV DNA compared to antigen. However, the possibility of non-replicating FeLV DNA existing in tissues other than peripheral blood could not be ruled out.

Previous studies suggest T but not B lymphocyte transformation by FeLV, however more sophisticated methods of lymphocyte phenotype identification are now available. Of 70

feline LSAs evaluated using IHC for T and B cell antigens, 67% were T cell and 27% were B cell. The B cell tumors were as likely at T cell tumors to contain FeLV DNA and antigen, however, both B and T cell tumors from older cats were more likely to contain FeLV DNA than demonstrable FeLV antigen.

To investigate FeLV genomic variance in relation to disease type, amplified enhancer regions from 33 FeLV-infected cats were sequenced and compared. No enhancer duplications within the 33 LTR sequences were found, nor were obvious correlations between nucleotide sequence and disease type. Sequences from antigen-negative LSAs contained few mutations relative to infectious virus suggesting that the LTR is unlikely to be responsible for lack of antigen expression in these tumors.

ABSTRACT

The feline leukemia virus (FeLV) causes degenerative and proliferative hemolymphatic diseases in domestic cats. Some cats with clinicopathologic evidence of FeLV infection have no detectable FeLV antigen within tissues. To test the hypothesis that FeLV provirus is present but not expressing antigen in some cats, a region of the FeLV long terminal repeat (LTR) was detected using the polymerase chain reaction (PCR) and compared to FeLV antigen detection using immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (ELISA), in tissues from cats with suspected FeLV-related disease.

In the retrospective study to investigate lymphosarcomas (LSAs) from 70 cats, FeLV DNA was detected significantly more often than antigen, particularly in tumors from cats ≥7 yr. Conclusions were that latent or defective FeLV may be more prevalent in LSAs from older cats. Older cats may have eliminated replicating FeLV, but retained non-replicating, but LSA-inducing strains of the virus.

The prospective study to investigate FeLV in peripheral blood from 68 sick cats with varying clinicopathologic evidence of FeLV-related disease, revealed no difference in the prevalence of FeLV DNA compared to antigen. The possibility exists that in test-negative cats with high suspicion of FeLV-related disease, non-replicating FeLV may have been present in tissues other

than peripheral blood cells.

Previous studies suggest T cell but not B cell transformation by FeLV. However in earlier reports, only mature B cell markers were used, and investigations involved mainly thymic (T cell) tumors. In this study, of 70 feline LSAs evaluated immunohistochemically using commercially available T and B cell antibodies, 67% were T cell and 27% were B cell. The B cell tumors were as likely as T cell tumors to contain FeLV DNA and antigen. Both B and T cell tumors from older cats were more likely to contain FeLV DNA than demonstrable FeLV antigen. Conclusions were that FeLV transforms B cells as well as T cells, although in older cats, LSA may be associated with non-replicating FeLV.

Studies with some type C retroviruses indicate that genomic variance within the LTR relates, in part, to disease manifestation. Therefore, the nucleotide sequences of LTR PCR products from natural cases of neoplastic and non-neoplastic FeLV-related disease were determined. Phylogenetic analysis of FeLV LTR sequences from tissues of 33 infected cats, showed genomic homology among 4 of 5 antigen-negative LSAs. Previously, enhancer duplications were found to be prevalent in neoplastic FeLV diseases, however no repeat sequences were found in this study. Point mutations relative to the common form FeLV were frequent, and most involved 5 single nucleotide sites both within and peripheral to nuclear protein binding motifs.

All LSAs, but particularly those not expressing antigen, contained the fewest LTR mutations compared to this region in other FeLV-related diseases. Mutations within the LTR enhancer region are unlikely to account for the lack of viral gene expression in antigen-negative LSAs. Functional studies will be required to determine how specific point mutations within the FeLV LTR relate to disease potential in infected tissues. The lack of consistent LTR changes among FeLV-induced disease groups indicates that genetic alterations within the LTR enhancer are at least not solely responsible for disease manifestation. Envelope, *gag*, and other regions of the viral genome, as well as cellular oncogenes and other host factors may act in concert to determine outcome in infected cats.

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A	adenine
ABC	avidin-biotin complex
AIDS	acquired immunodeficiency syndrome
AGE	agarose gel electrophoresis
BFU-E	burst-forming unit - erythroid
bp	base pair
C	cytosine
C3	complement component 3
CA	capsid
5'cap	5' 7-methylguanylate cap
CAT	chloramphenicol acetyltransferase
CBC	complete blood count
cDNA	complementary DNA
CFU-E	colony-forming unit - erythroid
CFU-F	colony-forming unit - fibroblast
CFU-GM	colony-forming unit - granulocyte-monocyte
CIC	circulating immune complex
CORE	simian virus 40-like core enhancer
dNTP	deoxyribonucleotide triphosphate
ddH_20	double distilled water
E-rosette	erythrocyte rosette
EAC	erythrocyte, antibody, complement
ELISA	enzyme-linked immunosorbent assay
env	envelope
FAIDS	feline acquired immunodeficiency syndrome
FeLV	feline leukemia virus
FeSV	feline sarcoma virus
FIP	feline infectious peritonitis
FIV	feline immunodeficiency virus
FLV-1	feline leukemia virus-specific binding site
FOCMA	feline oncornavirus-associated cell membrane antigen
G	guanine
GALV	gibbon ape leukemia virus
gp	glycoprotein
GPE	guinea pig erythrocyte
GRE	glucocorticoid response element
HIV	human immunodeficiency virus
IFA	indirect immunofluorescent antibody
IgG	immunoglobulin G
IHC	immunohistochemistry
IN	integrase
IR	indirect repeat
K	kilodalton
kb	kilobase
kbp	kilobase pair
L	leader
LSA	lymphosarcoma
LTR	long terminal repeat
LVb	leukemia virus factor b
MA	matrix

MCV	mean corpuscular volume
MoMuLV	Moloney murine leukemia virus
mRNA	messenger RNA
MuLV	murine leukemia virus
NC	nucleocapsid
NF1	nuclear factor 1
NP	non-producer
p	protein
PB	primer binding
PB^+	primer binding for plus strand DNA synthesis
PB^-	primer binding for minus strand DNA synthesis
PCR	polymerase chain reaction
PCV	packed cell volume
PEA2	polyomavirus A enhancer
poly A tail	polyadenylate tail
pp	phosphoprotein
PPT	polypurine tract
PR	protease
Pr	precursor protein
PRCA	pure red cell aplasia
RT	reverse transcriptase
R	terminal repeat
RNase H	ribonuclease H
SDS	sodium dodecyl sulfate
sIg	surface immunoglobulin
SSC	standard saline citrate
SU	surface
SV	simian virus
T	thymine
TM	transmembrane
Taq	*Thermus aquaticus*
TNF	tumor necrosis factor
tRNA	transfer RNA
U	uracil
U3	unique 3′ sequence
U5	unique 5′ sequence
UCR	upstream control region
UPGMA	unweighted pair group method with arithmetic mean
UVD	unintegrated viral DNA
vr	variable region
WBC	white blood cell
Ψ	packaging or encapsidation sequence

1. INTRODUCTION

The feline leukemia virus (FeLV) is a mammalian type C retrovirus and represents the most important pathogen of domestic cats (Essex, 1980; Hardy *et al.*, 1973b; Cotter *et al.*, 1975; Hardy, 1980a; Hardy, 1981d; Hardy, 1981b; Loar, 1993). Whether FeLV-related disease has been significantly reduced by recent vaccine development remains unknown (Loar, 1993; Essex *et al.*, 1985; Hardy, 1992). FeLV was first described in 1964 in transmission experiments where kittens were injected with passage material from a cat with multicentric lymphosarcoma (LSA). All 4 kittens developed LSA within 18 months and when tumor tissue was examined by electron microscopy, numerous virus-like particles were seen that resembled murine leukemia viruses (MuLVs) (Jarrett *et al.*, 1964a; Jarrett *et al.*, 1964b).

In 1970, an indirect immunofluorescent antibody (IFA) test was developed for detection of FeLV antigens in peripheral blood cells (Hardy *et al.*, 1973a). Large scale testing of cats followed, facilitating epidemiologic studies of the virus in the natural environment. These studies confirmed the contagious (horizontal) transmission of FeLV (Hardy *et al.*, 1973b; Essex *et al.*, 1985; Brodey *et al.*, 1970), and established FeLV as the main etiologic agent of not only LSA in the cat, but also neoplasms of

most other hemopoietic cells, and several cytosuppressive and immunosuppressive hemolymphatic disorders (Hardy *et al.*, 1973b; Hardy *et al.*, 1976; Cotter *et al.*, 1975; Hardy, 1981a; Mackey, 1975; Hardy *et al.*, 1976). In some regards, FeLV is a misnomer because cytosuppressive and immunosuppressive conditions leading to death, are much more common sequelae of persistent FeLV infection than are leukemia and LSA (Hardy *et al.*, 1973b; Hardy, 1981d; Hardy, 1980a; Hardy *et al.*, 1976; Hoover *et al.*, 1981a). Furthermore, FeLV-induced cytosuppressive diseases provided investigators with clues of a retroviral etiology of human acquired immunodeficiency syndrome (AIDS) (Mullins and Hoover, 1990).

The contagious (exogenous) FeLV probably originated by cross-species infection of an endogenous rodent retrovirus to ancestors of the domestic cat (Benveniste *et al.*, 1975; Todaro, 1980). In addition, endogenous FeLV-related sequences are present in the genomes of all domestic cats and related small feline species (Todaro, 1980; Benveniste *et al.*, 1975). Comparisons between endogenous FeLV-like sequences of cats and endogenous rodent type C viruses, as well as immunologic studies involving reverse transcriptase, support the hypothesis that these FeLV-related genes were transmitted from a rodent to cat ancestor (Benveniste *et al.*, 1975). Whether endogenous FeLV-like sequences originated by germ line integration of a complete exogenous virus followed by deletion, or by

integration of a preexisting, defective, deleted variant of the infectious virus is not clear (Soe *et al.*, 1983).

Although many endogenous FeLV-like sequences represent almost full-length copies, two domains diverge from the infectious exogenous FeLV. These include the 3' unique region (U3) of the RNA genome, which forms part of the proviral 5' and 3' long terminal repeats (LTRs) (Casey *et al.*, 1981; Kumar *et al.*, 1989; Berry *et al.*, 1988), and part of the *env* gene (encoding surface and transmembrane proteins) (Stewart *et al.*, 1986b; Overbaugh *et al.*, 1988b; Sheets *et al.*, 1993). Endogenous FeLV-related sequences do not encode infectious virus although subgenomic transcripts may be expressed (Soe *et al.*, 1985; McDougall *et al.*, 1994); however, they contribute to the generation of pathogenic FeLV variants through recombination with exogenous FeLV (Stewart *et al.*, 1986b; Neil *et al.*, 1991; Overbaugh *et al.*, 1988b; Tzavaras *et al.*, 1990; Mathes *et al.*, 1994).

The efficiently transmitted, ubiquitous FeLV is in itself, minimally to moderately pathogenic, but is the basis for the generation of all disease-producing FeLV forms (Neil *et al.*, 1991). These may arise by recombination with endogenous FeLV, mutation, duplication of enhancer regions, and/or altered expression of cellular genes (oncogenes) by transduction or insertional activation (Neil *et al.*, 1991; Miura *et al.*, 1989; Fulton *et al.*, 1990; Matsumoto *et al.*, 1992; Sheets *et al.*, 1993; Donahue

et al., 1991; Mathes *et al.*, 1994; Tsatsanis *et al.*, 1994; Rohn *et al.*, 1994). Pathogenicity and disease manifestation are thought to relate at least in part, to sequence variation particularly within the LTR and the *env* (Riedel *et al.*, 1986; Donahue *et al.*, 1988; Soe *et al.*, 1983; Tzavaras *et al.*, 1990; Golemis *et al.*, 1990; Fulton *et al.*, 1990; Sheets *et al.*, 1993; Donahue *et al.*, 1991; Plumb *et al.*, 1991; Matsumoto *et al.*, 1992; Rohn *et al.*, 1994), and less commonly, the *gag* region (encoding internal structural proteins) (Laprevotte *et al.*, 1984).

The incidence of FeLV infection rises progressively with time among outdoor cat populations, peaking at 3-5 years of age (Pedersen, 1990). Up to 50% of free-roaming, urban domestic cats may become infected during their lifetime, and up to 30% of these develop persistent infection and die within 3 years of diagnosis (Rogerson *et al.*, 1975; Jarrett, 1984; Hoover *et al.*, 1976). The immunologic response early in FeLV infection appears to be pivotal in determining outcome (Rojko *et al.*, 1979; Rojko and Olsen, 1984). Also, cats that are repeatedly exposed and/or exposed at an early age are most susceptible to developing persistent infection (Rogerson *et al.*, 1975).

Epidemiologic studies support FeLV involvement in some LSAs without evidence of viral replication and gene expression (Hardy *et al.*, 1980b; Francis *et al.*, 1981). Also, some cats with non-neoplastic FeLV-related disease may lack circulating viral antigen but have atypical or

sequestered infection defined as FeLV antigen expression localized to certain tissues (Hayes *et al.*, 1989; Lutz *et al.*, 1983). Other cats may develop latent infections and harbour non-replicating FeLV within their DNA; under certain circumstances, latent virus may become reactivated and presumably, cause FeLV-related disease (Pacitti and Jarrett, 1985; Madewell and Jarrett, 1983; Pedersen *et al.*, 1984; Rojko *et al.*, 1982; Post and Warren, 1980). Whether cats with sequestered or latent FeLV mount an immune response intermediate between those that recover fully and those that develop persistent viremia, remains to be determined.

Previously, latent infections were detected by administering immunosuppressive doses of corticosteroids to suspect cats or by treating bone marrow and/or lymphocyte cultures from suspect cats with corticosteroids. Using this technique which facilitates antigen expression, only replication-competent virus is detected, yet there is evidence for disease due to replication-defective FeLV (Braun *et al.*, 1985; Tzavaras *et al.*, 1990; Guilhot *et al.*, 1989; Overbaugh *et al.*, 1992). Molecular techniques to identify viral DNA rather than products of viral replication are applicable to the investigation of latent or replication-defective FeLV. With this in mind and using the polymerase chain reaction (PCR) to amplify a short exogenous FeLV U3 region distinct from endogenous FeLV, the current study was undertaken.

The prevalence of FeLV DNA was compared to the prevalence of FeLV antigen in archival LSAs from pet cats, and both were related to anatomic site of the tumor, patient age, and B or T lymphocyte phenotype. Viral antigen was also compared to viral DNA detection in peripheral blood samples from cats with high, moderate, or low clinicopathologic evidence of FeLV infection. Finally, in order to investigate a potential relationship between sequence variation in the LTR and disease manifestation, amplified FeLV U3 enhancer regions from tissues of cats with a variety of FeLV-induced diseases were sequenced and analyzed.

Herein follows a review of the FeLV literature emphasizing aspects relevant to this work, details of the hypotheses and objectives of the study, and the body of the research which is organized into a series of four manuscripts. The final section is a summary of the findings in relation to the current state and future directions of FeLV research.

2. LITERATURE REVIEW

2.1. Feline Leukemia Virus

2.1.1. Structure and Genome Organization

The FeLV is a typical mammalian type C retrovirus with a virion of about 125 nm diameter and a lipid bilayer envelope surrounding an icosahedral core which contains the nucleocapsid (Pedersen, 1990; Coffin, 1992). The FeLV virion structure is depicted in Figure 2.1. The core and envelope coassemble at the cell membrane during budding, with no visible cytoplasmic intermediates (Harrison, 1990; Coffin, 1992). The immature virion has ill-defined surface projections, and a large, open spherical core which condenses and centralizes upon maturation, presumably related to cleavage of the *gag* precursor protein (Pedersen, 1990; Coffin, 1992; Harrison, 1990). The surface projections are formed by the two protein subunit products of the *env* gene (Coffin, 1990; Coffin, 1992). The internal core is comprised of *gag*-encoded structural proteins and *pol*-encoded reverse transcriptase and integrase proteins, in association with the RNA genome (Luciw and Leung, 1992). The virion also contains several small nucleic acid molecules believed to be derived accidently from host cells, and of no consequence. However, a single molecule of host acquired prolyl transfer RNA (tRNA), specifically

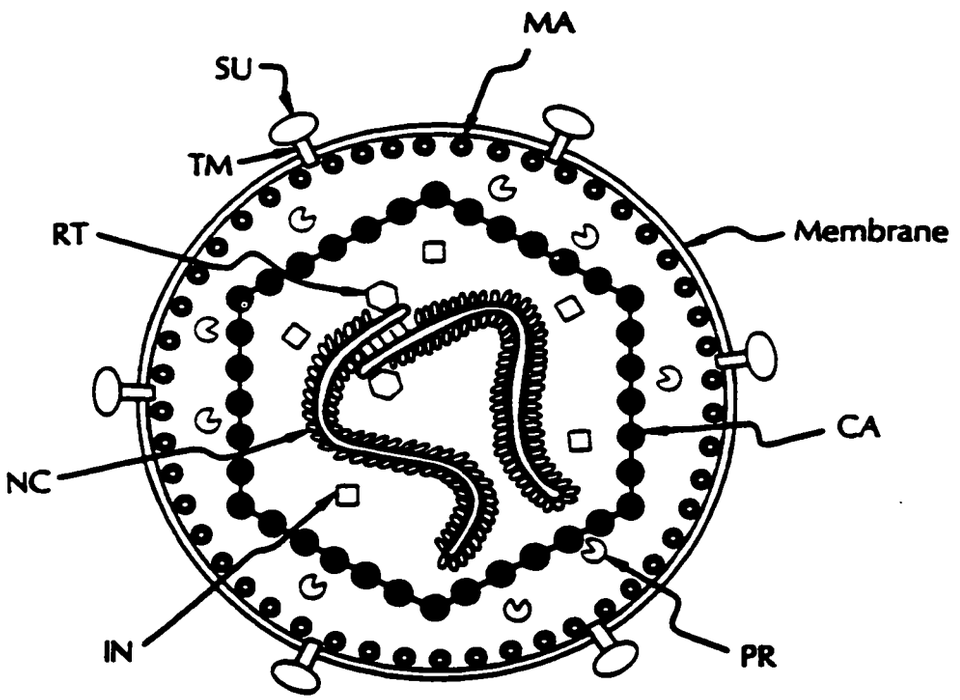

Figure 2.1. Structure of the mature retrovirion. The surface (SU) glycoprotein, which mediates binding of the virion to the host cell, and the transmembrane (TM) protein that anchors SU to the envelope are products of the *env* gene. The *gag* gene products are internal structural proteins. The MA protein forms a matrix immediately beneath the viral envelope. The CA protein forms the prominent capsid structure that is seen in electron micrographs and which surrounds the nucleocapsid complex composed of the RNA genome and the NC protein. The reverse transcriptase (RT) and integrase (IN), products of the *pol* gene, are also found within the capsid. The location of the protease (PR) in the mature virion is uncertain but evidence suggests a position between the capsid and membrane. (Reproduced from Wills, 1991, with permission).

associates with the viral RNA genome by base-pairing to the primer binding site near the 5′ end (Hampe *et al.*, 1983; Luciw and Leung, 1992; Donahue *et al.*, 1988). This primes the initiation of DNA synthesis during early infection (Coffin, 1992).

The FeLV RNA and proviral genomes are shown in Figure 2.2. The FeLV RNA genome consists of two identical molecules of positive sense, single stranded RNA. The two strands are bound noncovalently, and in the prototype FeLV subgroup A virus, each is about 8.4 kilobases (kb) in length (Donahue *et al.*, 1988). The viral RNA is modified similar to cellular messenger RNA (mRNA) with a 7-methylguanylate cap at the 5′ end (5′cap) and a 3′polyadenylate tail (poly A tail) (Coffin, 1990).

In virion RNA, a terminal repeat sequence (R) follows the 5′cap site and also occurs just before the 3′poly A tail. The 5′R is followed by the unique sequence, U5, and then the 18 base tRNA binding site, the primer binding (PB) site for minus strand DNA synthesis. The untranslated leader sequence (L) of about 200 bases is immediately 3′ to the PB site, and contains the splice donor site for generation of subgenomic mRNAs, and the encapsidation or packaging sequence (Ψ) which is involved in virion assembly (Neil and Onions, 1985; Bender *et al.*, 1987; Donahue *et al.*, 1988).

The genes encoding viral proteins, which account for about 7.6 kb of the RNA genome, follow in the

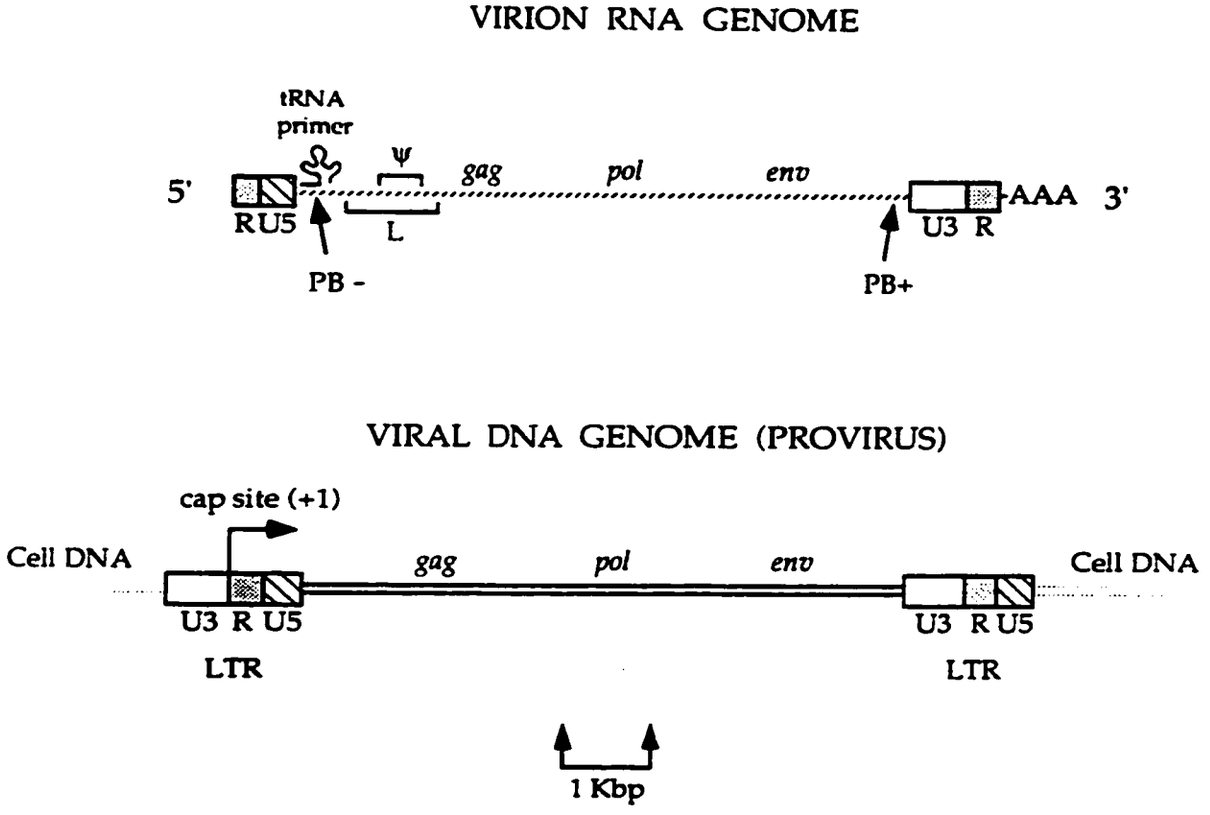

Figure 2.2. *Cis*-acting elements in genomic viral RNA. The 5′ end of viral RNA has a cap structure (7 Mg) and the 3′ end has a poly A tail. R is the short repeat at each end of the genome, U5 is a unique sequence element immediately after the 5′ R sequence, PB- is the primer site for minus strand DNA synthesis (tRNA binding site), L is the leader region before the start of *gag*,Ψ is the element required for assembly of viral RNA into virions, PB+ is the primer site for plus strand DNA synthesis, and U3 is a unique sequence at the 3′ end of the genome. Replication-competent retroviruses have genomes about 8 kb in length. This figure also reveals the relationship of the U3, R, and U5 elements in viral RNA with respect to the LTRs in linear viral DNA. Genes encoding virion polyproteins *gag*, *pol*, and *env* are also shown; the RNA and DNA genomes are co-linear. (Reproduced from Luciw, 1992, with permission).

characteristic order - *gag*, *pol*, and *env*, followed by the binding site for plus-strand DNA synthesis, and the U3 sequence. The U3 contains enhancer and promoter elements that control transcription of the integrated provirus by cellular RNA polymerase II (Luciw and Leung, 1992; Coffin, 1990; Varmus, 1988). In the RNA genome, the U3 is followed by a second copy of R and the poly A tail.

The main difference between the genomes of retroviral RNA and DNA is the formation of LTRs at each end of the provirus during reverse transcription and DNA synthesis (Hampe *et al.*, 1983). Each FeLV LTR is about 482 base pairs (bp) long, and is comprised of U3-R-U5 sequences representing about 340, 68, and 74 nucleotides, respectively (Donahue *et al.*, 1988; Hampe *et al.*, 1983).

2.1.2. Gene Products

The FeLV genome contains the *gag*, *pol*, and *env* open reading frames present in all retroviruses. No accessory genes, such as those involved in transcriptional control of human, simian, and bovine retroviruses, have been identified in FeLV (Mullins and Hoover, 1990). Genomic length viral mRNA encodes the *gag* and *pol* gene products, but the *env* protein is encoded by a singly spliced subgenomic viral mRNA of about 3 kb (Coffin, 1990; Papenhausen and Overbaugh, 1993; Mullins and Hoover, 1990). No other viral mRNAs are found in FeLV-infected cells (Mullins and Hoover, 1990; Papenhausen and Overbaugh, 1993).

Figure 2.3 depicts the coding sequences of the FeLV provirus in relation to the structural proteins of the virion. *Gag* and *pol* products are synthesized on free ribosomes as cytoplasmic proteins, whereas the *env* proteins are translated on membrane-bound polyribosomes (Coffin, 1990; Neil and Onions, 1985).

The most abundant *gag* gene product is a non-glycosylated 65 kilodalton (K) precursor polyprotein (Pr gag65) that is later cleaved by virus-encoded protease, into 4 major structural (capsid) proteins (Coffin, 1990; Mullins and Hoover, 1990; Thomsen *et al.*, 1992; Neil and Onions, 1985; Wills and Craven, 1991). These include: a 15K matrix (MA) protein (p15), 12K phosphoprotein (pp12) of unknown function, 27K capsid (CA) protein (p27), and 10K highly basic nucleocapsid (NC) protein (p10) (Hardy, 1992; Thomsen *et al.*, 1992). The MA protein lines the inner face of the viral envelope, and probably directs *gag* precursor proteins to the site of assembly on the plasma membrane (Wills and Craven, 1991; Coffin, 1992). The CA protein forms the hydrophobic core of the virion, and may form the casing housing reverse transcriptase (RT) and integrase (IN), which are needed for synthesizing and integrating the DNA intermediate into the host genome (Wills and Craven, 1991). The CA protein is the most readily detectable antigen and is the basis for most FeLV immunoassays (Coffin, 1992; Jarrett, 1991; Tonelli, 1991). The NC coats the two copies of the viral RNA genome, and appears to have

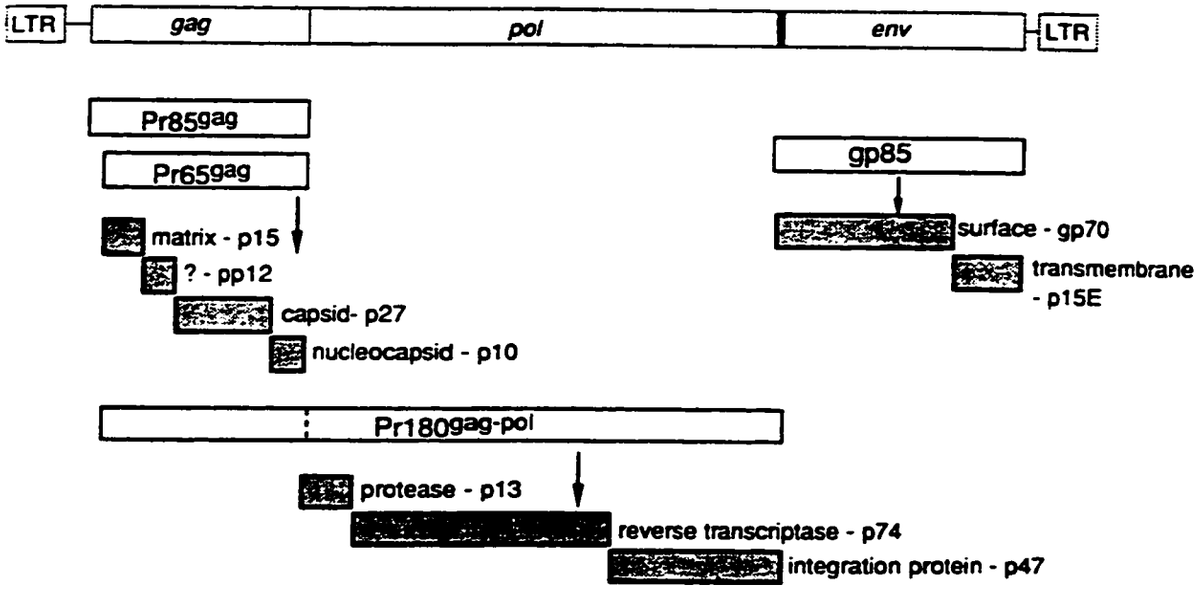

Figure 2.3. Deduced and known FeLV proteins. The coding segments for each protein are shown at the top of the figure. Mature proteins are shaded. The observed or, in the case of protease and integration proteins, deduced molecular weights of the mature proteins are listed adjacent to their expected functions. The functions of some of the *gag* and *pol* viral proteins have not been independently verified; rather they are identified by virtue of their gene order and homology to their murine virus counterparts. (Reproduced from Mullins, 1990, with permission).

a role in packaging the viral genome into budding virus (Wills and Craven, 1991; Coffin, 1992).

In addition to the non-glycosylated *gag* protein, a glycosylated *gag* precursor [gp75 *gag* (Neil and Onions, 1985), or Pr85 *gag* (Mullins and Hoover, 1990)] is also synthesized, processed, and shed from the cell surface (Neil *et al.*, 1980; Laprevotte *et al.*, 1984). The glycosylated protein is less abundant than the non-glycosylated form presumably because the gp75 *gag* protein is initiated at an AUG codon which is surrounded by sequence configuration less favourable for translation (Neil and Onions, 1985). The significance of glycosylated *gag* proteins in FeLV infection is not clear, but the 40K protein released from infected cells is precipitable with antiserum to p27 and may be involved in anti-*gag* antibody production and recovery, or immune complex formation and disease progression (Neil *et al.*, 1980).

Gag and *pol* are encoded by the same translational reading frame, but separated by a stop codon. The *pol* gene is expressed as a *gag-pol* fusion protein due to glutamyl tRNA suppression of the TAG stop codon resulting in glutamine residue incorporation within the 5′ pol-protease gene (Donahue *et al.*, 1988; Mullins and Hoover, 1990). The 180K *gag-pol* polyprotein (Pr180 *gag-pol*) is cleaved to PR (p13), RT (p74), and IN (p47) (Donahue *et al.*, 1988; Mullins and Hoover, 1990). Processing of precursor to mature proteins is by protease, and the first 4 amino acids

of protease are, in fact, derived from the *gag* gene (Yoshinaka *et al.*, 1985). Reverse transcriptase is necessary for viral DNA synthesis through its RNA directed polymerase property, and removal of RNA template during DNA synthesis with its ribonuclease H (RNase H) activity (Coffin, 1992; Coffin, 1990). The synthetic and nucleolytic activities are in genetically separable domains in the order RT-RNase H (Coffin, 1992), with the polymerase covering about two-thirds of the amino terminal (Coffin, 1990). The IN protein is the smaller, carboxyl terminal cleavage product of the *pol* gene protein (Coffin, 1990). The IN protein provides enzymatic activities for integration of the viral DNA into the host DNA, including nucleolytic activities to trim the 3′ ends of viral DNA and to cleave the host DNA, as well as joining the viral and cellular DNA (Coffin, 1992).

The *env* gene product is an 80-85K precursor protein which is modified by glycosylation and cleavage into surface (SU) (gp70) and transmembrane (TM) (p15E) proteins which remain associated by disulfide and noncovalent bonds (Coffin, 1992; Mullins and Hoover, 1990; Neil and Onions, 1985). The larger gp70 SU protein contains the receptor-binding function and stimulates the major neutralizing antibodies in the host (Coffin, 1990). The smaller p15E TM protein is not glycosylated, is further processed to p12E (Neil and Onions, 1985), and has three domains. The TM external domain is attached to the SU protein and contains

an amino terminal hydrophobic region necessary for membrane fusion. The membrane-spanning domain is also hydrophobic and anchors the envelope protein complex to the virus envelope. The cytoplasmic domain is not necessary for viral replication in all cases, and its function is not clearly established (Coffin, 1992; Coffin, 1990). Deduced FeLV p15E proteins are highly conserved, however, gp70 amino acid sequences are divergent (Donahue *et al.*, 1988) and form the basis for A, B, and C subgroup classification (Rosenberg *et al.*, 1980; Luciw *et al.*, 1985; Donahue *et al.*, 1988).

2.1.3. Replication

The FeLV is thought to follow the replication cycle of all retroviruses as shown in Figure 2.4. Mature FeLV particles can infect only cells with receptors for the envelope gp70, however, cellular receptors for FeLV are not well characterized (Neil *et al.*, 1991; Takeuchi *et al.*, 1992; Ghosh *et al.*, 1992). Epidemiologic studies have shown that cats are infected oronasally by viral entry into local epithelial and lymphoid cells of the head and neck (Hardy, 1992). Once FeLV is bound to the host cell membrane, viral and cellular membranes fuse, and the virion core is released into the cytoplasm (Luciw and Leung, 1992). Fusion is controlled by the external hydrophobic domain of the TM (p15E) envelope protein, perhaps through a conformational change induced by binding of the gp70 SU protein to its receptor (Luciw and Leung, 1992). Genomic

unintegrated viral DNA (complexed with viral integrase and *gag* proteins) to the nucleus, and integration into host cell DNA to produce a provirus. Viral DNA is detected in the cytoplasm at about 2 hr after infection, full-length linear viral DNA molecules start to appear in the nucleus about 4-6 hr later, and proviral DNA is first detected about 12 hr after infection. The late phase starts with synthesis of viral transcripts from the provirus in the nucleus at about 15 hr after infection and continues through to release of progeny virions (about 24 hr after infection). Full-length viral transcripts serve as genomic RNA and as mRNA for *gag* and *pol* polyproteins; *env* gene mRNA is generated from the full-length viral transcript by splicing. Free cytoplasmic polysomes are the site of translation of mRNA for viral polyproteins derived from the *gag* and *pol* genes; these polyproteins subsequently associate with genomic viral RNA to form an intracellular core structure. The mRNA for *env* polyprotein is translated on membrane-bound polysomes; *env* polyprotein is modified by glycosylation and proteolytic cleavages mediated by host cell enzymes and then inserted into the cell plasma membrane. Through the budding process, immature cores are engulfed by the cell plasma membrane and thus acquire a lipid bilayer membrane which contains *env* proteins. Proteolytic processing of *gag*, and *pol* polyproteins takes place during the budding step and continues in newly released particles (immature virions) to produce mature infectious virions. The time course indicated above for major events in a single replication cycle is based on observations in actively growing tissue culture cells that have not been synchronized with respect to the cell cycle. (Reproduced from Luciw, 1992, with permission).

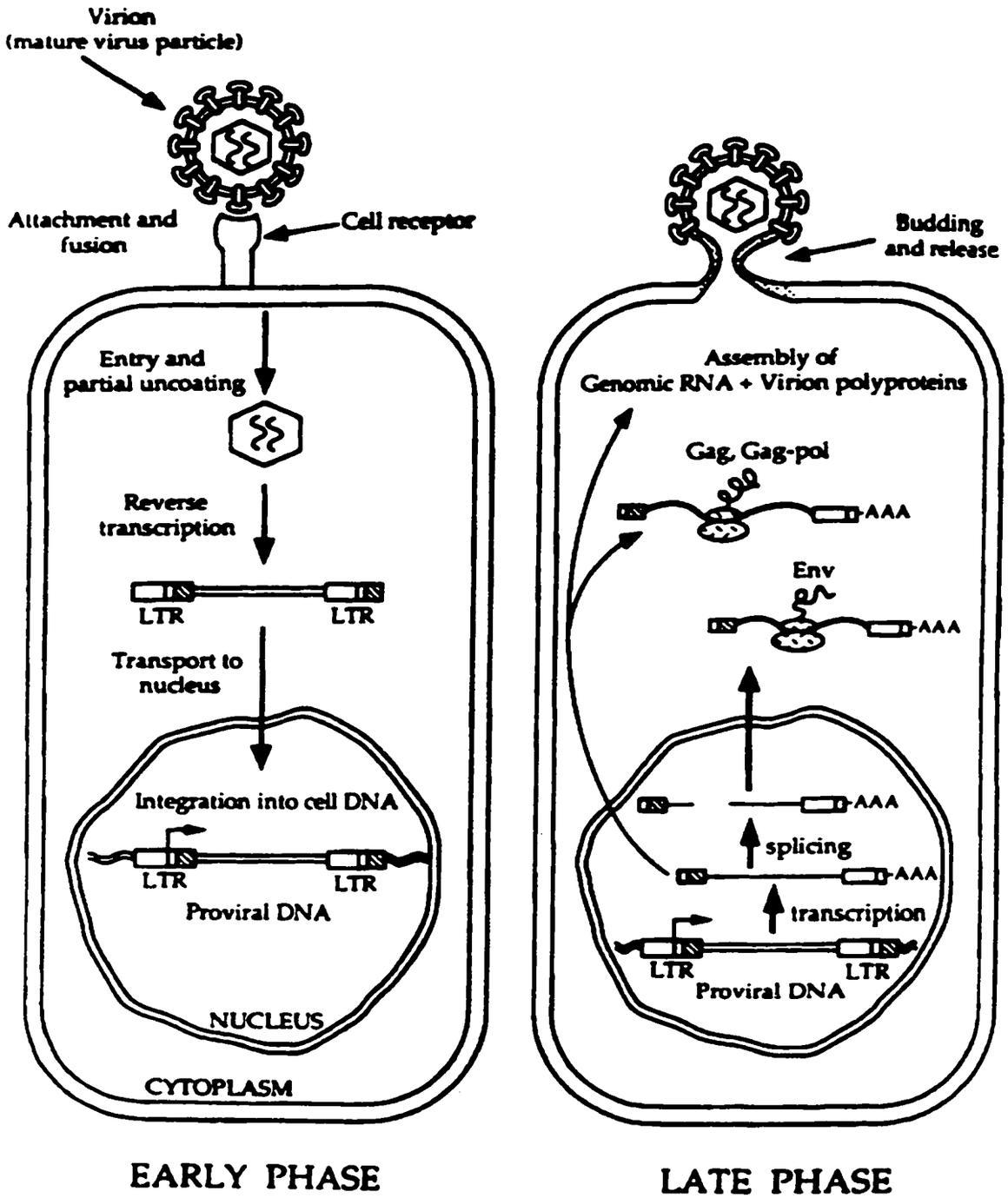

Figure 2.4. Major features of the replication cycle of a prototype retrovirus. The early phase of replication involves attachment of a virion to a receptor on the cell surface, entry and uncoating, viral DNA synthesis by reverse transcription in the cytoplasm, transport of

viral RNA may remain associated with CA, NC, RT, IN, and perhaps PR proteins in a cytoplasmic nucleoprotein particle (Luciw and Leung, 1992).

Viral DNA synthesis occurs by reverse transcription starting at the tRNA primer near the 5′ end of the RNA genome, as shown in Figure 2.5. The prolyl tRNA binds to the PB site, a complementary region of the viral genome. The RT elongates from the 3′ end of the tRNA to the 5′ end of viral RNA to produce minus strand DNA for the region between the PB site and the 5′ end of the viral RNA, including R and U5. The RNase H activity of RT degrades R and U5 from the 5′ end of viral RNA genome so that the newly synthesized single stranded R DNA is available for base pairing to the complementary R at the 3′ end of either the same viral RNA molecule or the associated identical strand. This pairing of the DNA with R at the opposite end represents the first template "jump". Negative strand synthesis then proceeds through to the PB site at the 5′ end, since R and U5 have been removed by RNase H.

A plus strand DNA copy of the newly synthesized minus strand DNA is then generated. Plus strand synthesis begins at a binding site, known as the polypurine tract (PPT), immediately 5′ to U3, after RNase H cleavage of the RNA template. Reverse transcriptase synthesizes plus strand DNA by elongation 5′ to 3′ so that U3-R-U5 and PB are copied. A modified base in the tRNA molecule sets the precise 3′ end of this segment of positive strand DNA.

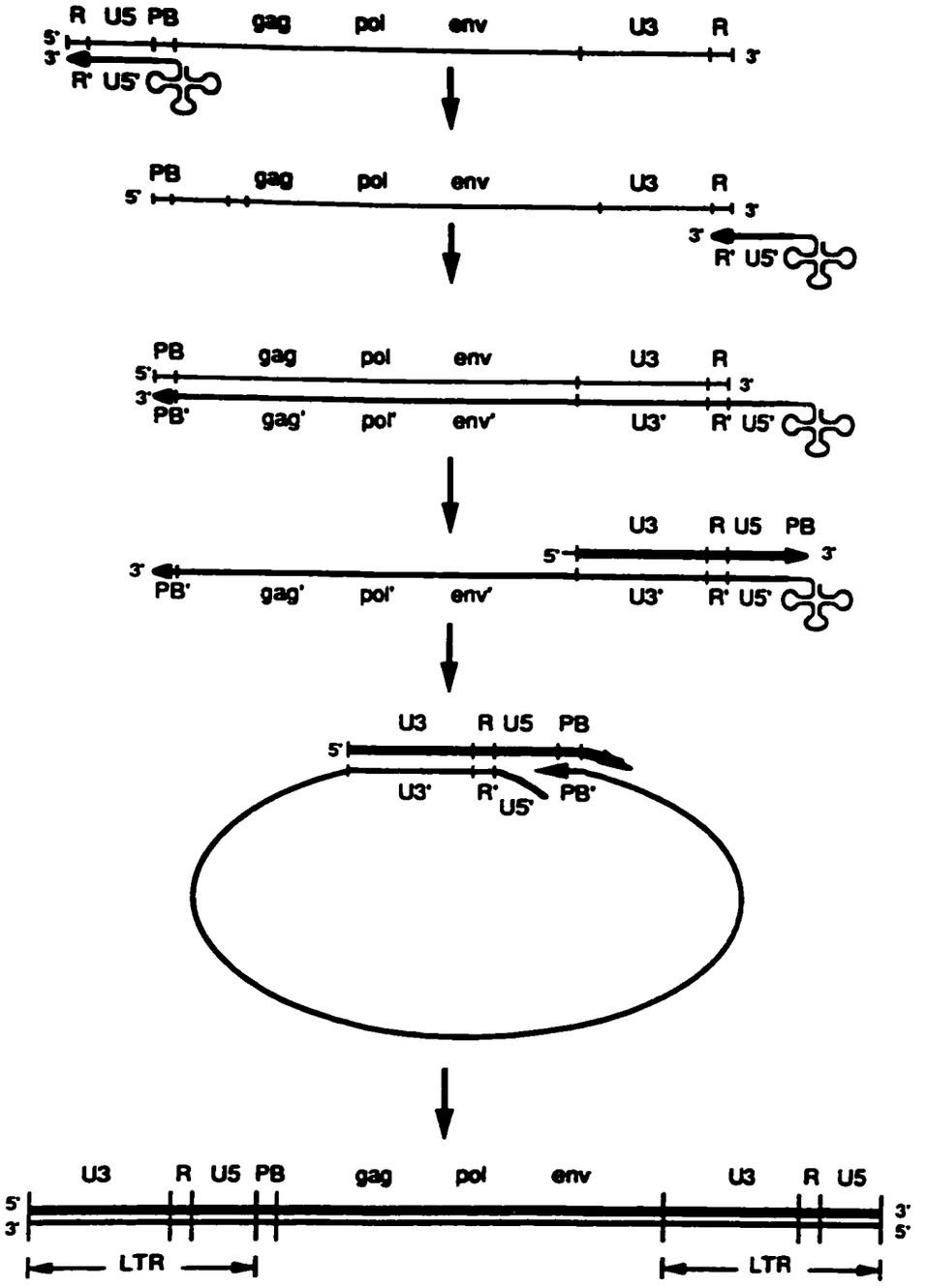

Figure 2.5. Mechanism of viral DNA synthesis. Thin lines depict RNA, medium lines depict negative strand DNA, and thick lines denote positive strand DNA. Negative sense sequences are also indicated by a "prime". (Reproduced from Coffin, 1990, with permission).

RNase H degrades the tRNA so PB can then form another primer pair, the second template "jump", with its complement at the 3′ end of the negative strand. Genomic RNA is degraded at the same time to make way for the plus strand DNA. The final product is duplex linear DNA with identical LTRs and blunt termini, which is in a cytoplasmic nucleoprotein complex containing IN and other virion proteins. This complex is subsequently transported into the nucleus for integration into the host cell genome to produce the provirus (Luciw and Leung, 1992; Coffin, 1990; Varmus, 1988; Temin, 1993).

Viral integration first involves removal of the 3′ terminal two bases, probably TT, from either end of viral DNA by a dimer or tetramer of the IN protein (Figure 2.6). Then IN makes a staggered cut in cellular DNA and catalyzes single-strand joining of the recessed dinucleotide CA 3′ ends of viral DNA to these cellular DNA 5′ overhangs of 4-6 bases (Luciw and Leung, 1992; Coffin, 1990). Host DNA repair enzymes, or possibly IN, remove mismatched AA dinucleotide overhangs at 5′ viral ends and seal single-stranded regions in target DNA (Kulkosky and Skalka, 1994). Therefore, direct repeats occur at the site of integration which are characteristic for the infecting virus and not the cell type (Luciw and Leung, 1992; Coffin, 1990). Although nucleotide sequence does not appear to determine integration site, there are preferred sites or "hot spots" in the host cell genome. The need for cellular DNA

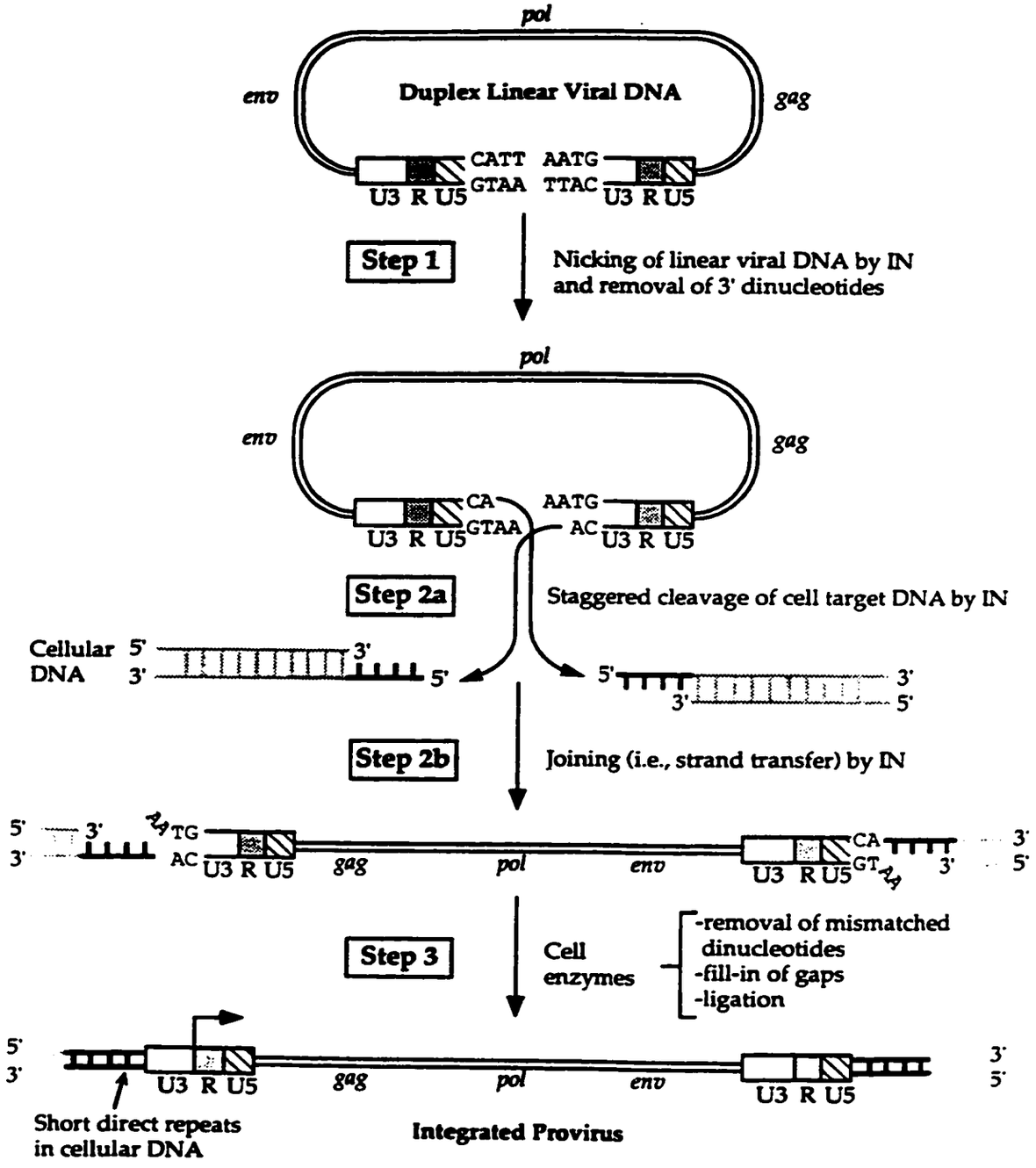

Figure 2.6. Model for integration of viral DNA into the host cell genome. This figure emphasizes the interactions of the ends of linear viral DNA molecules with host cell DNA during the integration process. (Reproduced from Luciw, 1992, with permission).

replication and structural accessibility of target DNA are probably most important in site selection for viral integration (Kulkosky and Skalka, 1994).

The 5′ proviral LTR, particularly the 5′ U3 region, contains the promoter to initiate viral RNA synthesis. Basal promoter activity is provided by the interaction of cellular DNA-binding proteins with the CCAAT and TATA boxes; in FeLV, these sequences are located 71 and 30 bp, respectively, upstream of the transcription start site (the U3-R junction). Bound cellular factors form a complex that facilitates cellular RNA polymerase II attachment and initiation of viral transcription. Transcription from the basal promoter can be enhanced or activated by upstream *cis*-acting sequences. As shown for FeLV in Figure 2.7, these may include the simian virus (SV)40-like core enhancer (CORE), nuclear factor 1 (NF1) site, and a FeLV-specific binding site (FLV-1) similar to the PEA2 binding site of the polyomavirus A enhancer (Fulton *et al.*, 1990). Several additional motifs may be relevant for the regulation of FeLV LTR activity including, the upstream control region (UCR), leukemia virus factor b (LVb), and glucocorticoid response element (GRE) (Plumb *et al.*, 1991).

The 5′ LTR initiates synthesis of the full length viral transcript that enters one of three pathways: genomic RNA for packaging into virions, messenger RNA for translation into *gag* and *pol* encoded polyproteins, and precursor for subgenomic *env* viral transcripts (Luciw and

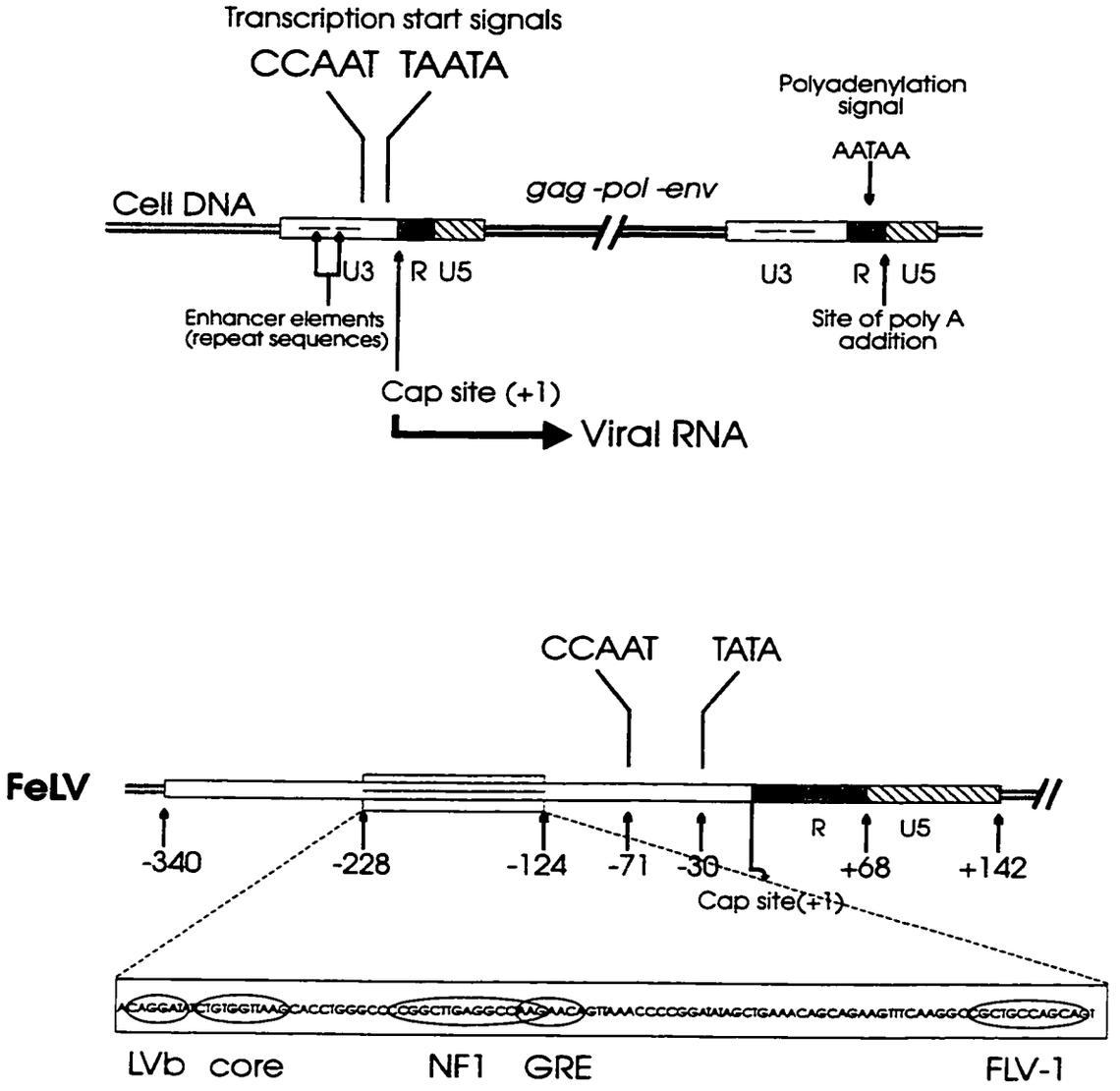

Figure 2.7. Transcriptional control elements in the retroviral LTR. The integrated provirus for a prototype retrovirus is shown in the upper figure together with several *cis*-acting elements in the U3, R, and U5 domains of the LTR that play roles in regulating viral transcription. In the lower figure, binding motifs for cellular transcription factors are shown for the 5' LTR of FeLV. The cap site is the location of the 5' end of viral transcripts. (Reproduced in part, from Luciw, 1992, with permission; FeLV sequence and motifs for nuclear protein binding sites are from Fulton, 1990).

Leung, 1992). As described earlier, the 5′ ends of newly synthesized viral transcripts are capped and methylated by host cell enzymes, similar to cellular mRNA (Luciw and Leung, 1992).

2.1.4. Subgroups

Replication competent FeLV isolates can be classified into three subgroups (Sarma and Log, 1973; Jarrett *et al.*, 1973). Subgroup phenotype is determined by the viral envelope and probably reflects recognition of three distinct host cell receptors by FeLV (Neil *et al.*, 1991). The A, B, and C subgroups have historically been determined by virus interference, neutralization, and host-range assays (Jarrett *et al.*, 1973; Sarma and Log, 1973; Kristal *et al.*, 1993).

Interference assays involve inoculating feline cells with an extract from the infected feline tissue of interest. After establishment of FeLV in the culture, cells are superinfected with pseudotype viruses containing the genome of a sarcoma virus and FeLV A, B, or C envelopes. The sarcoma virus causes visible transformation of cultured cells unless infection is resisted by blockade of SU receptors in cultures infected with FeLV of the same subgroup (Jarrett, 1980; Sarma and Log, 1973). These studies confirmed earlier suspicions that isolates of B and C subgroups were virus mixtures with subgroup A virus as one component (Sarma and Log, 1973).

Virus neutralization tests with subgroup neutralizing

antisera allowed classification of FeLV into the same subgroups as determined by interference tests. This parallelism suggested the same envelope antigens were involved in both viral interference and antiviral antibody production (Sarma and Log, 1973). Despite these findings, cross-neutralization among FeLV subgroups, particularly subgroup A and C viruses, was later demonstrated (Russell and Jarrett, 1978a). When several strains within each subgroup were examined, strain specificity corresponded to subgroup specificity only for subgroup A viruses, suggesting homogeneity within the A group, but heterogeneity within B and C subgroup viruses. This was early evidence that FeLV-A may be the prototype virus, and B and C may have arisen by mutation or recombination. Although the antigens involved in neutralization and components determining subgroup were presumably associated with the envelope gp70, the two components were suggested to occupy different parts of the envelope topographically or functionally (Russell and Jarrett, 1978a).

Host range assays reflect receptor specificity of FeLV subgroups. FeLV-A isolates generally grow only in feline cells, whereas B and C subgroups replicate in cell cultures from several other species. FeLV-B has the widest host range, but C is uniquely capable of growing in guinea pig cells (Ghosh *et al.*, 1992; Jarrett, 1980; Jarrett *et al.*, 1973; Hardy, 1992).

In nature, FeLV-A is frequently found alone, however,

subgroup B and C viruses are always associated with subgroup A virus (Jarrett, 1980; Sarma and Log, 1973; Jarrett *et al.*, 1973; Jarrett *et al.*, 1978). Sequence comparisons of the three gp70 domains, show that subgroups A and C share about 88% homology, compared to about 80% homology with subgroup B (Donahue *et al.*, 1988). FeLV-A is minimally to moderately pathogenic, efficiently transmitted, and present in all field isolates (Neil *et al.*, 1991; Jarrett, 1980; Jarrett *et al.*, 1978). FeLV-B is present in 40-50% of isolates, but is overrepresented in leukemic cats (>60%). Sequence analysis provides evidence that the FeLV-B phenotype results from recombination of FeLV-A and endogenous FeLV, such that a portion of the 5' *env* domain in FeLV-B is obtained from the endogenous virus (Stewart *et al.*, 1986b; Kumar *et al.*, 1989; Neil *et al.*, 1987).

FeLV-C viruses are rare and isolation is invariably from anemic cats (Neil *et al.*, 1991; Abkowitz *et al.*, 1987b; Onions *et al.*, 1982). Limited sequence comparisons indicate that FeLV-C is very closely related to FeLV-A, and may arise by mutation within the 5' variable region (vr 1) of the FeLV-A *env* gene encoding gp70 (Brojatsch *et al.*, 1992). Studies using virus chimeras of FeLV-C and the FeLV-A prototype, demonstrated that subgroup C receptor specificity localized to the amino terminus 87 to 92 amino acids of gp70. Determinants for growth in guinea pig cells colocalized to the same region located within vr 1 of gp70

(Brojatsch *et al.*, 1992). Another study localized determinants of C subgroup phenotype and host range to a single cysteine bound domain of gp70 encompassing vr 1. This sequence varied among other subgroup C isolates suggesting that divergent primary sequences in *env* can confer the same host cell surface receptor specificity (Rigby *et al.*, 1992).

Generally, subtle alterations in the FeLV SU, particularly the amino terminal region, impart multiple significant functional differences which distinguish virus variants (Kristal *et al.*, 1993). FeLV-A maintains the virus in nature, although most of the diseases caused by FeLV are associated with novel strains which are generated from FeLV-A in various ways (Jarrett, 1992). These strains often kill their host but are not transmitted and therefore, of little consequence in the general cat population (Jarrett, 1992; Neil *et al.*, 1991).

Few studies have been done to identify and characterize the cellular receptors for FeLV subgroups. However, evidence shows that FeLV-B uses the same widely distributed cell surface receptor as gibbon ape leukemia virus (GALV) (Takeuchi *et al.*, 1992), and FeLV-A binds to feline T lymphocytes through a 70K cell surface protein, its presumptive receptor (Ghosh *et al.*, 1992). Research to determine the nature and distribution of cellular receptors for FeLV will contribute greatly to our knowledge of FeLV disease pathogenesis.

2.1.5. Epidemiology

Epidemiologic studies indicate about 2% of the estimated 50 million pet cats in the United States are infected with FeLV (Hardy, 1990; Hardy, 1981b), and the prevalence in Canada may be similar. FeLV is transmitted horizontally primarily from the saliva of infected cats to the oronasal mucosal membranes of others. The virus is fragile in the environment, therefore, close contact through licking, biting, and/or sharing of dishes promotes spread (Hardy, 1992). Transplacental transmission occurs resulting in fetal resorption, abortion, or persistent viremia in kittens (Onions, 1987). Kittens may also be infected by nursing from a carrier queen (Hardy, 1980b; Hardy *et al.*, 1976).

Estimates are that up to 50% of free-roaming, urban domestic cats become infected at some point in their lifetime (Rogerson *et al.*, 1975). However, data using antibody to feline oncornavirus-associated cell membrane antigen (FOCMA) as an indicator of prior exposure to FeLV, may be misleading since the origin of FOCMA is controversial and may represent expressed endogenous FeLV *env* sequences (Neil *et al.*, 1991).

Up to 30% of known infected cats become persistently viremic, particularly if repeatedly exposed or exposed at a young age (Rojko *et al.*, 1979; Hoover *et al.*, 1981b). Most persistently viremic cats die within 3 years of diagnosis (Hardy, 1980a; McClelland *et al.*, 1980; Francis and Essex,

1980; Mullins and Hoover, 1990). About 60% of exposed cats develop sufficient antiviral immunity to restrict FeLV replication, and virus expression ends by about 4-8 weeks after exposure (Rojko and Kociba, 1991; Hoover and Mullins, 1991; Rojko *et al.*, 1979; Hoover *et al.*, 1977). However, 30-40% of these recovered cats may harbour latent FeLV in some cells, and be at low risk of illness due to reactivation of the virus (Madewell and Jarrett, 1983; Pedersen *et al.*, 1984; Pacitti and Jarrett, 1985). Although uncommon, some cats with well established infections appear to generate a belated but effective immune response and recover (Hoover and Mullins, 1991). Least commonly, perhaps 5% of exposed cats develop sequestered FeLV infection in which virus replicates in localized sites with accompanying low level/intermittent antigenemia and viremia. With time, these cats may recover or become persistently viremic (Hayes *et al.*, 1989; Lutz *et al.*, 1983).

Macrophages in oronasal lymphoid tissue may form an early target for FeLV infection (Rojko *et al.*, 1979). Antigen-positive mononuclear cells within the blood infect other lymphoid tissues and eventually, the bone marrow. In lymph nodes, viral replication is often first detected in germinal centers. With persistent infection, bone marrow replication of FeLV is followed by release of free virus into the circulation as well as cell associated viremia, particularly involving neutrophils and platelets. Viremia

leads to dissemination of FeLV to epithelial tissues of salivary gland, oropharynx, bladder, mammary gland, pancreas, and intestine, followed by shedding of virus from these tissues (Rojko *et al.*, 1979; Onions, 1987).

2.1.6. Immunology

The consequences of FeLV infection depend in part, on immune response and age of the individual cat. The immune responses leading to recovery versus persistent, latent, or sequestered FeLV infection are not well studied or understood. Complement-associated non-immune factors and various macrophage functions are age-dependent, and help explain the increased resistance to viremia associated with maturity of the host (Rojko and Olsen, 1984; Hoover *et al.*, 1976; Rojko and Kociba, 1991). Macrophages are less permissive for virus replication with increasing maturity, however treatment with hydrocortisone removes this inherent protection (Hoover *et al.*, 1981b).

Generally, protection against cell-free viremia is conferred by virus neutralizing antibodies to envelope glycoproteins. Whereas, cytotoxic T lymphocytes and antibody-dependent cell-mediated cytotoxicity may be important in eliminating FeLV-infected bone marrow or tumor cells (Rojko *et al.*, 1982; McCarty and Grant, 1983).

Multiple epitopes on gp70 are involved in neutralization; some are shared by all subgroups, others are shared by two subgroups, and some are subgroup specific (Onions, 1987). Virus-neutralizing antibodies to FeLV

subgroup A gp70 are particularly important in protection against viremia or reactivation of latent infection (Rojko and Kociba, 1991; Hardy *et al.*, 1976; Russell and Jarrett, 1978b; Jarrett and Russell, 1978). However, termination of viremia has been associated with appearance of antibodies of many different types and activities (Charreyre and Pedersen, 1991; Grant *et al.*, 1980). In fact, the sometimes poor correlation between humoral immune responses and recovery indicates that other types of immune responses are probably involved in recovery from FeLV infection (Charreyre and Pedersen, 1991; Hawks *et al.*, 1991). Some apparently FeLV-recovered cats fail to maintain lifelong immunity and later become reinfected (Charreyre and Pedersen, 1991). Also, non-neutralizing antibodies, particularly to p27, some gp70 epitopes, and p15E, may contribute to immune complex formation and disease progression (Snyder *et al.*, 1985; Hardy *et al.*, 1976; Hardy, 1992).

Some cats have antibody that reacts with antigen on the surface of transformed lymphocytes and fibrocytes (FOCMA antibody). Although traditionally FOCMA was considered to be an FeLV-induced antigen, there is now evidence that FOCMA is a tumor specific antigen resulting from expression of the endogenous FeLV *env* gene (Neil *et al.*, 1991). The cell-surface protein (p70) is related to but structurally distinct from FeLV-C/Sarma gp70, and is expressed on the surface of all neoplastic feline

lymphocytes regardless of the presence of exogenous FeLV (Snyder *et al.*, 1983). FeLV-C/Sarma differs from other subgroup C viruses which arise from mutation of FeLV-A, in that its *env* gene resembles endogenous FeLV rather than FeLV-A in the antigenic carboxyl terminus of gp70 (Russell and Jarrett, 1978a). Previously FOCMA antibody was used as a marker of prior FeLV exposure, and significant antibody titers implied protection from FeLV-induced tumors but not FeLV-induced non-neoplastic disease (Swenson *et al.*, 1990; Hardy *et al.*, 1976). The FOCMA system is more complex than appreciated previously (Snyder *et al.*, 1983), and the prognostic significance of various FOCMA antibody titers is now less clear.

Acquired immunosuppression is the most frequent consequence of FeLV infection in the cat. Immunosuppression is targeted primarily to the cell-mediated immune system and may relate to the viral p15E envelope protein. A defect in T helper function results in decreased immunoglobulin G (IgG) responses and decreased proliferative responses to T cell mitogens (Ogilvie *et al.*, 1988). The T helper cell and associated cytokine defects adversely affect cytotoxic lymphocyte and macrophage functions (Good *et al.*, 1990).

In a study examining lymphocyte subsets in cats with natural FeLV infections, B lymphocyte numbers were normal, but CD4+ (helper) and CD8+ (suppressor) T lymphocytes were both reduced. Experimental infection of kittens with an

immunosuppressive FeLV strain resulted in decline in B cell numbers during the first week post infection, followed by a steady increase to twice the preinfection level by week 17, and a return to normal B cell numbers by week 20. As in the naturally infected cats, CD4+ and CD8+ lymphocyte numbers both declined (Tompkins *et al.*, 1991). To some extent, however, the effect on certain lymphocyte subpopulations is strain specific, as other investigators have found a selective decrease in CD4+ T lymphocytes and accompanying inversion of the CD4+ to CD8+ ratio (Quackenbush *et al.*, 1990).

FeLV also inhibits interleukin 2 and macrophage activating factor. Gamma interferon production is suppressed by inactivated FeLV, and may in turn result in depressed macrophage responses (Good *et al.*, 1990). Neutrophil dysfunction can occur with both persistent and latent FeLV infections and may contribute to the establishment of opportunistic infections (Lafrado and Olsen, 1986).

2.1.7. Laboratory Detection

2.1.7.1. Virus Isolation

The FeLV was first identified by examination of infected tissues using electron microscopy (Mackey *et al.*, 1972; Laird *et al.*, 1968). Next cell free homogenates of leukemic cells from cats in LSA cluster households, were inoculated into tissues cultured from normal kittens. Viral infection of cell cultures was visualized by electron

microscopy, and replication confirmed by the presence of virus budding from cell surfaces (Jarrett *et al.*, 1968).

Following these initial studies, various methods were used to confirm FeLV growth in cell cultures. A virus assay is described that involves coculturing of feline embryonic cells and a cat cell line carrying the genome of a murine sarcoma virus. Cell free sample is then added and cultures examined at various intervals thereafter for transformation (Jarrett *et al.*, 1982a; Fischinger *et al.*, 1974). FeLV in the test sample causes discrete focal lesions identified as piled up, loosely attached cells which can be removed by washing (Fischinger *et al.*, 1974).

Other methods of virus isolation involve inoculation of uninfected feline cells of various types, with serum, plasma, or cell free homogenates from the test cat; cultured cells are then examined by fixed cell immunofluorescence for FeLV *gag* products (Grant *et al.*, 1984; Ubertini *et al.*, 1971), or culture supernatant is tested by enzyme-linked immunosorbent assay (ELISA) for FeLV p27 (Pedersen *et al.*, 1984). Alternatively, bone marrow aspirates from test cats can be cultured directly and supernatants tested afterwards by ELISA for FeLV p27 (Pedersen *et al.*, 1984).

Although of upmost importance in initial study of the biology of FeLV, electron microscopy, *in vitro* virus assay, and virus isolation techniques are too time-consuming, technically demanding, and costly for routine diagnostic

purposes.

2.1.7.2. Indirect Immunofluorescence Assay

Central to large scale studies of the epidemiology of FeLV was development of an immunodiagnostic assay for virus detection. The logical targets for immunodetection are the internal *gag* protein products common to all FeLV subgroups and produced in great excess during active infection. However, because cats do not develop high antibody titers to these internal structural proteins, antibody was raised in other species and tests were developed for antigen detection (Hardy, 1971).

A specific rabbit anti-FeLV *gag* serum was first produced in 1968 (Hardy, 1971; Hardy *et al.*, 1969). Although high titer rabbit antisera may react with all FeLV proteins since immunization is with ether-disrupted FeLV, generally, p27 *gag* protein is considered to be the predominant immunogen (Lutz *et al.*, 1980).

The first FeLV immunodiagnostic test was a double immunodiffusion test (Hardy, 1971; Hardy *et al.*, 1969), followed by a complement-fixation test which proved impractical (Sarma *et al.*, 1971). The IFA test was then developed for FeLV detection in peripheral blood leukocytes, platelets, and bone marrow cells (Hardy *et al.*, 1973a; Hardy *et al.*, 1973b; Ubertini *et al.*, 1971). Alcohol fixed peripheral blood or bone marrow smears are reacted with rabbit (or other species) anti-FeLV serum in the IFA test (Hardy, 1981b). The alcohol fixation disrupts

cell membranes so that reagents can react with cytoplasmic antigen. A secondary goat (or other species) fluorescein-conjugated serum is then applied to identify and visualize any IgG that is bound to FeLV antigens on the slide. Yellow-green punctate fluorescence in the cytoplasm of neutrophils, eosinophils, lymphocytes, and platelets in peripheral blood, and in nucleated hemopoietic cells in the bone marrow, indicates a positive IFA test for FeLV antigens (Hardy and Zuckerman, 1991a). Positive IFA test results correlate 98% of the time with virus isolation, and indicate that the cell is replicating FeLV (Hardy, 1981b). Blood smears that are too thick, or smears from severely leukopenic cats can usually not be interpreted (Hardy and Zuckerman, 1991a).

Compared with the immunodiffusion test and virus isolation, the IFA test is considered most accurate, easiest to perform, and least time-consuming (Hardy and Zuckerman, 1991a). However, the IFA test requires specialized equipment, technical expertise (Hardy and Zuckerman, 1991a), and occasionally, subjective judgement in interpreting results (Lutz *et al.*, 1983).

FeLV *gag* products have also been identified in tissues of naturally and experimentally infected cats by an IFA procedure applied to methanol-fixed paraffin-embedded tissue sections (Rojko *et al.*, 1978). These studies together with IFA assays of peripheral blood cells and bone marrow, helped establish the basic pathogenesis of FeLV

infection in cats (Rojko *et al.*, 1978; Hardy, 1981b).

2.1.7.3. Enzyme-linked Immunosorbent Assay

The ELISA for soluble FeLV p27 in plasma or serum, is now the most widely used and simplest FeLV diagnostic assay (Hoover and Mullins, 1991). Several commercial FeLV ELISA kits are available for use in reference laboratories or private veterinary clinics (Tonelli, 1991). Most ELISAs use monoclonal antibody to p27 immobilized on a solid phase; test serum, plasma, whole blood, tears, or saliva is added and any FeLV p27 present will bind to the antibody. Washing removes unbound reactants, and a substrate/chromogen mixture is added which enhances the sensitivity by acting as an amplifying system. Colour intensity is proportional to the amount of p27 present (Tonelli, 1991; Lutz *et al.*, 1980; Sodikoff, 1979; Kahn *et al.*, 1980).

Since introduction of the FeLV ELISA kit in about 1979 (Hardy and Zuckerman, 1991b; Lutz *et al.*, 1980; Sodikoff, 1979), there have been several reports comparing IFA, ELISA, and/or virus isolation for FeLV detection (Lutz *et al.*, 1980; Kahn *et al.*, 1980; Hirsch *et al.*, 1982; Lutz *et al.*, 1983; Lopez *et al.*, 1990; Jarrett *et al.*, 1982a; Hardy and Zuckerman, 1991b; Jarrett *et al.*, 1982b). Controversy exists over the relative sensitivity and specificity of the IFA test and ELISA. Although samples negative by ELISA are almost always negative using IFA, a significant proportion of samples are ELISA-positive but IFA-negative (discordant

results) (Hirsch *et al.*, 1982; Lutz *et al.*, 1980; Jarrett *et al.*, 1982b). Initially, specificity varied among commercial ELISA kits due to non-specific detection of anti-mouse reactivity by some tests, or technical variability in performing and interpreting the test (Lutz *et al.*, 1980; Lopez *et al.*, 1990). Many manufacturers corrected these problems to improve specificity of their product (Lopez *et al.*, 1990).

Most persistently ELISA-positive/IFA-negative cases may have latent or sequestered FeLV infection (Lutz *et al.*, 1980). These discordant cases may not be a threat to other cats, but the true significance for the individual cat and its contacts is not yet fully understood (Jarrett *et al.*, 1982b; Lutz *et al.*, 1980). In one clinical study, 6 of 13 discordant cases had hematologic disorders generally associated with FeLV infection (Hirsch *et al.*, 1982).

Some researchers suggest that discordant results are due to false positive ELISAs, and that all ELISA-positive results should be immediately confirmed by IFA testing (Hardy and Zuckerman, 1991b). Such recommendations disregard the fact that the ELISA and the IFA test detect FeLV antigen in different blood compartments, and sometimes reflect different forms of FeLV infection. Also, results cannot be entirely explained by false positive ELISAs in many studies (Lutz *et al.*, 1983; Lopez *et al.*, 1990; Jarrett *et al.*, 1982a; Hirsch *et al.*, 1982; Lutz *et al.*, 1980). Development of the FeLV ELISA has provided insight

into the complexities of the biology of FeLV that were not identified by the IFA test.

2.1.7.4. Enzyme Immunohistochemistry

Enzyme immunohistochemistry (IHC) is now widely used in veterinary medicine for the characterization and identification of several cell types, tumors, infectious agents, and immune-mediated lesions (Haines and Clark, 1991; Haines and Chelack, 1991; Berrington *et al.*, 1994; Spangler *et al.*, 1994; Breuer *et al.*, 1994; Pace *et al.*, 1994; Ackermann *et al.*, 1994). The technique is applicable to formalin-fixed paraffin-embedded tissues which are readily available in pathology archives, and the resulting permanent stain can be conveniently visualized by ordinary light microscopy. Also, tissues can be stained simultaneously for routine histologic examination to precisely localize immunohistochemical positivity (Haines and Chelack, 1991; Haines and Clark, 1991). Enzyme IHC is adaptable to automation and therefore useful not only for individual case diagnosis, but also for large-scale retrospective studies (Haines and Clark, 1991; Brigati *et al.*, 1988).

The sensitivity of enzyme IHC is enhanced by indirect immunostaining in which the unlabeled primary antibody reagent binds to the antigen of interest in the tissue. A second enzyme-labeled antibody to IgG then binds to the primary antibody. When enzyme substrate is added, colour development occurs. The amplification of indirect

immunostaining can be augmented further by employing the avidin-biotin complex (ABC) method in which the secondary antibody is biotin labeled. Preformed ABCs are then added which bind to tissue-associated secondary antibody through free biotin-binding sites on the avidin molecules. The biotin molecules are usually peroxidase-labeled so that when the diaminobenzidine substrate for peroxidase is added, brown colour develops. The ABC technique allows detection of scarce or formalin altered antigens (Haines and Chelack, 1991).

There are few reports of enzyme IHC for FeLV detection (Reinacher, 1989; Reinacher and Theilen, 1987; Reinacher, 1987). An indirect immunoperoxidase procedure for use in methanol-fixed tissues is described using bovine anti-FeLV serum as the primary antiserum, and peroxidase-labeled rabbit anti-bovine IgG serum as the second antiserum (Reinacher and Theilen, 1987). Anti-FeLV monoclonal antibodies directed against gp70 and p27 have also been used when results with polyclonal bovine anti-FeLV serum were uncertain (Reinacher and Theilen, 1987). Nonspecific staining occurs occasionally with either polyclonal or monoclonal primary antibody. Also, false negative results can occur particularly with monoclonal antibodies which rely on preservation in fixed tissues of a single epitope. However, use of a cocktail of monoclonal antibodies and/or antibodies directed against highly conserved antigens, will minimize such false negative results (Haines and Chelack,

1991).

Enzyme IHC for FeLV detection is particularly suited to the investigation of sequestered/localized infection when fixed tissues are available. Similarly when used in conjunction with viral DNA detection, enzyme IHC is applicable to studying tissue distribution of latent or replication-defective forms of FeLV. Together with traditional serum ELISA and/or peripheral blood IFA testing, controversial issues such as discordant results and FeLV antigen-negative LSA, may be resolved.

2.1.7.5. Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an *in vitro* method for the primer-directed enzymatic amplification of specific DNA sequences (Erlich *et al.*, 1988; Lynch and Brown, 1990; Jackson, 1990; Rodu, 1990). The procedure was invented in 1983 (Mullis, 1990), applied to sickle cell anemia diagnosis in 1985 (Saiki *et al.*, 1985), and described biochemically in 1987 (Mullis and Faloona, 1987). There are now several thousand reports including reviews, applications, improvements, precautions, and modifications of the method; PCR is now considered to be one of the most important technical advances in molecular genetics of the past decade (Eisenstein, 1990; Persing, 1991).

The PCR involves repeated cycling of three steps performed at different temperatures. In the first step, the double-stranded DNA template is denatured (rendered single-stranded) by heating to about 94°C. The annealing

step occurs at 37-60°C and involves binding of the two oligonucleotide primers to complementary single-stranded sample DNA. Warming of the reaction mixture to about 72°C allows DNA polymerase to function efficiently to incorporate nucleotides onto the 3' ends of the primers by a template-specific pairing of adenine (A) with thymine (T) and guanine (G) with cytosine (C). The three steps are repeated perhaps 20-35 times and the quantity of target DNA increases exponentially with each cycle. The amplified fragment is usually detected by ethidium-stained gel electrophoresis (Kumar, 1989; Jackson, 1990; Rodu, 1990; Lynch and Brown, 1990). However, several other methods may be used to increase the sensitivity and/or confirm the specificity of the product including Southern, slot, or dot hybridization with radiolabeled or non-radiolabeled probes, restriction enzyme analysis, direct sequencing, or cloning followed by sequencing (Jackson, 1990).

The PCR technique is very sensitive and can tolerate small quantities of poor quality template DNA or RNA (Jackson *et al.*, 1990). Fresh, frozen, fixed, and ancient tissues have all been used successfully in PCR (Sninsky, 1990; Crisan *et al.*, 1990; Coates, 1991). Nucleic acid preparation ranges from highly purified to minimally processed (Sninsky, 1990). However, several procedures involve a proteinase K/non-ionic detergent digestion step followed by heat inactivation of the enzyme, and/or nucleic acid purification by an organic extraction step (Jackson *et*

al., 1990; Crisan *et al.*, 1990; Greer *et al.*, 1991). Generally shorter target fragments can more successfully be amplified from poor quality/degraded template than can longer regions (Coates, 1991).

Guidelines for the design of optimal primers include: 20-30 bases in size, 50% or the average GC content of the target nucleic acid, lack of predicted secondary structure, proper orientation with respect to each other and target DNA, perfect complementarity to target especially at the 3' end (with exceptions depending on the PCR goals), and minimal complementarity to known non-target sequences (Sninsky, 1990; Kumar, 1989). However, primers not meeting these guidelines have been effective in PCR, and primers meeting all these guidelines have been ineffective. Empirical testing remains the final criterion required to determine the efficiency of primers chosen (Sninsky, 1990).

The introduction of a thermostable DNA polymerase derived from *Thermus aquaticus* (*Taq* polymerase) obviated the need for adding fresh polymerase with each cycle, and made automation and increased efficiency of the PCR possible (Kumar, 1989; Saiki *et al.*, 1988). Other DNA polymerases with varying degrees of thermostability, reaction requirements, economy, and fidelity are now available (Salhi *et al.*, 1990; Lundberg *et al.*, 1991; Lohff and Cease, 1992; Rao and Saunders, 1992); however, *Taq* polymerase probably remains the most widely used.

The PCR is successful when the correct choices,

concentrations, and combinations of the following are made: target genomic DNA or complementary DNA (cDNA), 2 synthetic oligonucleotide primers, DNA polymerase, the 4 deoxyribonucleotide triphosphates (dNTPs), a divalent metal ion (Mg^{2+} usually), a thermal cycle of about 2-4 minutes repeated 20-35 times, all in a sealed tube containing a total of 25-200 uL of buffered reaction mixture (Bloch, 1991; Saiki *et al.*, 1988). "Standard" conditions for PCR are often successful, for example, 2.5 units *Taq* polymerase, 200 uM of each dNTP, 2.5 mM $MgCl_2$, 1 uM primers, 1X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), < 1 ug template, and sterile distilled water to 100 uL (Sninsky, 1990; Kumar, 1989; Innis *et al.*, 1990; Linz *et al.*, 1990). However, efficiency of the reaction may be improved by evaluating annealing temperature and time, denaturation temperature and time, enzyme concentration, $MgCl_2$ concentration, and primer concentration (Sninsky, 1990; Linz *et al.*, 1990).

Since inception, the PCR has had many applications including molecular virology and in particular, the diagnosis and study of human retroviral diseases (Laure *et al.*, 1988; Sninsky and Kwok, 1990; Young *et al.*, 1990; Sninsky, 1990; Sönnerborg *et al.*, 1990; Shibata *et al.*, 1991; Kwok, 1992). With respect to FeLV research, PCR has been used to amplify FeLV LTR enhancer regions from hemopoietic tumors and non-neoplastic diseases for sequence determination (Fulton *et al.*, 1990; Matsumoto *et al.*,

1992), and to determine the splice donor and acceptor sequences for the FeLV *env* mRNA (Papenhausen and Overbaugh, 1993). In the LTR studies, sources of DNA for PCR were cloned provirus from LSAs, or primary cells from naturally FeLV-infected cats with various neoplastic and non-neoplastic diseases. Nucleotide sequencing was performed using cloned PCR product as the source of DNA template (Fulton *et al.*, 1990; Matsumoto *et al.*, 1992). Direct sequencing of double-stranded PCR products for FeLV LTR mutational analysis has not been reported. Because of the relatively high error rate of *Taq* DNA polymerase, the sequencing of individual subcloned PCR products increases the incidence of DNA sequence artifacts (Cao and Brosius, 1993). Therefore, direct sequencing of PCR products offers the opportunity to determine DNA sequences with greater accuracy than traditional cloning methods (Thomas and Kocher, 1993). There have not been reports relating PCR to antigen detection for FeLV using peripheral blood in a clinical setting, or using formalin-fixed paraffin-embedded feline LSAs in retrospective studies.

2.2. Feline Leukemia Virus-related Disease

2.2.1. Non-neoplastic Disease

2.2.1.1. Cytosuppressive Hematologic Disease

Hemopoiesis is probably suppressed to varying degrees in most FeLV infections, and clinical signs related to cytopenia are common (Mackey *et al.*, 1975a; Hoover and Mullins, 1991; Hardy, 1981a). Bone marrow suppression may

be transient during initial FeLV infection, or a terminal event in persistently infected cats (Pedersen *et al.*, 1977). Bone marrow suppression syndromes are estimated to be the primary consequence of persistent FeLV infection in 25-30% of cases (Mullins and Hoover, 1990). The specific hemopoietic cell line(s) affected relates in part, to virus strain and therefore, genotype (Hoover and Mullins, 1991; Dean *et al.*, 1992; Brojatsch *et al.*, 1992; Neil *et al.*, 1990; Hoover *et al.*, 1990).

About 70% of anemic cats are infected with FeLV (Cotter *et al.*, 1975; Hardy *et al.*, 1973a), and anemia is second only to immunosuppression as a manifestation of natural progressive FeLV infection (Rojko *et al.*, 1986). Although anemia is most commonly a primary effect of FeLV infection, it may be secondary to FeLV-induced neoplasia or immunosuppression (Hardy, 1981a; Jarrett, 1984). Most experimental studies of FeLV-related anemia have involved FeLV-C which is known to induce erythroid hypoplasia. Although FeLV-C is isolated from only 1% of infected cats, subgroup C virus is found in over 1/3 of cats with anemia (Jarrett, 1992). The anemia due to FeLV-C is usually nonregenerative, normocytic, and normochromic; bone marrow examination typically reveals selective loss of erythroid progenitors but normal thrombopoiesis and granulopoiesis, much like pure red cell aplasia (PRCA) of humans (Riedel *et al.*, 1986; Onions *et al.*, 1982; Riedel *et al.*, 1988; Jarrett *et al.*, 1984).

Despite consistent findings with experimental FeLV-C infection, the types of anemia seen in natural FeLV infections are much more diverse. These include regenerative anemia often with hemolysis, and nonregenerative macrocytic anemia with megaloblastic changes in erythroid precursors (Weiser and Kociba, 1983; Hirsch and Dunn, 1983). Bone marrow abnormalities include erythroid hyperplasia, erythroid hypoplasia, dyserythropoiesis, dyshemopoiesis, and generalized hypoplasia (known by the misnomer, aplastic anemia). Disturbed hemopoiesis resulting in any combination of peripheral cytopenias may preceed myeloproliferative disease (Madewell *et al.*, 1979; Raskin and Krehbiel, 1985; Pedersen *et al.*, 1977; Mackey, 1975).

The pathogenesis of erythroid hypoplasia with FeLV remains unknown, although several effects of FeLV on hemopoiesis have been investigated. Experimentally, bone marrow fibroblast colony-forming units (CFU-F) which form part of the stroma and likely have a regulatory role in hemopoiesis, are decreased in progressor cats that develop anemia compared to CFU-F in regressor cats (Wellman *et al.*, 1988). The FeLV p15E but not p27, suppresses uninfected feline CFU-F and erythroid colony-forming units (CFU-E) *in vitro*, suggesting a role for envelope protein(s) in both stromal and erythroid precursor abnormalities (Wellman *et al.*, 1988; Wellman *et al.*, 1984). Also evidence shows *in vitro* exposure to FeLV directly depresses development of

CFU-E (Rojko *et al.*, 1986; Abkowitz *et al.*, 1987a) and the more primitive erythroid burst-forming units (BFU-E) (Rojko *et al.*, 1986; Abkowitz *et al.*, 1987b; Testa *et al.*, 1983). The suppression of CFU-E correlates with the presence of infectious rather than inactivated FeLV (Rojko *et al.*, 1986).

The decrease in erythroid precursors may relate to immune elimination of infected cells, to a defective hemopoietic microenvironment, or a direct block of erythroid precursor differentiation (Onions *et al.*, 1982). Both erythroid and granulocyte-macrophage progenitors are infected by FeLV-C (Testa *et al.*, 1983; Abkowitz, 1991) yet granulocyte-macrophage colony-forming units (CFU-GM) are not suppressed, indicating selective block of erythroid differentiation. The BFU-E from cats infected with FeLV-C but not non-C strains, are abnormally sensitive to complement-mediated lysis, therefore, complement may also play a role in the pathogenesis of FeLV-induced erythroid hypoplasia (Abkowitz *et al.*, 1987b). Another suggestion is that FeLV-C gp70 may bind to erythroid progenitors through the receptor for an essential erythropoietic differentiation factor, thus preventing further cellular development (Jarrett, 1992; Abkowitz, 1991).

Viral infection of macrophages may stimulate release of cytokines such as prostaglandin E-2, interleukin-6, and tumor necrosis factor-alpha (TNF-α). Cytotoxicity mediated by bone marrow mononuclear cells in co-cultures with FeLV-C

infected feline fibroblasts, has been documented. Therefore, a cytopathic effect directed towards erythroid progenitors could be mediated by macrophages through release of TNF-α (Khan *et al.*, 1992).

Because the hematologic profiles seen with FeLV infection are diverse, bone marrow disturbances probably involve several mechanisms. The development of novel variant viruses within individual cats may explain some of the differences noted in natural FeLV infection (Hoover and Mullins, 1991). As previously mentioned, these more pathogenic variants often result in death of the host with no further transmission in nature (Jarrett, 1992; Neil *et al.*, 1991).

Other FeLV-related cytosuppressive conditions have been described (Madewell *et al.*, 1979; Lester and Searcy, 1981; Cotter *et al.*, 1975; Hardy, 1981a; Cotter, 1979) but the pathogenic mechanisms remain poorly understood. In one study of 100 FeLV-positive anemic cats, about 1/3 were also leukopenic, usually due to neutropenia and lymphopenia. Platelet counts were decreased in 18% of cats, and these cats were often leukopenic (Cotter, 1979). Macrothrombocytes accompanying thrombocytopenia may suggest impaired platelet volume regulation (Boyce *et al.*, 1986). An FeLV-associated enteritis with features of feline panleukopenia has been described in cats immune to the panleukopenia virus (Hardy, 1981a), however, the hematologic findings in affected cats have been disputed

(Reinacher, 1987). Cyclic hemopoiesis involving neutrophils, monocytes, erythrocytes, and platelets is yet another documented manifestation of FeLV infection (Swenson *et al.*, 1987).

Studies show that marrow fibroblast presursors are a major target for FeLV *in vivo*, however, gp70 is expressed at low level on the surface of these cells. These fibroblast precursors may escape immune surveillance and provide a reservoir of virus during active or latent infection. Since stromal cells contribute to the hemopoietic microenvironment, cytopenias and other hematologic diseases could result from infection of marrow fibroblast precursors with FeLV (Linenberger and Abkowitz, 1992b). Other possible mechanisms of FeLV-induced leukopenia and thrombocytopenia include those proposed for erythroid hypoplasia, such as direct cytotoxic effect of FeLV towards granulocytic and megakaryocytic precursors, or interference by gp70 with membrane receptors required for growth and differentiation.

2.2.1.2. Immunosuppressive Disease

A frequent consequence of FeLV infection is immunodeficiency characterized by leukopenia, lymphoid depletion, and secondary microbial infections (Hardy, 1990; Anderson *et al.*, 1971; Perryman *et al.*, 1972; Trainin *et al.*, 1983; Cotter *et al.*, 1975). In fact, immunodeficiency syndrome is estimated to be the primary consequence of persistent FeLV infection in 40-50% of cases (Mullins and

Hoover, 1990). FeLV-associated immunosuppression has been recognized since natural isolates of the virus were first used in experimental infections (Anderson *et al.*, 1971; Mackey *et al.*, 1972). The long latency subgroup A FeLV is generally associated with gradual development of both humoral and cell-mediated immunologic defects (Quackenbush *et al.*, 1990; Trainin *et al.*, 1983; Wernicke *et al.*, 1986). However, more pathogenic variants may emerge within individual cats to accelerate or precipitate profound immunodeficiency (Thomas and Overbaugh, 1993; Hoover and Mullins, 1991).

Generally, FeLV isolates cause a decrease in leukocyte numbers and lymphocyte responses to mitogens within the first 2 months after infection (Pedersen *et al.*, 1977; Rojko *et al.*, 1979; Tompkins *et al.*, 1991; Hoover *et al.*, 1981a). Most immune system abnormalities reported are consistent with T lymphocyte defects (Cockerell and Hoover, 1977; Cockerell *et al.*, 1976a; Mathes *et al.*, 1978; Quackenbush *et al.*, 1989; Trainin *et al.*, 1983; Wernicke *et al.*, 1986). These T cell alterations include thymic atrophy and T cell depletion of lymphoid tissues (Perryman *et al.*, 1972; Anderson *et al.*, 1971), delayed allograft rejection (Perryman *et al.*, 1972), depletion of a colony forming T cell subset (Quackenbush *et al.*, 1989), decreased blastogenic responses to T cell mitogens and antigens (Cockerell and Hoover, 1977; Cockerell *et al.*, 1976a; Cockerell *et al.*, 1976b), p15-induced suppression of

lymphocyte responses *in vitro* (Mathes *et al.*, 1978; Mathes *et al.*, 1979) [later recognized as the transmembrane protein, p15E (Snyderman and Cianciolo, 1984)], and impaired primary humoral antibody responses (Wernicke *et al.*, 1986; Trainin *et al.*, 1983). The defect in humoral immunity is likely mediated by impairment of a subpopulation of T helper cells involved in antibody production (Pardi *et al.*, 1991).

Leukopenia, including neutropenia, also contributes to increased susceptibility to opportunistic infections. In addition to affecting neutrophil numbers, FeLV depresses neutrophil chemotaxis (Kiehl *et al.*, 1987), chemiluminescence (Dezzutti *et al.*, 1990), and phagocytosis (Lafrado and Olsen, 1986). Even non-viremic FeLV-infected (latently infected) cats exhibit suppressed neutrophil function which may relate to the presumed site of latency in bone marrow myelomonocytic precursors (Lafrado and Olsen, 1986).

Pathogenic mechanisms leading to FeLV-induced immunodeficiency remain poorly understood. The transmembrane protein, p15E, has been shown to suppress monocyte, lymphocyte, natural killer cell, and neutrophil functions *in vitro* (Harris *et al.*, 1989). The mechanism of p15E suppression may be at the cell membrane level through blocking signal transfer (Olsen *et al.*, 1987), or perhaps through altering receptor-mediated calcium mobilization in the cell (Harris *et al.*, 1989; Wright *et al.*, 1989). Since

p15E is hydrophobic, interference with cell surface mobility, receptor binding, and/or signal transfer across the cell membrane could explain its activity. However, p15E is highly conserved among all FeLVs so acute immunodeficiency associated with certain strains is unlikely to relate to this protein unless the configuration of p15E with gp70 varies in these isolates (Quackenbush *et al.*, 1990). Also, most studies of p15E activity have been done *in vitro* using purified protein; the immunosuppressive role of p15E in natural FeLV infections is more controversial (Wardley *et al.*, 1992). Furthermore, studies involving inactivated FeLV vaccine indicate that p15E does not have a negative impact on vaccine effectiveness, in fact, the transmembrane protein may enhance vaccine efficacy (Hoover *et al.*, 1991; Wardley *et al.*, 1992) perhaps through its association with gp70 to form immunogenic epitopes.

Studies of specific immunodeficiency-inducing FeLV strains, and development of markers for lymphocyte subsets in the cat have enabled more direct investigations of effects of the virus on the immune system (Quackenbush *et al.*, 1990; Tompkins *et al.*, 1991). Persistent infections with less immunosuppressive FeLV strains cause gradual declines in both $CD4^+$ and $CD8^+$ T cells (Quackenbush *et al.*, 1990; Tompkins *et al.*, 1991). With more immunosuppressive FeLV isolates, decreases in lymphocyte numbers occur early and persist (Quackenbush *et al.*, 1990). The first isolated

FeLV strain causing feline acquired immunodeficiency syndrome (FeLV-FAIDS) was from tumor tissue from a pet cat with thymic LSA. This strain produces acute immunodeficiency when given to kittens, and chronic immunodeficiency when given to weanling or adult cats. The disease is characterized by persistent viremia, weight loss, lymphoid hyperplasia followed by severe lymphoid depletion, chronic diarrhea, and opportunistic infections (Hoover *et al.*, 1987). FeLV-FAIDS is replication defective and requires help from the replication competent, common form FeLV-A (Overbaugh *et al.*, 1988a). Disease correlates with the presence of large numbers of FeLV-FAIDS replication intermediates (unintegrated viral DNA) within lymphoid, bone marrow, and intestinal epithelial cells (Mullins *et al.*, 1986). A proposed conformational change in gp70 may result in failure to develop superinfection interference leading to continual reinfection and therefore, cytopathicity (Donahue *et al.*, 1991; Mullins *et al.*, 1989).

Early depletion of $CD4^+$ T cells, possibly a $CD4^+$ helper T cell subset, is proposed to explain some of the immunologic defects in FeLV-FAIDS (Quackenbush *et al.*, 1990), however, the FeLV-Rickard strain causes a T cell lymphopenia consisting of decreased $CD4^+$ and $CD8^+$ T cells (Tompkins *et al.*, 1991). Presumably different FeLV strains have tropism for different cell types which may relate to variance within the viral genome, particularly the envelope

gp70. Emerging pathogenic strains in natural FeLV infections are likely genomically diverse and somewhat unique to each case; cellular targets for the virus are likely also diverse so that lymphocyte subset alterations vary (Tompkins *et al.*, 1991).

2.2.1.3. Other Diseases

Reproductive, neurologic, and other diseases which are not primarily cytosuppressive or neoplastic, are estimated to occur in 5-10% of persistently FeLV infected cats (Mullins and Hoover, 1990). Generally these less common manifestations of FeLV infection, have not been well characterized or studied.

Reproductive disorders associated with natural FeLV infections include infertility, fetal resorption, and abortion (Cotter *et al.*, 1975; Hardy, 1981a). Although viremic queens conceive and embryos implant, fetuses frequently become nonviable during the first month of pregnancy and the placenta involutes. If the fetuses are resorbed rather than aborted, the queen may appear to be infertile. Kittens born congenitally infected, may die acutely or develop severe immunosuppressive disease (Hoover and Mullins, 1991). Experimental studies of viremic pregnant cats show that FeLV induces early fetal death and congenital fetal infection. Also, congenital latent fetal infection may occur during pregnancy in latently infected cats (Hoover *et al.*, 1983; Rojko *et al.*, 1982). Queens can infect their kittens through both the placenta and

colostrum (Hardy *et al.*, 1969; Hoover *et al.*, 1983; Pacitti *et al.*, 1986). The pathogenesis of FeLV-induced abortions and resorptions is unknown. However, as for MuLVs, FeLV may integrate into and thus inhibit, an essential gene (α_1-collagen, for example) in the early embryo (Hardy, 1990).

Neurologic deficits often involving the spinal cord and peripheral nerves rather than the brain have been described with FeLV infection (Hardy, 1981a; Reinacher and Theilen, 1987). Infected cats may develop fore and/or hind limb weakness and eventual paralysis. Although LSA has been found infiltrating or compressing the spinal cord or peripheral nerves in many cats (Brightman *et al.*, 1977; Spodnick *et al.*, 1992), some have no evidence of LSA (Hardy, 1981a). FeLV has been cultured from brain tissue and the brain proposed to be a site of latency in an experimental study involving 7 cats (Haffer *et al.*, 1987).

FeLV-specific circulating IgG immune complexes (CIC) have been identified in the plasma of persistently viremic cats with or without LSA (Snyder *et al.*, 1982; Snyder *et al.*, 1985; Hardy, 1980a). These CIC have been implicated in development of hypocomplementemia, cell-mediated and humoral immune system defects (Snyder *et al.*, 1982; Kobilinsky *et al.*, 1979), and glomerulonephritis (Cotter *et al.*, 1975; Anderson and Jarrett, 1971; Hardy, 1981a). Although 4 of 5 cats with fatal glomerulonephritis were FeLV-infected in one survey (Cotter *et al.*, 1975), glomerulonephritis was not associated with persistent FeLV

infection in large-scale studies of necropsied cats (Reinacher, 1989). Several groups have treated FeLV-infected cats, particularly those with LSA, by extracorporeal immunoadsorption to remove CIC. Enhanced antibody response to FeLV gp70 (Snyder *et al.*, 1984), and clinical improvement/regression in cats with LSA (Snyder *et al.*, 1985; Jones *et al.*, 1984; Engelman *et al.*, 1987; Liu *et al.*, 1984b; Liu *et al.*, 1984a) have been reported. The major contribution of CIC to FeLV-related disease, may be through immunosuppression, rather than clinically significant glomerulonephritis.

Other diseases or conditions that have been associated with FeLV infection include: hepatitis and liver degeneration (Reinacher, 1989; Reinacher and Theilen, 1987), and enteritis with intestinal but not hemolymphatic histologic features of feline panleukopenia (Reinacher, 1987). Several bacterial, viral, parasitic, and mycotic agents have also been associated with FeLV infection, and are generally considered to be opportunistic due to host immunosuppression (Cotter *et al.*, 1975; Hoover and Mullins, 1991; Hardy, 1981a).

2.2.2. Neoplastic Disease

2.2.2.1. Lymphoid Tumors

2.2.2.1.1. Phenotype

Lymphosarcoma is a consequence of viremia in 10-15% of FeLV-infected cats (Mullins and Hoover, 1990). About 30% of all feline tumors are LSA (Hardy *et al.*, 1980a; Hardy,

1981d; Crighton, 1969; Dorn *et al.*, 1967; Hardy, 1978; Dorn *et al.*, 1968), and FeLV is incriminated in about 70% of cases using virus isolation or antigen detection (Hardy *et al.*, 1980b; Francis *et al.*, 1979; Hardy and MacEwen, 1989). Generally, virus positive T cell tumors are considered to account for the majority of FeLV-induced tumors (Hardy and MacEwen, 1989; Mullins and Hoover, 1990), and virus positive B cell tumors are not commonly seen (Rojko *et al.*, 1989).

The phenotype of feline LSAs has been determined in several ways. Earliest characterization of feline LSAs was by descriptive histopathology relating anatomic distribution of neoplastic cells to specific compartments in lymphoid tissues. Therefore, neoplastic lymphocytes within Peyer's patches and germinal centers of mesenteric lymph nodes suggested B cell transformation. Whereas, neoplastic lymphocytes within paracortical or thymus-dependent areas in thymic or multicentric LSA suggested T cell transformation (Mackey and Jarrett, 1972). Feline LSAs have also been classified histocytologically on the basis of cell size, nuclear cleavage, and histologic architecture (nodular or diffuse), to determine if correlations could be made with other tumor and patient data, and ultimately, prognosis (Valli *et al.*, 1981).

B cells are characterized by surface immunoglobulin (sIg) and receptor sites for the complement component, C3. Therefore, B cells may be detected by immunofluorescence

and erythrocyte, antibody, complement (EAC) rosette formation (Taylor *et al.*, 1975). T cells have no sIg or C3 receptors, but do have receptors for red blood cells of certain other species. Feline T lymphocytes form non-immune rosettes (E-rosettes) with guinea pig erythrocytes (GPE) but not sheep erythrocytes (Taylor *et al.*, 1975; Mackey *et al.*, 1975; Mackey *et al.*, 1975b; Cockerell *et al.*, 1976b).

Phenotypes of feline LSAs have been determined using E-rosette formation with GPE for T cell identification, and sIg and/or EAC rosette formation for B cell identification (Holmberg *et al.*, 1976; Cockerell *et al.*, 1976b; Hardy *et al.*, 1977; Mackey *et al.*, 1975b; Mackey *et al.*, 1975; Taylor *et al.*, 1975). In a study of 14 naturally occurring feline LSAs where FeLV status was not reported, 8 thymic tumors were T cell, 2 alimentary tumors were B cell, and 3 multicentric tumors displayed neither B nor T cell surface markers (Holmberg *et al.*, 1976).

In another study, the phenotype of 36 feline LSAs, 27 of which were FeLV-positive by IFA, was determined. Twelve of 12 thymic tumors were T cell, 10 of 18 multicentric tumors were T cell, 3 of 5 intestinal tumors were B cell, and the remaining 11 tumors were either mixed phenotype (both T and B cell markers present) or null cell (no surface markers present). Unfortunately, the distribution of the FeLV IFA results in relation to tumor phenotype was not reported (Hardy *et al.*, 1977). Attempts to

differentiate feline T and B lymphocytes using differential responses to mitogens, and cell surface morphology by scanning electron microscopy, have been unsuccessful (Holmberg *et al.*, 1976).

Rosette formation with GPE is now known to be nonspecific for T cell identification in the cat since feline monocytes and neutrophils also readily rosette GPE (Wellman *et al.*, 1986). More recent characterization of feline LSAs has employed monoclonal and polyclonal antibodies to feline and murine T and B cells, immunofluorescence assays for surface antigen expression, rosette-forming assays with GPE, and cytochemistry. Five primary feline LSAs and 6 feline LSA cell lines, all thymic and/or multicentric, were examined. Two cell lines were FeLV antigen negative, 3 primary LSAs were from cats with latent FeLV, and the rest were FeLV antigen positive. Results of phenotyping assays suggested that FeLV transforms several cell types including prothymocytes, null cells, and possibly, helper T cells or monocyte/macrophages. Transformation of mature B cells was excluded by lack of sIg expression (Rojko *et al.*, 1989).

Previous studies are limited by the low numbers of naturally occurring LSAs examined (Holmberg *et al.*, 1976; Rojko *et al.*, 1989; Cockerell *et al.*, 1976b), a bias towards examining thymic tumors which are more likely to be T cell (Rojko *et al.*, 1989; Cockerell *et al.*, 1976b; Mackey *et al.*, 1975b), and the use of a B cell marker which

detects only a relatively mature developmental stage (Rojko *et al.*, 1989; Hardy *et al.*, 1977; Holmberg *et al.*, 1976). Together, these reports underscore the need for: phenotypic characterization of spontaneous LSAs from all recognized anatomic sites and patient age groups, marker(s) for less mature B cells, and FeLV assessment by viral antigen and DNA within tumor tissue. Application of these assays to formalin-fixed, paraffin-embedded necropsy tissues would ensure that large numbers of anatomically well-characterized LSAs were assessed.

2.2.2.1.2. Anatomic Site

Feline lymphoid tumors are commonly classified according to anatomic distribution of the primary lesions. The 3 major forms of LSA are mediastinal, alimentary, and multicentric (Crighton, 1969).

Mediastinal LSA is thought to arise from T cells of the thymus, and frequently involves regional lymph nodes. Affected cats are usually <3 yr, and 75-80% are FeLV antigen-positive (Jarrett, 1984; Hardy, 1981d; Hardy, 1978).

Alimentary LSA involves the gastrointestinal wall, most commonly in the small intestine, cecum, or colon, but also the stomach or rectum (Jarrett, 1984). Mesenteric lymph nodes and less commonly kidneys and liver, may be infiltrated with neoplastic lymphocytes (Hardy, 1981d). Alimentary LSAs occur more often in older cats averaging 8 yr (Jarrett, 1984; Meincke *et al.*, 1972; Hardy, 1981d), and

10-33% are FeLV antigen-positive (Hardy, 1981d; Cotter *et al.*, 1975; Jarrett, 1984).

Multicentric LSA has a more generalized distribution usually involving multiple lymph nodes, visceral, and/or nonvisceral organs (Hoover and Mullins, 1991). Cats with multicentric LSA average 4 yr, and about 80% are FeLV antigen-positive (Hardy, 1981d).

Lymphocytic leukemia is often considered a manifestation of multicentric LSA and classified as such (Hardy, 1981d; Shelton *et al.*, 1990; Hardy *et al.*, 1980a). However, some classify lymphocytic leukemia according to a fourth distinct anatomic category (Cotter *et al.*, 1975; Mackey and Jarrett, 1972), or include it in a miscellaneous group along with cutaneous, ocular, and neurologic LSA (Meincke *et al.*, 1972).

Studies which include necropsies and histopathology in addition to clinical findings, are more likely to accurately classify LSA by anatomic site. With advanced disease, determination of primary tumor site may be impossible, resulting in more frequent assignment to the multicentric category. Inconsistencies in defining anatomic tumor sites of feline LSA and possible regional differences, contribute to discrepancies among prevalence of anatomic forms reported (Mackey, 1975; Loar, 1984). Nevertheless, in general, multicentric LSA including lymphocytic leukemia, is probably most common followed by anterior mediastinal, alimentary, then miscellaneous

anatomic sites (Hardy, 1981d; Hardy *et al.*, 1977; Rojko and Hardy, 1994). Young cats, particularly with multicentric or mediastinal LSA, are more likely to be FeLV antigen-positive than older cats with alimentary LSA (Rojko and Hardy, 1994; Hardy, 1981d; Jarrett, 1984).

Discrepancies exist in reports correlating tumor site with response to chemotherapy. One study showed highest remission rates and longest median survival times with multicentric LSA, and poorest prognosis with mediastinal LSA (Jeglum *et al.*, 1987). However, cats most likely to achieve complete response were those with mediastinal, peripheral lymph node, or solitary LSAs in another report (Cotter, 1983). Others noted no differences in response to therapy or survival times with anatomic location of LSA, although clinical staging of extent of disease and FeLV antigen-positive status were found to be important variables (Mooney *et al.*, 1989).

Anatomic classification of feline LSAs is useful for descriptive purposes, however, clear and consistent correlations with prognosis and response to therapy have not been shown. Differences may be determined using more sophisticated tumor phenotyping, tests for viral antigen and DNA within the tumor, and molecular analysis of oncogene expression and viral integration sites, in conjunction with traditional descriptive pathology.

2.2.2.1.3. Virus-negative Tumors

About 70% of cats with LSA have circulating FeLV

antigen and/or infectious virus (Hardy *et al.*, 1980b; Hardy *et al.*, 1977; Cotter *et al.*, 1975; Francis *et al.*, 1979). In these cats, both neoplastic and non-neoplastic cells usually contain integrated proviral FeLV which is replicating and releasing viral antigens and infectious virus, locally and into the peripheral circulation (Hardy *et al.*, 1977; Hardy *et al.*, 1980b; Rojko *et al.*, 1981; Casey *et al.*, 1981). Although results of virus isolation and IFA usually concur (Hardy *et al.*, 1980b), some cats without circulating FeLV antigen have culturable or reactivatable virus within their tumors (Hardy *et al.*, 1980b; Rojko *et al.*, 1989). This suggests a role for sequestered and/or latent FeLV in development of some feline LSAs.

Early attempts to demonstrate exogenous FeLV proviral sequences within virus-negative LSAs were unsuccessful (Casey *et al.*, 1981; Levin *et al.*, 1976; Okabe *et al.*, 1978). Recently however, with the PCR technique, FeLV DNA was detected in tumor tissue from 1 FeLV ELISA-negative cat with alimentary LSA (Matsumoto *et al.*, 1992). Whether the FeLV in this tumor was latent, sequestered, or replication-defective is not known.

Epidemiologic studies indicate similarities between cats with FeLV-positive LSA and those with virus-negative/non-producer (NP) LSA. Development of NP LSA in pet cats is associated with exposure to FeLV (Hardy *et al.*, 1980b; Francis *et al.*, 1981; Francis *et al.*, 1979).

Clinical studies show cats with NP LSA are often older, with a mean age of about 7 yr compared to 3 yr for cats with FeLV-positive LSA (Hardy *et al.*, 1980b; Francis *et al.*, 1979; Niman *et al.*, 1977), and/or they often have the alimentary form of LSA (Hardy *et al.*, 1980b; Cotter *et al.*, 1975; Hardy, 1987).

FOCMA expression on NP LSA cells supported the hypothesis of FeLV involvement in NP LSA (Hardy *et al.*, 1980b; Francis *et al.*, 1979; Francis *et al.*, 1981). However, as discussed earlier, FOCMA may be an endogenous gp70-like, rather than an FeLV-induced protein of neoplastic lymphocytes (Neil *et al.*, 1991), and its presence may not be relevant to LSA induction by FeLV.

Several explanations have been offered for FeLV involvement in NP LSA. Some cats may resist FeLV viremia but retain integrated FeLV provirus in hemolymphatic cells rendering them susceptible to neoplastic transformation. Following infection, replication-defective FeLV variants may arise perhaps through recombination with endogenous FeLV sequences (Hardy *et al.*, 1980b; Neil *et al.*, 1991). Also, FeLV may damage or alter host DNA causing transformation without itself becoming stably integrated, a so-called "hit and run" phenomenon (Hardy *et al.*, 1980b). Alternatively, provirus may be eliminated completely from the host cell genome following oncogenic activation, indicating that continued presence of viral genetic information is not necessary for tumor growth (Mullins and

Hoover, 1990).

Latent FeLV infection is present in some cats with NP LSA, as shown by reactivation of FeLV upon bone marrow culture (Rojko *et al.*, 1989). A possible role of sequestered FeLV in NP LSA induction, has not been investigated. Host-specific genetic predisposition to LSA development with FeLV infection, has also not been fully investigated (Mullins and Hoover, 1990).

At least some of the NP LSAs are probably sporadic and/or caused by agents other than FeLV. However, the availability of sensitive techniques for detecting viral DNA in relation to antigen expression within tumor tissues, is likely to incriminate FeLV in a significant proportion of NP LSAs.

2.2.2.2. Non-lymphoid Tumors

Hemopoietic neoplasms represent 33% of all feline tumors, and about 10% of these are non-lymphoid (Dorn *et al.*, 1968). FeLV replicates in all nucleated bone marrow cells of cats (Hardy, 1980b; Hardy *et al.*, 1973a), and circulating viral antigen is present in 70-90% of cats with erythrocytic (including erythroleukemic), granulocytic, monocytic, megakaryocytic, or myelomonocytic tumors (Blue *et al.*, 1988; Cotter *et al.*, 1975; Hardy, 1981d).

The pathogenesis of FeLV-related non-lymphoid tumors is not well studied but mechanisms, such as oncogene activation, may be similar to those for LSA (Hoover and Mullins, 1991). Other possibilities include interaction of

modified or recombinant viral proteins with specific growth factor receptors, or direct intracellular induction of proliferation of hemopoietic precursors by viral proteins (Neil *et al.*, 1987). Enhanced growth factor release by FeLV-infected bone marrow stromal cells (particularly fibroblasts), is another possible mechanism of hemopoietic progenitor cell proliferation (Linenberger and Abkowitz, 1992a; Abkowitz *et al.*, 1986; Linenberger and Abkowitz, 1992b).

An FeLV strain (FeLV-GM1), containing a mixture of subgroup A and B viruses, has been isolated from a naturally occurring case of myeloid leukemia. This strain causes myeloproliferative changes when given to newborn kittens, and the defective recombinant B component may play an indirect but important role in accelerated myeloid leukemogenesis (Tzavaras *et al.*, 1990).

Cats with hemopoietic neoplasms often have nonregenerative anemia and/or other cytopenia(s), hypercellular bone marrow, and circulating tumor cells (Hardy, 1981d). Tumors are sometimes difficult to classify using morphologic criteria alone, particularly when poorly differentiated or displaying heterogeneous characteristics. Cytochemical (Grindem *et al.*, 1985; Facklam and Kociba, 1986) and, as in human leukemia diagnosis, immunocytochemical assays (Hammer *et al.*, 1992; Perkins and Kjeldsberg, 1993) may help to accurately classify tumors that do not display obvious morphologic characteristics of

normal differentiated cells.

Aggressive multicentric fibrosarcomas in young FeLV-infected cats may be associated with a replication-defective FeLV known as feline sarcoma virus (FeSV). FeSVs are recombinant FeLVs which have captured one of at least 7 cellular proto-oncogenes, and are completely dependent on helper FeLV for their replication (Essex, 1980; Hardy, 1980c; Rojko and Hardy, 1994). About 2% of all feline fibrosarcomas may be attributed to FeLV/FeSV infection (Hardy, 1980c), and although the disease can be reproduced experimentally in cats and several other species, natural transmission of FeSV is unlikely because each FeSV is generated *de novo* by FeLV (Rojko and Hardy, 1994; Hardy, 1981c).

Virus particles resembling FeLV have been observed budding from chondrocytes in the cartilage caps of osteochondromas in cats (Pool and Carrig, 1972; Pool and Harris, 1975). Osteochondromas are benign tumors arising from the surface of bone formed by endochondral ossification. When many such lesions are present, the descriptive terms osteochondromatosis and multiple cartilaginous exostoses are used (Pool, 1990). Although the pathogenesis of osteochondromatosis in cats is unknown, hematogenous localization of FeLV into random periosteal sites followed by periosteal proliferation in response to the virus, has been proposed (Pool, 1990).

2.3. Molecular Basis of Feline Leukemia Virus-related Disease

2.3.1. Role of Envelope Sequences

FeLV gp70 sequences confer receptor binding properties which can be distinguished for the 3 subgroups using antigenic, superinfection interference, and host range assays (Mullins and Hoover, 1990; Hoover *et al.*, 1990; Wunsch *et al.*, 1983; Jarrett *et al.*, 1973; Sarma and Log, 1973; Kristal *et al.*, 1993). The *env* gene encodes the surface gp70 and the transmembrane p15E, and while p15E is highly homologous among FeLV subgroups, polymorphism exists in gp70 (Donahue *et al.*, 1988).

Generation of FeLV-B viruses from FeLV-A occurs both *in vitro* (Overbaugh *et al.*, 1988b) and *in vivo* (Boomer *et al.*, 1994; Rohn *et al.*, 1994). Five variable regions (vr) within gp70 have been identified based on alignments of coding sequences of subgroup A, B, and C proviral clones (Neil *et al.*, 1991; Kumar *et al.*, 1989). FeLV-B viruses have acquired vr 1-4 from endogenous FeLV, although the minimal content of endogenous FeLV which defines a subgroup B virus has not been determined (Neil *et al.*, 1991; Kumar *et al.*, 1989). Each FeLV-B is unique in the exact point at which recombination between FeLV-A and endogenous FeLV occurs during its generation (Neil *et al.*, 1987; Stewart *et al.*, 1986b). However, recombination at sites of identity between the exogenous and endogenous FeLV sequences is likely (Boomer *et al.*, 1994) since template jumping during

DNA synthesis would require base pairing to complementary sequences.

A 5' *env* domain, seemingly acquired from endogenous FeLV, is shared by all FeLV-B described suggesting that this region plays a role in FeLV-B receptor recognition (Boomer *et al.*, 1994). FeLV-B uses the same cell surface receptor as GALV (Takeuchi *et al.*, 1992), and FeLV-B, like GALV, tends to be directly associated with leukemogenesis (Jarrett and Russell, 1978; Rojko *et al.*, 1979). The GALV and FeLV-B receptors have broad species and tissue distribution, and envelope-receptor interactions may enhance proliferation of infected cells in order to increase virus spread (Takeuchi *et al.*, 1992).

In another study, although some thymic LSAs in cats infected with FeLV-A contained subgroup B variants, variants with defective *env* genes predominated (Rohn *et al.*, 1994). This finding lead to the speculation that deleted or truncated subgroup A-like *env* gene products may be involved in tumor induction. Cells containing proviruses with defective *env* genes may lack functional envelope proteins on their surface and therefore, evade the host immune response. Alternatively, cells infected with *env*-defective proviruses may be susceptible to superinfection and greater risk of proto-oncogene transduction or insertional activation by increased proviral insertions (Rohn *et al.*, 1994).

The prevalence of FeLV-B in LSAs has been questioned

since FeLV-B can be generated quickly *in vitro,* and earlier studies utilized *in vitro* assays to determine subgroup specificity (Overbaugh *et al.,* 1988b). Therefore, a direct approach using PCR was employed to examine the prevalence of recombinant or FeLV-B genomes in feline LSAs. Recombinants between endogenous and exogenous *env* sequences were found to be heterogeneous and common in naturally occurring FeLV-related LSAs (Sheets *et al.,* 1993). Mutations were found in the vicinity of the major neutralizing epitope of the gp70 protein; this epitope, located between vr 3 and vr 4, is strictly conserved among the three exogenous FeLV subgroups. Mutations of the major neutralizing epitope are known to permit escape from virus neutralization which may be important for virus latency and/or leukemogenicity (Sheets *et al.,* 1992; Sheets *et al.,* 1993; Elder *et al.,* 1987).

A FeLV subgroup B-like variant has been detected in brain, bone marrow, and lymph node from a specific pathogen free cat that developed neurological disorders more than 2 yrs after inoculation with FeLV-A. The subgroup B-like variant was replication defective and apparently acquired a major part of the gp70 gene from endogenous FeLV. Whether this variant contributed to the neurological disorders experienced by the cat is not known (Boomer *et al.,* 1994).

Recently, truncated endogenous FeLV proviral transcripts were detected within feline LSAs and a variety of normal feline tissues. Expression of a 35K truncated

env product correlated with endogenous FeLV RNA levels, and was negatively correlated with susceptibility to infection with FeLV-B. The truncated envelope protein is stably shed from cells and may block receptors for FeLV-B, which offers an explanation of the natural resistance of cats to FeLV-B in the absence of FeLV-A (McDougall *et al.*, 1994). The effect of constitutive expression of the endogenous FeLV antigen on immune responses to exogenous FeLV is unknown (McDougall *et al.*, 1994).

Many investigations of the role of *env* gene sequences in FeLV-related disease have focused on the strains inducing fatal immunodeficiency (FeLV-FAIDS) and fatal erythroid hypoplasia (FeLV-C). The prototype FeLV-FAIDS variant, 1161C, is a replication-defective virus with lymphocytopathic and enterocytopathic determinants localized to gp70, although LTR mutations may also enhance disease expression (Overbaugh *et al.*, 1988a; Neil *et al.*, 1991; Donahue *et al.*, 1991). Differences between gp70 sequences of 1161C and the prototype helper FeLV, 1161E, include 17 single nucleotide changes resulting in 11 scattered amino acid changes, an 18 bp deletion at vr 1 (near the amino terminus), and an 18 bp insertion at vr 5 (near the carboxyl terminus) (Overbaugh *et al.*, 1988a; Neil *et al.*, 1991). These genomic *env* changes result in delayed posttranslational processing of the 1161C gp70 (Poss *et al.*, 1989; Poss *et al.*, 1990) rendering it larger and antigenically distinct compared to the 1161E protein (Poss

et al., 1989).

Further analysis has shown 1161C precursor gp70 accumulates intracellularly due to retention of glucose residues on oligosaccharide side chains. A direct link between posttranslational modifications of gp70 protein and pathogenicity suggests that accumulation of gp70 in the rough endoplasmic reticulum or Golgi apparatus may mediate lymphocytopathicity (Poss *et al.*, 1990).

Studies using FeLV helper and immunodeficiency-inducing chimeric viruses *in vivo,* have demonstrated that a 7 amino acid region near the carboxyl terminus of 1161C gp70 is sufficient to mediate the immunodeficiency characteristic of this variant. This 7 amino acid region includes the 6 amino acid insertion and an adjacent amino acid substitution within vr 5 of the protein (Kizaki *et al.*, 1991; Quackenbush *et al.*, 1990). However, the chimera containing the entire *env* gene and 3'LTR of 1161C causes enhanced T lymphocyte killing and viral replication indicating that other regions of gp70 and the LTR are also involved in FeLV-FAIDS pathogenesis (Donahue *et al.*, 1991).

Subgroup phenotype of FeLV-FAIDS 1161C has not been fully determined although superinfection interference assays indicate recognition of subgroup A receptor (Neil *et al.*, 1991). Although delayed, processing of 1161C gp70 eventually does occur. Perhaps only the processed protein recognizes the subgroup A receptor, whereas precursor gp70 accumulates within cells rather than being expressed on

infected cell surfaces. The clinical onset of immunodeficiency correlates with replication of FeLV-FAIDS variant detectable as unintegrated linear viral DNA (UVD) within bone marrow, lymphoid tissues, and intestine (Mullins *et al.*, 1986; Hoover *et al.*, 1987). The UVD causes T cell killing and may result from FeLV superinfection which is permitted rather than blocked by the mutated 1161C gp70 protein (Donahue *et al.*, 1991). Determinants for the superinfection interference defect are similar to those for T cell killing, and the more cytopathic the virus, the less capable the gp70 is of establishing interference (Reinhart *et al.*, 1993).

The cytopathic properties of FeLV variants in feline T cell cultures parallel the ability to induce immunodeficiency disease *in vivo* (Thomas and Overbaugh, 1993; Overbaugh *et al.*, 1988b; Overbaugh *et al.*, 1992; Donahue *et al.*, 1991). Therefore, an *in vitro* system was used to investigate differences in latency period between FeLV 1161C and FeLV 1161B (Overbaugh *et al.*, 1992) which are both replication defective and cause fatal immunodeficiency with help from replication competent FeLV-A (Thomas and Overbaugh, 1993). The longer latency period between infection and disease with 1161B compared to 1161C, mapped to 4 predicted amino acid differences within p15E of 1161B. Cytopathicity which eventually occurred with 1161B was associated with generation of a recombinant virus encoding the gp70 of 1161B, and p15E of helper virus

(Thomas and Overbaugh, 1993). Presumably the predicted amino acid differences in the TM protein (p15E) of 1161B prevented expression of a functional variant SU glycoprotein (gp70) required for cytopathicity (Thomas and Overbaugh, 1993). The external domain of the TM protein attaches to the SU glycoprotein and contains an amino terminal hydrophobic region necessary for membrane fusion (Coffin, 1990). Variation within this region could disturb the gp70-p15E configuration which may be crucial for receptor binding and virus entry.

Purified gp70 from FeLV 1161E and FeLV 1161C each coprecipitate 70K surface proteins on feline T cells, yet 1161C gp70 has a defect that results in T cell killing and failure to prevent superinfection. Multiple cellular components are probably involved in FeLV binding and penetration, and although 1161E and 1161C may both use a primary receptor, secondary receptors may be variant specific (Reinhart *et al.*, 1993).

Nucleotide sequence analysis of the *env* gene of FeLV-C/Sarma, the prototype anemogenic virus, compared to a subgroup B FeLV, has revealed 5 regions of predicted amino acid differences, and fewer potential glycosylation sites for FeLV-C/Sarma gp70 (Riedel *et al.*, 1986). Experiments involving chimeric viruses of FeLV-C/Sarma and FeLV-A colocalized the pathogenic and host range determinants of FeLV-C/Sarma to a 3'*pol*-5'*env* region of the genome, particularly a region encoding 241 amino acids of the amino

terminus of gp70 (Riedel *et al.*, 1988). Moreover, receptor specificity of FeLV-A/Glasgow-1 can be converted to that of FeLV-C by exchange of vr 1 of the gp70 coding region, a cysteine bounded domain which differs by a 3 codon deletion and 9 adjacent substitutions (Rigby *et al.*, 1992).

Only 1 complete sequence of FeLV-C, the Sarma strain, is known (Luciw *et al.*, 1985; Riedel *et al.*, 1986), however, sequence analysis of vr 1 from limited numbers of naturally occurring subgroup C isolates shows that each is unique in this region (Rigby *et al.*, 1992). Although chimeras generated by exchanging vr 1 of FeLV-A with vr 1 fragments of FeLV-C, have FeLV-C host range and subgroup characteristics, they are less age-restricted than FeLV-C/Sarma for infection in kittens. Also, the anemia in neonates infected with FeLV-C vr 1 chimeras compared to FeLV-C/Sarma, is delayed, regenerative, and accompanied by early depletion of granulocyte-monocyte precursors, suggesting that sequences besides those specifying the subgroup C phenotype, are involved in infectivity and pathogenicity (Rigby *et al.*, 1992).

Although FeLV-C/Sarma and FeLV-A 1161E have equivalent tropism for nucleated erythroid, myeloid, and lymphoid cells in the bone marrow, selective elimination of erythroid progenitor cells is specific to the FeLV-C/Sarma (Dean *et al.*, 1992). This indicates that anemogenesis is not due to a unique tropism of FeLV-C/Sarma for erythroid cells, but a unique cytopathic effect on erythroid cells

mediated by the amino terminus of gp70 (Dean *et al.*, 1992). Interference with the transferrin or other erythroid differentiation factor receptors by FeLV-C/Sarma has been suggested (Abkowitz, 1991), however, the pathogenic mechanisms remain unknown.

2.3.2. Role of Long Terminal Repeat Sequences

Although extensive homologies exist between LTRs of endogenous and exogenous FeLV, regions within the U3 diverge and form the basis of a hybridization probe to differentiate the two (Casey *et al.*, 1981). Clues to explain relative inefficiency of transcription from the endogenous FeLV LTR are likely to reside in the U3 sequences. Major differences include variations in sets of 14-19 bp direct repeats, altered location of the SV40 core enhancer-like sequence, and 3 regions of nonhomologous sequence. Although endogenous FeLVs contain promoter and enhancer elements within U3, transcription is thought to be tightly regulated by proximal host DNA sequences (Berry *et al.*, 1988).

Exogenous FeLVs do not contain the 3 different sets of direct repeats which lie outside the putative enhancer motifs in endogenous U3 regions. Large direct repeats of 0.85-1.3 kilobase pair (kbp) in retroviral vectors are unstable (Rhode *et al.*, 1987). Although the direct repeats in endogenous FeLV are only 14-19 bp, deletion of these sequences and acquisition of new sequences in U3 may have conferred replication efficiency and selective advantages

to exogenous FeLVs (Berry *et al.*, 1988). Moreover, spacing of enhancer motifs relative to each other may be important for efficient enhancer function (Golemis *et al.*, 1990). The endogenous FeLV U3 is 30-60 bp larger than its exogenous counterpart, and the SV40-like sequence of endogenous U3 is 90 bp downstream relative to its position in the exogenous FeLV U3 (Berry *et al.*, 1988). These differences between endogenous and exogenous FeLV LTRs relate, in part, to the 3 direct repeats in the endogenous virus, and offer an explanation for less efficient transcriptional activity of the endogenous LTR.

When published sequences are aligned for U3 regions of several type C mammalian retroviruses, consensus motifs of the transcriptional enhancers for this set of viruses can be determined (Golemis *et al.*, 1990). These include binding sites for LVb, the SV40 core-like element, NF1, and the GRE. Some type C retroviruses contain 2 or more tandem copies of enhancer sequences which include these binding sites in both copies of the repeat (Golemis *et al.*, 1990).

Studies involving mutated enhancer sequences of the Moloney murine leukemia virus (MoMuLV) have helped determine some of the functions of these motifs in this virus (Boral *et al.*, 1989; Johnson *et al.*, 1987; Thornell *et al.*, 1988; Speck and Baltimore, 1987; Speck *et al.*, 1990b; Speck *et al.*, 1990a). For instance, several mutations in LVb and GRE sites of MoMuLV, decreased transcription in all cell lines examined. However, certain

mutations in the SV40-like core of MoMuLV affected transcription in lymphocytes specifically, whereas mutations in NF1 decreased transcription in fibroblasts (Speck *et al.*, 1990b). Also, certain point mutations in either the LVb or SV40 core-like sites of the MoMuLV enhancer resulted in increased latency before disease induction and development of altered types of leukemia (Speck *et al.*, 1990a). These studies suggest transcriptional regulation of the virus correlates with the presence or absence of certain DNA-binding proteins within tissues. Abundance of enhancer binding protein(s) in certain tissues could increase transcription leading to neoplastic transformation of that tissue. Lack of binding of cellular proteins to negative regulatory elements could actually accelerate transcription and disease induction. Decreased transcription may result in prolonged latency before disease induction.

In addition to the highly conserved enhancer framework (LVb, SV40-like core, NF1, and GRE sites) present in most mammalian type C viruses (Golemis *et al.*, 1990; Speck and Baltimore, 1987), the FLV-1 site is located downstream from GRE in FeLV LTRs (Fulton *et al.*, 1990). FLV-1 contains an indirect repeat which is similar to the PEA2 binding site of the polyomavirus A enhancer, and is thought to be a negative regulatory element that binds a protein factor in fibroblast but not T cell tumor extracts (Fulton *et al.*, 1990).

The inverted repeat (IR) sequence, TGAAAGACCCC, in the 5' region of U3, is highly conserved in mammalian type C retroviruses, including FeLV, and is essential for replication (Panganiban and Temin, 1983). A second motif, CGCCAAT, within the upstream conserved region located 18-27 bp downstream of the IR, is conserved and for several murine viruses represents a negative regulatory element (Flanagan *et al.*, 1989). This second motif appears to be less well conserved in exogenous than endogenous FeLV LTRs.

Highly conserved sequences of unknown function have been identified between the CCAAT and TATA motifs of the promoter region in many mammalian type C retroviruses (Golemis *et al.*, 1990). At least one of these, CGCGCTT, is present in both endogenous and exogenous FeLV, but its role in transcription, if any, remains to be determined.

Little is known about specific functional roles of putative enhancer elements in FeLV. Although several potential binding sites for nuclear proteins have been identified in FeLV LTRs, *in vitro* footprint analysis has demonstrated protein binding only to the SV40-like core, NF1, and FLV-1 sites (Fulton *et al.*, 1990).

In several mammalian type C retroviruses, the GRE motif is a binding site for steroid hormone receptors which correlates with transcriptional activation by dexamethasone. Sequences flanking the GRE site are thought to affect activation by glucocorticoids since not all enhancers with GRE sites are transcriptionally activated by

dexamethasone (DeFranco and Yamamoto, 1986; Overhauser and Fan, 1985). Failure to demonstrate protein binding to the GRE site in FeLV is interesting in light of the ability to convert latent to replicating FeLV *in vivo* with dexamethasone treatment (Pedersen *et al.*, 1984). Immunologic as well as transcriptional factors may be involved in maintenance of the latent FeLV-infected state, even though serum antibody levels are not different between latently infected and recovered cats (Madewell and Jarrett, 1983).

A point mutation in the FeLV NF1 binding motif reduces NF1 binding and transcriptional activity *in vitro*, however, the degree of LTR impairment varies with cell type. Transcriptional activity appears to be less impaired in FeLV-induced T LSA cell lines which also exhibit decreased NF1 activity (Plumb *et al.*, 1991).

Enhancer duplications were common in 8 FeLV LTRs cloned directly from 3 naturally occurring thymic LSAs, and in 3 FeLV LTRs cloned from cell lines derived from 3 induced thymic tumors. Duplications were unique to each isolate but consistently included at least the SV40-like core and adjacent LVb motif (Fulton *et al.*, 1990). In another study involving a T cell leukemia cell line (FT-1) with a rearranged cellular oncogene, c-myc, downstream of integrated FeLV, the FeLV LTR contained 3 copies of the complete enhancer framework, exclusive of the FLV-1 site (Miura *et al.*, 1989).

FeLV LTRs have been analyzed in a more heterogeneous group of hemopoietic tumors and a few non-neoplastic FeLV-related diseases. Based on electrophoresis of PCR products, enhancer duplications were predicted in 8 of 8 thymic LSAs, 3 of 8 LSAs from other sites, 2 of 3 myeloid tumors, and 1 of 6 non-neoplastic diseases. FeLV U3 sequences were determined from 1 myeloid and 5 lymphoid tumors, and only the SV40-like core element was present in all the enhancer repeats (Matsumoto *et al.*, 1992). The novel FLV-1 site was invariably excluded from enhancer duplications which may support its role as a negative regulatory element (Fulton *et al.*, 1990).

To determine if enhancer duplications arise *de novo* during infection, FeLV U3 regions were compared in tissues from 9 cats that died after infection with molecularly cloned FeLVs containing a single enhancer. Enhancer regions from the 4 cats that died with T cell thymic LSA contained single base changes including point mutations in the core and FLV-1 sequences. Two of the 4 tumors contained duplications of LVb and core sites. The 5 cats with non-neoplastic FeLV-related disease retained the single enhancer unit of the infecting virus (Rohn and Overbaugh, 1995).

Although duplicated FeLV enhancer sequences may be more prevalent in cats with FeLV-related tumors, particularly thymic LSA, functional assays of LTRs with duplicated enhancer sequences show only modest increases in

activity resulting from the duplicated sequences (Plumb *et al.*, 1991). FeLVs with duplicated enhancers might be expected to have a growth advantage over those with single units. However, when comparing PCR products within individual cases with concurrent single and duplicated enhancers (Matsumoto *et al.*, 1992; Rohn and Overbaugh, 1995), bands with single enhancers appear more intense than those representing duplicated sequences in 12 of 16 cases. With PCR, detection of the double enhancer product is possible when it represents 2% or more of the LTR species (Rohn and Overbaugh, 1995).

Elucidating the role of FeLV enhancer elements in disease manifestation will involve sequence analysis of FeLV LTRs from larger numbers of natural cases of FeLV-related neoplastic and nonneoplastic disease. More extensive functional assays of FeLV LTR alterations *in vitro* and *in vivo*, and investigations of LTR mutations and/or duplications in relation to oncogene activation, will follow.

2.3.3. Role of Oncogenes

Natural variants of FeLV have been described which contain the cellular genes *fes, fems, abl, sis, kit, fgr* + actin, K-*ras*, *myc*, and *tcr*. All have been derived from fibrosarcomas except *myc* and *tcr*-containing variants which have been isolated from T cell LSAs [reviewed in (Mullins and Hoover, 1990; Neil *et al.*, 1991; Rezanka *et al.*, 1992; Hardy, 1981c; Hardy, 1990; Rojko and Hardy, 1994)].

An estimated 10-30% of naturally occurring FeLV positive T cell LSAs are associated with activation of the *myc* oncogene by FeLV transduction (Mullins *et al.*, 1984; Neil *et al.*, 1984; Levy *et al.*, 1984; Miura *et al.*, 1987), enhancer insertion (Neil *et al.*, 1987; Neil *et al.*, 1984; Levy *et al.*, 1984; Miura *et al.*, 1987; Mullins and Hoover, 1990), promoter insertion (Forrest *et al.*, 1987), or by events not directly linked to viral integration (Miura *et al.*, 1987; Forrest *et al.*, 1987). Cellular *myc* encodes a DNA-binding phosphoprotein which is involved in transcriptional regulation (Varmus, 1988). Transduction of c-*myc* appears to predominate in field cases of LSA, whereas, proviral insertion within or near c-*myc* may be more common in experimentally induced LSAs (Mullins and Hoover, 1990).

The probability of *cis*-activation of cellular oncogenes is higher with increased viral entry into target cells and increased viral transcription. The use of highly leukemogenic virus strains and young less immunocompetent cats in experimental infections may promote rapid spread of virus and increased opportunity for *cis*-activation of cellular genes (Mullins and Hoover, 1990). Intermediates in the generation of virus/oncogene recombinants have not been observed. However, the presumed stepwise process of oncogene capture (transduction), deletion of viral gene sequences, and generation of a replication defective virus, probably occurs slowly as does tumor induction in natural

FeLV infections (Mullins and Hoover, 1990; Mullins *et al.*, 1984).

Almost all of the coding sequences of c-*myc*, exons 2 and 3, are retained in most recombinant FeLV/*myc* viruses which have been studied (Braun *et al.*, 1985; Doggett *et al.*, 1989; Forrest *et al.*, 1987; Neil *et al.*, 1987). The exon 1 content of v-*myc* in FeLV/*myc* isolates varies. However, the loss of noncoding sequences of exon 1 in several v-*myc*, suggests that these sequences are not required for transformation, in fact, loss of these sequences may increase stability of the transcript and favor transformation (Stewart *et al.*, 1986a).

Despite evidence that the *myc* protein carboxyl terminal is involved in DNA binding, a v-*myc* protein with a truncated carboxyl terminal in the FeLV/*myc* isolate, FTT-*myc*, remains active (Doggett *et al.*, 1989). In general the feline v-*myc* genes are highly conserved relative to c-*myc* (Neil *et al.*, 1987), suggesting that altered *myc* expression without *myc* mutations contributes to tumorigenesis of affected tissues (Doggett *et al.*, 1989).

The 5′ and 3′ virus/oncogene junctions are unique in each FeLV/*myc* isolate studied, however, there is clustering particularly of the 5′ recombinational junctions. Whether this is a result of FeLV recombinational "hot spots" or factors required for oncogene expression, is not known (Neil *et al.*, 1991). The 2 cluster points for 5′ virus/oncogene junctions are within the 3′ end of *gag* or

within the 3′ end of *pol*, immediately downstream of the splice acceptor site. The 3′ virus/oncogene junctions are within the 3′ half of *pol* or within *env* (Neil *et al.*, 1991). The 3′ FeLV-*myc* junctions precede the major c-*myc* polyadenylation signal. Exclusion of polyadenylation signals in transduced *myc* may prevent premature cleavage of recombinant viral transcripts (Forrest *et al.*, 1987), thereby allowing expression of FeLV-*myc* as a full-length transcript available for packaging by helper FeLV (Rezanka *et al.*, 1992).

Experimental infection of neonatal kittens with *myc*-containing FeLV may, but does not invariably, induce short latency thymic LSA (Levy *et al.*, 1988). Also, *in vitro* infection of bone marrow cells or thymocytes with FeLV/*myc* viruses does not result in tumor formation. These findings suggest at least one event besides *myc* transduction by FeLV is required for T cell tumorigenesis *in vivo* (Terry *et al.*, 1992).

A naturally occurring thymic LSA containing full length helper FeLV, recombinant FeLV/*myc*, and recombinant FeLV/*tcr* (T cell antigen receptor β-chain gene) has been investigated (Fulton *et al.*, 1987; Terry *et al.*, 1992). The 2 transductions occurred independently, and v-*tcr* did not have a readily transmissible oncogenic function. Thymic LSAs induced by direct inoculation of neonatal kittens with the 3 virus complex, or with molecularly cloned FeLV-*tcr*, did not contain clonally positioned v-*tcr*

sequences. Most T cell tumors induced with the 3 virus complex manifested v-*myc* or activated c-*myc*, and had rearranged endogenous T cell antigen receptor β-chain genes suggesting transformation of T cells at a discrete stage of differentiation (Terry *et al.*, 1992). The oncogenic significance of the transduced *tcr* gene has not yet been determined.

Integration of FeLV at *flvi*-1 sites was identified in 4 of 7 naturally occurring feline LSAs that were non T cell, splenic in origin, and which contained a smaller number of proviral integrations than found in other LSAs. The *flvi*-1 domain may represent a previously unidentified oncogene, and the cascade of events leading to transformation of non T lymphocytes may be small when FeLV integration occurs at *flvi*-1 (Levesque *et al.*, 1990).

The *flvi*-2 locus is another target of insertional activation in natural and experimental FeLV-induced thymic LSA. Inserted proviruses are downstream and in the same transcriptional direction as *flvi*-2, and about 50% of the time alterations of c-*myc* accompany *flvi*-2 activation (Levy and Lobelle-Rich, 1992; Levy *et al.*, 1993b). The locus *flvi*-2 is a feline homolog of *bmi*-1, whose product is a DNA binding protein which has been implicated as a *myc*-collaborator in oncogenesis (Levy and Lobelle-Rich, 1992; Levy *et al.*, 1993a). However, activation of *flvi*-2 occurs in FeLV-induced thymic LSA with or without *myc* activation. Proviral insertion at *flvi*-2 is likely an early event in

oncogenesis although concurrent *myc* alteration may accelerate tumor development (Levy *et al.*, 1993b).

In a study of 63 LSAs and tumor cell lines of probable T cell origin, *myc* was involved in 32% (transduced in 21% and insertionally activated in 11%), and proviral insertions were present at *flvi*-2 in 24%, at *fit*-1 in 8%, and at *pim*-1 in 5% (Tsatsanis *et al.*, 1994). Two loci were affected in 19% of tumors, and 3 loci were involved in 5% of tumors. Involvement of the *fit*-1 locus was most commonly seen in tumors with activated *myc*, suggesting *fit*-1 insertions may occur late in tumor development. Rearrangement of the T cell receptor β-chain, a standard marker of T cell differentiation, was strongly associated with activation of *myc*, and with insertional activation of *flvi*-2, suggesting a window of susceptibility to T cell transformation. A subset of thymic LSAs lacked T cell receptor rearrangement and involvement of *myc*, *flvi*-2, *fit*-1, or *pim*-1 (Tsatsanis *et al.*, 1994).

Rearrangement of c-*myc* in FeLV-induced thymic LSA is most commonly associated with FeLV enhancer insertion upstream and in the opposite direction to *myc* (Forrest *et al.*, 1987; Neil *et al.*, 1987; Rezanka *et al.*, 1992). Moreover, one such FeLV isolate which activates c-*myc* by enhancer insertion, originates from a feline T cell leukemia cell line, contains 3 copies of the putative enhancer region, and is integrated about 1 kbp upstream and in the opposite transcriptional orientation of c-*myc* (Miura

et al., 1989).

Activation of c-*myc* by promoter insertion has also been suspected in a thymic tumor with truncated FeLV inserted within intron 1 of *myc* (Forrest *et al.*, 1987). A complex rearrangement at the 3' end of c-*myc* within another thymic tumor has been described, but linkage between the rearranged *myc* and FeLV sequences was not found (Forrest *et al.*, 1987).

The mechanism of FeLV proviral insertional activation, and functional roles of *flvi*-1, *flvi*-2, *fit*-1, and *pim*-1 loci have not been investigated in detail. Nor has the possibility of tumor suppressor gene inactivation been adequately studied in FeLV-induced tumors. Most reports involving the role of oncogenes in FeLV-related tumor pathogenesis, have focused on thymic (T cell) LSAs, whereas FeLV-induced neoplasms including LSAs from other anatomic sites, B cell tumors, and sarcomas of other hemopoietic cells remain largely unexplored.

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3. OBJECTIVES

Domestic cats with persistent FeLV infection often succumb to hemopoietic neoplasms, cytopenias, or immunodeficiency. Cats with historical, clinical, and pathologic evidence of FeLV-related disease may lack expression of viral antigens in peripheral blood, bone marrow, or other tissues. Latent or replication-defective FeLV variants cannot be detected by traditional assays which rely upon viral transcription and translation of protein products. The PCR for amplification of FeLV DNA, in conjunction with serum ELISA or IHC for FeLV antigens, allows comparison of prevalence of FeLV DNA versus antigens in fresh or fixed feline tissues.

A region of the FeLV LTR which contains the putative transcriptional enhancer elements, is ideal for amplification by PCR. This LTR region is generally conserved among exogenous FeLV subgroups but distinct from endogenous FeLV-like sequences. Also, subtle differences or repeated sequences within the enhancer region of FeLV are likely to relate, at least in part, to disease manifestation. Therefore, sequence analysis of PCR products may provide insights into FeLV disease pathogenesis.

Lymphosarcomas induced by FeLV are generally

considered to be T cell. However, previous investigations of FeLV-induced LSAs have been limited by relatively crude assays to detect T and B cell phenotypes, and a bias toward study of thymic tumors which are likely to be T cell. Polyclonal and monoclonal antibodies for identification of T and B lymphocytes, respectively, in paraffin-embedded, formalin-fixed feline tissues, are now available.

The detection of FeLV DNA using PCR, and FeLV antigen and lymphocyte phenotype using immunohistochemistry, would enable more accurate description of feline LSAs than previously possible. Archival feline LSAs are well-characterized with respect to anatomic location and histopathology, and large numbers of naturally occurring tumors from various sites and patient ages are usually available. Meaningful data could be obtained by investigating such a diverse yet representative sampling of feline LSAs.

Detection of FeLV in peripheral blood of cats with evidence of FeLV-related disease, using PCR compared to ELISA, may help determine if some cats harbour non-replicating but disease-producing FeLV within peripheral blood cells.

Sequence analysis of putative enhancer regions in a wide range of FeLV-induced diseases may provide insights into the relationship between the FeLV LTR and neoplastic versus degenerative disease, and replicating (antigen-positive) versus nonreplicating (antigen-negative) FeLV.

Therefore, the hypotheses of this study were:

1. that most, if not all, feline LSAs are caused by FeLV which may or may not be replicating.
2. that FeLV induces B as well as T cell LSAs.
3. that cats with clinicopathologic evidence of FeLV-related disease but without detectable viral antigen, harbour latent or replication-defective forms of FeLV.
4. that sequence differences within the FeLV enhancer region correlate with disease manifestation in individual FeLV-infected cats.

The specific research objectives that enabled testing of the hypotheses were as follows:

1. to determine the prevalence of FeLV DNA using PCR, and antigen using IHC, in formalin-fixed tumors from 70 cats with LSA, and to relate these findings to tumor anatomic site, patient age, and antemortem FeLV ELISA results.
2. to determine the T and B lymphocyte phenotype of 70 formalin-fixed feline LSAs, and to relate these findings to FeLV detection by PCR and IHC, patient age, and tumor anatomic site.
3. to determine the prevalence of FeLV DNA using PCR, and antigen using ELISA, in peripheral blood samples from 68 cats with high, moderate, or low clinicopathologic evidence of having FeLV-related disease.
4. to sequence and analyze PCR products containing the putative FeLV enhancer region, from 33 cats with diverse proliferative and degenerative FeLV-related diseases.

4. FELINE LEUKEMIA VIRUS DETECTION BY IMMUNOHISTOCHEMISTRY AND POLYMERASE CHAIN REACTION IN FORMALIN-FIXED, PARAFFIN-EMBEDDED TUMOR TISSUE FROM CATS WITH LYMPHOSARCOMA

4.1. Abstract

The prevalence of feline leukemia virus (FeLV) antigen and DNA was assessed in formalin-fixed, paraffin-embedded tumor tissues from 70 cats with lymphosarcoma (LSA). Tissue sections were tested for FeLV gp70 antigen using avidin-biotin complex (ABC) immunohistochemistry (IHC); DNA was extracted and purified from the same tissue blocks for polymerase chain reaction (PCR) amplification of a 166 base pair region of the FeLV long terminal repeat (LTR). Results were related to antemortem FeLV enzyme-linked immunosorbent assay (ELISA) for serum p27 antigen, anatomic site of LSA, and patient age. Viral DNA was detected by PCR in 80% of cases and viral antigen by IHC in 57% of cases. Seventeen cases were PCR-positive and IHC-negative; one case was PCR-negative and IHC-positive. Clinical records included FeLV ELISA results for 30 of 70 cats. All 19 ELISA-positive cats were positive by PCR and IHC; of the 11 ELISA-negative cats that were negative by IHC, 7 were positive by PCR. When evaluated according to anatomic site, FeLV DNA and antigen were detected less frequently in intestinal LSAs than in multicentric and mediastinal

tumors. Lymphosarcoma tissues from cats <7 yr were several fold more likely to be positive for FeLV antigen by IHC than were tumors from cats ≥7 yr. However, there was no significant difference in PCR detection of FeLV provirus between LSAs from cats <7 yr and those ≥7 yr. Immunohistochemical detection of FeLV antigen in formalin-fixed, paraffin-embedded tissues agreed with antemortem FeLV ELISA results, however, FeLV DNA could be detected by PCR in some LSA tissues with no demonstrable FeLV antigen, particularly in cats ≥7 yr. These provirus-positive, antigen-negative cases may represent infection with latent or replication-defective FeLV.

4.2. Introduction

Feline leukemia virus is an exogenous retrovirus causing several diseases in domestic cats, including tumors of most hemopoietic cells, aplastic anemia, myeloproliferative disorders, and immunosuppression (Donahue *et al.*, 1988; Blue *et al.*, 1988; Harvey *et al.*, 1978; Casey *et al.*, 1981; Neil, 1985; Reinacher and Theilen, 1987; Riedel *et al.*, 1986; Soe *et al.*, 1983; Stewart *et al.*, 1986; Tzavaras *et al.*, 1990; Overbaugh *et al.*, 1988b; Hardy and MacEwen, 1989; Hoover and Mullins, 1991; Hoover *et al.*, 1981; Hardy *et al.*, 1976; Donahue *et al.*, 1988; Jacobsen *et al.*, 1985; Pedersen, 1990; Cotter *et al.*, 1975; Cotter, 1992).

Lymphosarcoma represents 30% of all tumors in cats and about 70% of feline LSAs contain FeLV antigen and/or infectious virus (Kitchell, 1989; Hardy and MacEwen, 1989; Cotter *et al.*, 1975; Francis *et al.*, 1979). Cats with LSA in which FeLV is not detected are usually $\geq$7 yr and often have the intestinal form of the tumor (Hardy *et al.*, 1980; Hardy and MacEwen, 1989; Hardy, 1987).

Immunohistochemistry, particularly immunofluorescence, has been used extensively for viral diagnosis, including FeLV (Hardy *et al.*, 1973). Traditionally this technique has been applied to detect viral antigens in fresh or frozen tissues, but now enzyme-based IHC includes detection of antigens in formalin-fixed, paraffin-embedded tissues (Haines and Clark, 1991). The ability to detect viral

antigens in fixed tissues is advantageous for ease of sample submission, improved morphologic detail of tissues, and in combination with immunoenzyme detection systems, production of a permanent stain visible with ordinary light microscopy. However, while IHC is sensitive for identification of viral antigens in productive infections, latent viral infections are not discovered.

The polymerase chain reaction is a molecular technique which can be applied to the amplification of viral DNA (Innis *et al.*, 1990). The PCR has been used to amplify a region of the FeLV LTR so that sequence variation in the LTRs of FeLV proviruses from T-cell LSAs could be investigated (Fulton *et al.*, 1990). Other researchers have compared enhancer duplication within the FeLV LTR in cells from FeLV-infected cats with neoplastic and non-neoplastic disease, by PCR amplification of the enhancer region followed by nucleotide sequencing (Matsumoto *et al.*, 1992). There are no reports of application of the PCR in a clinical setting for the diagnosis of FeLV infection, nor are there reports of PCR application to formalin-fixed, paraffin-embedded tissues for FeLV detection. The PCR is potentially more sensitive than IHC in detecting FeLV infection when virus load is low, the infection is latent, or a replication-defective virus is present. The purpose of this study was to apply these two techniques, PCR and enzyme-based IHC, to formalin-fixed, paraffin-embedded tissues for detection of FeLV in cats with LSA. The basic

objective was to determine if FeLV DNA is associated with antigen-negative feline LSA. The prevalence of FeLV DNA and antigen in tumor tissue was related to patient age, anatomic tumor site, and antemortem ELISA for serum FeLV antigen.

4.3. Materials and Methods

4.3.1. Case Selection

Seventy feline LSA cases diagnosed according to histopathologic criteria (Valli, 1985), were retrieved from the pathology records at the Western College of Veterinary Medicine. The tumors were grouped by anatomic site as follows: multicentric, mediastinal, intestinal, and miscellaneous. Lymphocytic leukemia was included in the multicentric category. One to four paraffin blocks containing tumor tissue from various sites were used from each case. Medical records were reviewed to tabulate patient data, clinical history, and antemortem ELISA results.

4.3.2. Immunohistochemistry

The avidin-biotin complex (ABC) method for IHC staining of formalin-fixed, paraffin-embedded tissues has been previously described (Haines and Chelack, 1991). The following modifications were made for FeLV antigen detection. Nonspecific adherence of proteins to tissues was blocked by flooding sections with phosphate buffered saline supplemented with 4.0% serum from normal rabbits, the species in which the secondary antiserum was raised. Goat anti-FeLV gp70 primary antiserum (National Cancer Institute, Bethesda, Maryland) was applied to tissues followed by a biotinylated secondary antiserum, rabbit anti-goat immunoglobulin (Vector Laboratories, Burlingame, California), and an ABC peroxidase detection system

(Vectastain Elite ABC, Vector Laboratories, Burlingame, California). Primary antiserum was applied to duplicate serial sections of each tissue, diluted 1:4000 and 1:8000. The secondary antiserum was applied diluted 1:200. Serial sections of each tissue were tested with similar dilutions of an irrelevant goat serum substituted for anti-FeLV antiserum.

4.3.3. Samples for Polymerase Chain Reaction

Samples were prepared for PCR as previously described (Innis *et al.*, 1990). From the same paraffin blocks used for IHC, 10 um sections were cut to yield a minimum of 1 cm^2 of tissue. Sections were deparaffinized by extraction twice with xylene, then rinsed once with 95% ethanol, centrifuged, the liquid decanted, and the remaining ethanol evaporated under vacuum. Samples were resuspended in 200 uL of digestion buffer [50 mM Tris (pH 8.5), 1 mM EDTA, 0.5% Tween 20] containing 200 ug/mL of Proteinase K (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Maryland), and incubated at 37°C overnight. Samples were then extracted twice with phenol-chloroform-isoamyl (25:24:1), once with chloroform-isoamyl (24:1), and precipitated with ethanol according to standard technique (Sambrook *et al.*, 1989). The dried samples were resuspended in 50 uL of HPLC grade water (BDH Inc., Toronto, Ontario). A 1-10 uL sample volume was used as template for the PCR.

4.3.4. Polymerase Chain Reaction

Primers targeting a 166 base pair (bp) segment of the

FeLV U3 LTR region (Fulton *et al.*, 1990; Matsumoto *et al.*, 1992) were synthesized (University of British Columbia, Oligonucleotide Synthesis Laboratory, Department of Biochemistry, Vancouver, British Columbia). The primer sequences were as follows:
5'-TTACTCAAGTATGTTCCCATG-3' (sense) and
5'-CTGGGGAGCCTGGAGACTGCT-3' (anti-sense). A computation was performed at the National Center for Biotechnology Information (NCBI) using the experimental GENINFO BLAST Network Service to assess degree of homology between these primers and other reported sequences. The BLAST service accesses GenBank, GenBank Update, EMBL Data Library, EMBL Update, Vector subset of GenBank, Kabat Sequences of Nucleic Acid of Immunological Interest Release, Eukaryotic Promoter Database Release 34, and Database of Expressed Sequence Tags Release 1.1. Reaction mixtures for the PCR consisted of 1X PCR Buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5 mM of $MgCl_2$, 200 uM of each deoxynucleoside triphosphate (dNTP), 1.25 units of Taq DNA polymerase (GeneAmp PCR Core Reagents, Perkin Elmer Cetus, Norwalk, Connecticut), 50 pmol of each primer, 1 to 10 uL of DNA template, and HPLC grade water (BDH Inc., Toronto, Ontario) to a volume of 50 uL. The reaction mixture was overlaid with 50 uL of liquid paraffin. The PCR was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, Connecticut) as follows: initial denaturation of 3 min at 94 C followed by 35 cycles of: denaturation - 1 min at 94

C, primer annealing - 1 min at 52 C, and primer extension - 2 min at 72 C. Samples were cooled to 4 C or frozen at -20 C until further analysis.

A 10 uL aliquot of the aqueous PCR product was mixed with 3 uL of stop buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and electrophoresed through a 2% agarose minigel (Ultra Pure Agarose Electrophoresis Grade, GIBCO BRL, Life Technologies, Inc. Gaithersburg, Maryland) in 0.5X TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) at 80-100 volts for 30-60 min. Either a 100 bp or 123 bp DNA size marker (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Maryland) was included in each gel. Gels were stained with ethidium bromide (Sigma Chemical Company, St. Louis, Missouri) for 15 min, and photographed (Polaroid film 667, Polaroid Corporation, Cambridge, Massachusetts) under UV transillumination (Foto UV 300 DNA Transilluminator, Fotodyne, Inc. New Berlin, Wisconsin). Sensitivity of the PCR had been determined using serially diluted cloned FeLV [FeLV clone 61E (Donahue *et al.*, 1988; Overbaugh *et al.*, 1988a), a gift from Dr. J. Overbaugh, School of Medicine, Department of Microbiology, University of Washington, Seattle, Washington]. When evaluated by visualization of the ethidium bromide stained agarose gel, the lower limit of detection for cloned virus in a background of both fresh feline genomic DNA and DNA derived from paraffin-embedded, formalin-fixed feline tissue was

between 3.2 fg and 160 fg. Generally, Southern hybridization enabled detection of PCR products to one dilution beyond that which could be visualized on agarose gels.

4.3.5. Southern Transfer and Hybridization

Specificity of the PCR for the FeLV U3 LTR was confirmed by Southern transfer and hybridization using a PCR synthesized digoxigenin-labeled probe directed to the 166 bp target. The template for the labeling reaction was a 1.1 kilobase (kb) *Eco*RI-*Hin*dIII fragment of pexU3, a plasmid containing two direct repeats of the 5'-end 249 bp of the FeLV-B-GA LTR (a gift from Dr. James I. Mullins, Stanford University School of Medicine, Stanford, California). This fragment was gel purified and then used as the source of FeLV DNA in the PCR labeling reaction. The PCR to generate labeled probe was modified as follows (Misra *et al.*, 1992; Lanzillo, 1990; Lion and Haas, 1990): the final reaction volume was doubled to 100 uL, the final concentration of the dTTP was reduced to 150 uM, and 50 uM of 1mM digoxigenin-11-2'-deoxyuridine-5'-triphosphate (Dig-11-dUTP) (Boehringer Mannheim Canada Ltd, Laval, Quebec) was added. Therefore, the labeled probe was 166 bp the same as the other FeLV PCR products except about every fourth dTTP was substituted with digoxigenin-labeled dUTP. A 10 uL aliquot of digoxigenin-labeled PCR product was used to probe one 100 cm^2 filter.

For hybridization, PCR products were transferred from

agarose gels to nylon membranes (Gene Screen Plus Hybridization Transfer Membrane, Biotechnology Systems NEN Research Products, Dupont, Boston, Massachusetts) using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Richmond, California) according to the manufacturer's instructions (Trans-Blot SD DNA Blotting Kit Instruction Manual, BioRad, Richmond, California). At the end of the procedure, membranes were cross-linked in a UV linker (UV Stratalinker 1800, Stratagene, La Jolla, California) at 1200 microjoules for 30 sec (autocrosslink mode). Hybridization and immunological detection of digoxigenin were done according to the manufacturer's instructions (DNA Labeling and Detection Kit Nonradioactive, Boehringer Mannheim Canada Ltd, Laval, Quebec).

4.3.6. Controls for Polymerase Chain Reaction

Template DNA for positive controls was from cloned FeLV 61E initially, and later from cloned virus and/or paraffin-embedded, formalin-fixed tissues from clinical cases that consistently yielded intensely staining appropriate sized bands on agarose gel electrophoresis (AGE). Template DNA for negative controls was derived from uninfected cultured feline T cells (3201 cells, a gift from Dr. J. Overbaugh, School of Medicine, Department of Microbiology, University of Washington, Seattle, Washington) and/or from blood, fresh or formalin-fixed bone marrow, or fresh or formalin-fixed tissue samples from cats

with no hematologic, clinical, or pathologic evidence of FeLV-related disease. Reagent controls containing no DNA were also included in each PCR.

4.3.7. Sequencing of Amplified Products

Representative PCR products were sequenced using the AmpliTaq Cycle Sequencing Kit (Perkin Elmer Cetus, Norwalk, Connecticut) according to the manufacturer's instructions. The sequence of PCR products not hybridizing to the FeLV probe was analyzed for homology to reported sequences; this computation was performed at the NCBI using the experimental GENINFO BLAST Network Service.

4.3.8. Interpretation of Polymerase Chain Reaction Results

Results from clinical cases were accepted and interpreted only when positive and negative controls yielded the expected results on AGE. An intensely staining 166 bp product on AGE was recorded as a positive result for PCR detection of FeLV provirus. Southern hybridizations were done randomly on these cases. The PCR was repeated at least twice on cases yielding equivocal or negative results on AGE, using the original DNA extract as template. Southern hybridizations were done on PCR products from the second and third amplifications of these samples. Results were recorded as positive, negative, or indeterminate. A positive result from those cases that had repeated PCR and Southern hybridizations, was defined as obtaining positive, negative, or equivocal 166 bp bands on AGE of 1 or both repeat PCRs, plus positive signals on both Southern

hybridizations. An indeterminate result represented samples with equivocal or no evidence of the 166 bp FeLV product on AGE, and discordant results on the two hybridizations (one positive and one negative). A negative result was assigned to those cases with negative or equivocal bands on the two PCRs plus negative or equivocal bands on both Southern hybridizations.

4.3.9. Statistical Analysis

To assess the level of agreement between tests (PCR and IHC, PCR and ELISA, and IHC and ELISA) a quotient, called kappa, was calculated. Kappa is a measure of agreement beyond that which might be expected due to chance. No agreement beyond chance yields a kappa of 0, and a kappa of 1 indicates perfect agreement. Moderate agreement is indicated by a kappa of at least 0.4-0.5 (Martin *et al.*, 1987). Indeterminate results were not included in the calculation.

To assess the relationship between age group and PCR result, the Fisher exact test (Daniel, 1990) was used. This test can be applied when cell frequencies in a 2x2 contingency table are too low to use the Chi-square test. The calculation was made by computer program (Epi Info USD Incorporated, Stone Mountain, Georgia), and indeterminate PCR results were not included. The relationship between age group and IHC result was determined using the Chi-square test (Daniel, 1990; Martin *et al.*, 1987). The critical Chi-square value for significance at the 5% level

is 3.84 (Martin *et al.*, 1987). When a significant relationship was found between test result and age group, the strength of the association was measured by calculating the odds ratio (Martin *et al.*, 1987) from the 2x2 contingency table. Exact confidence limits for the odds ratio were calculated as previously described (Mehta *et al.*, 1985).

4.4. Results

Immunohistochemical staining of tissue sections from 70 cases of feline LSA revealed FeLV gp70 antigen in 40 cases. Viral antigen was usually most apparent in neoplastic lymphoid cells, often within foci of cells infiltrating multiple organs and tissues, as shown in Figure 4.1. There was no specific staining when sections were tested substituting an irrelevant serum for the FeLV antisera.

Although the search of the various nucleotide databases using the BLAST Network Service detected homology between the primers and FeLV LTRs, the program identified no matches between the primers and other reported sequences.

In PCR testing, in addition to the expected 166 bp FeLV genome product, a band of lesser intensity representing DNA of about 210 bp was commonly seen (Figure 4.2). Sometimes there was also a band of about 100 bp, particularly in those cases where no FeLV product band was present or where the FeLV band was faint (Figure 4.2). The 166 and 210 bp bands hybridized to the FeLV U3 LTR probe in Southern hybridizations; the 100 bp band did not hybridize to the probe (Figure 4.2). Occasionally samples yielded multiple larger sized bands in addition to the 166 and 210 bp products; these also hybridized to the FeLV U3 LTR. Positive and negative controls yielded expected results in all cases for which PCR and Southern hybridization results

Figure 4.1. Formalin-fixed, paraffin-embedded liver section from a cat with lymphosarcoma. The tissue has been stained for FeLV gp70 antigen. Dark brown-staining foci of infected neoplastic lymphocytes are present. Avidin-biotin complex immunoperoxidase with hematoxylin counterstain. Bar = 100 um.

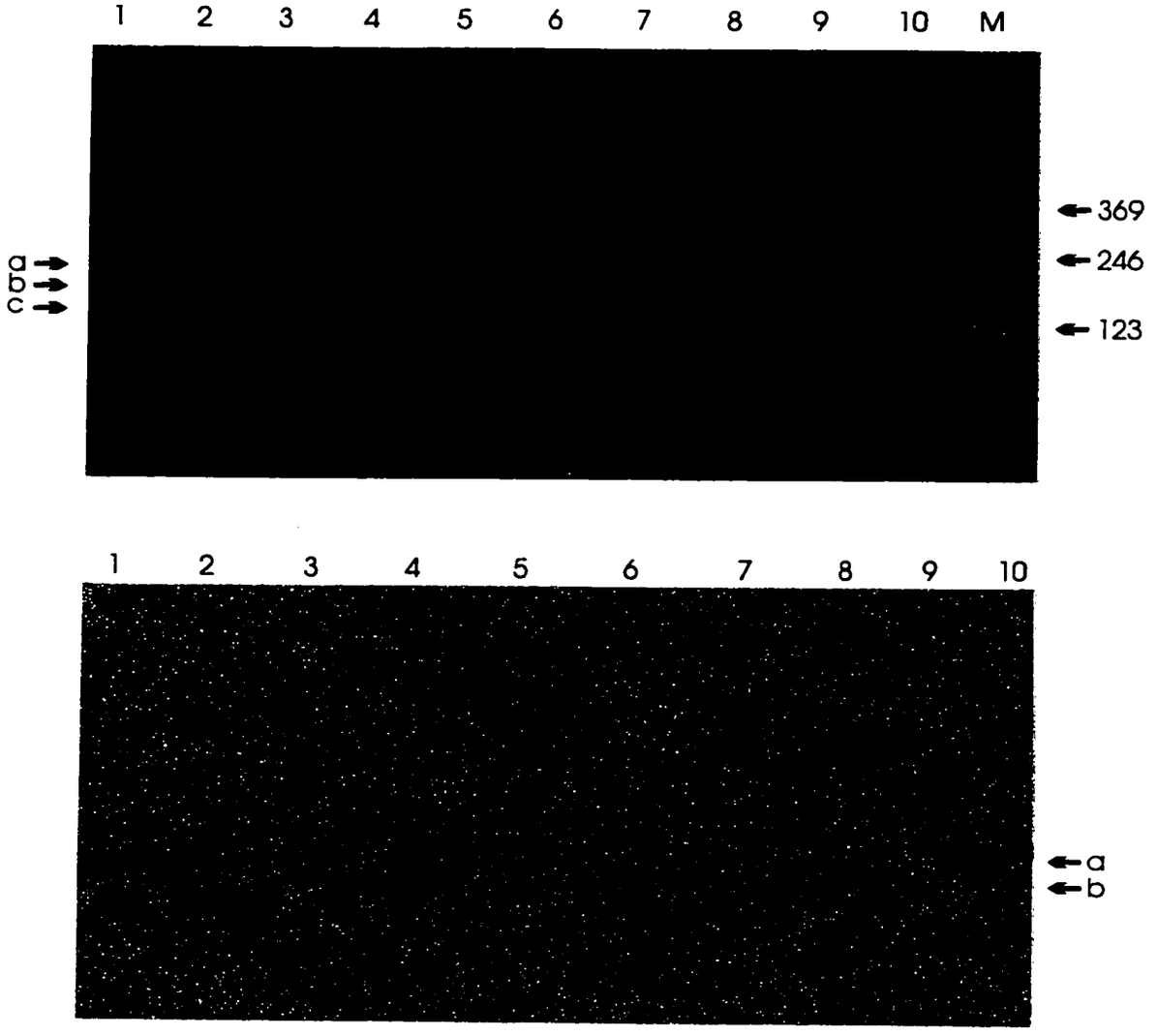

Figure 4.2. Ethidium bromide-stained agarose gel (top) and Southern hybridization (bottom) of typical PCR products amplified from feline LSA-derived DNA. In the top figure, lane M is a 123 bp DNA ladder. The a, b, and c arrows represent 210 bp, the expected 166 bp, and 100 bp products, respectively. Lane 1 was considered equivocal for FeLV. Lanes 2,4,7 and 8 appeared positive for FeLV, and lanes 3,5,6,9 and 10 appeared negative for FeLV. The bottom figure demonstrates hybridization of the PCR products shown above, using a digoxigenin-labeled FeLV probe. The a and b arrows indicate hybridization to the 210 and 166 bp products, respectively. There was no hybridization to the 100 bp product. Lanes 2,3,4,7 and 8 were positive for FeLV, whereas lanes 5,6,9 and 10 were negative. Lane 1 was equivocal and accepted as negative for exogenous FeLV.

were accepted and reported.

The nucleotide sequences of the 166 bp and apparent 210 bp bands were one and the same, and showed near homology to the sequence for FeLV 61E within this region (data not shown). The sequence of the 100 bp band showed complementarity to the FeLV target only for 13 bases within the sense primer, which was not chosen as the sequencing primer.

In total, 56 of 70 cases (80%) were positive for FeLV by PCR compared to 40 of 70 (57%) by IHC (Table 4.1). Seventeen of the 56 cases that were positive by PCR, were negative by IHC; 1 of the 7 cases that was negative by PCR, was positive by IHC. The 7 cases that were indeterminate by PCR were negative by IHC. The kappa quotient to compare PCR and IHC test results, was 0.29 indicating a low level of agreement between the two tests.

In 30 of the 70 cases antemortem FeLV ELISAs had been performed. The 19 cases positive by ELISA were also positive by PCR and IHC (Table 4.2). The 11 ELISA-negative cases were also negative by IHC, however, 7 ELISA-negative cases were positive by PCR. The kappa quotient to compare PCR and ELISA results, was 0.29 indicating a low level of agreement between the two tests; kappa to compare IHC and ELISA results was 1 which indicates perfect agreement between these two tests.

Thirty-seven of the 42 cats (88%) <7 yr were positive by PCR, and 34 of these cats (81%) were positive by IHC

Table 4.1. FeLV detection by PCR and IHC in tumor tissues from 70 cats with lymphosarcoma

	IHC +	IHC-	Totals
PCR +	39	17	56/70 (80%)
PCR -	1	6	7/70 (10%)
PCR I[a]	-	7	7/70 (10%)
Totals	40/70 (57%)	30/70 (43%)	

[a] I = indeterminate PCR results
Kappa to assess agreement between PCR and IHC results = 0.29

Table 4.2. FeLV detection by PCR, IHC, and ELISA in tumor tissues (PCR and IHC) and serum (ELISA) from 70 cats with lymphosarcoma

		ELISA +	ELISA -	ELISA nd[a]
PCR + (n = 56)	IHC +	19	0	20
	IHC -	0	7	10
PCR - (n = 7)	IHC +	0	0	1
	IHC -	0	2	4
PCR I[b] (n = 7)	IHC +	0	0	0
	IHC -	0	2	5

[a] nd = not done
[b] I = indeterminate PCR results
Kappa to assess agreement between PCR and ELISA results = 0.29
Kappa to assess agreement between IHC and ELISA results = 1

(Table 4.3). Eighteen of the 26 cats (69%) ≥7 yr were positive by PCR, whereas 5 of the 26 (19%) were positive by IHC. The Fisher exact calculation (P value = 0.23) indicated that there was no significant difference in PCR results between young and old groups of cats; LSA tissues from older cats (≥7 yr) were equally likely to be positive for FeLV DNA as were those from younger cats (<7 yr). However, IHC results were significantly different between young and old cats (Chi-square = 25.01, P<.05; odds ratio = 17.85, 95% confidence interval = 4.51,76.37); LSA tissues from young cats (<7 yr) were more likely to be positive for FeLV antigen than were those from older cats (≥7 yr).

The distribution of PCR and IHC positive results according to anatomic site of LSA is shown (Table 4.4). The multicentric form was most common followed by the mediastinal and intestinal forms. Proportionately fewer intestinal LSAs were positive for FeLV by PCR and IHC than were the multicentric and mediastinal LSAs.

The anatomic locations of LSA in the 26 cats ≥7 yr were: multicentric - 17, mediastinal - 1, intestinal - 5, miscellaneous - 3. Three of the 5 cats with intestinal tumors were positive by PCR while 1 of the 5 was positive by IHC and this one was not positive by PCR. For the 3 cats <7 yr with intestinal LSA, 2 were positive for FeLV by PCR and IHC, and ELISAs were not done; the third was indeterminate on PCR and negative on IHC and ELISA.

Table 4.3. FeLV detection by PCR and IHC according to age group in 70 cats with lymphosarcoma

Age group (yr)	PCR +	IHC +
< 7 (n = 42)	37 (88%)	34 (81%)
≥ 7 (n = 26)	18 (69%)	5 (19%)
unknown (n = 2)	1 (50%)	1 (50%)

Relationship of PCR result to age group, Fisher exact test, $p = 0.23$ (not significant)
Relationship of IHC result to age group, Chi-square = 25.01, $p<0.05$; odds ratio = 17.85, 95% confidence interval = 4.51 - 76.37

Table 4.4. FeLV detection by PCR and IHC according to anatomic site of lymphosarcoma in 70 cats

Anatomic site	PCR +	IHC +
Multicentric (n = 48)	39 (81%)	31 (65%)
Mediastinal (n = 11)	9 (82%)	6 (55%)
Intestinal (n = 8)	5 (63%)	3 (38%)
Miscellaneous (n = 3)	3 (100%)	0

4.5. Discussion

About 30% of cats with LSA are negative for FeLV by antigen detection and/or virus isolation; however, epidemiologic studies suggest that exposure to FeLV is associated with the development of LSA in these cats (Mullins *et al.*, 1984; Hardy *et al.*, 1980; Hardy, 1987; Koshy *et al.*, 1979; Rojko *et al.*, 1989; Francis *et al.*, 1981; Hardy and MacEwen, 1989). Some studies have attempted to further define this association. In one hybridization experiment however, no exogenous FeLV U3 sequences could be found in LSAs from 6 FeLV antigen-negative cats (Casey *et al.*, 1981). Other research investigating the role of latency showed that FeLV could be reactivated from cultured bone marrow cells but not from blood or tumor cells in 2 cats with FeLV antigen-negative LSA (Rojko *et al.*, 1982). Although studies of FeLV infection suggest that latency may be a phase of recovery that is generally short-lived, the latent period may persist for months or years in some cats (Pedersen *et al.*, 1984; Pacitti and Jarrett, 1985). These cats may be at increased risk for the development of virus-negative LSA as defined by absence of viral antigen and infectious virus. Explanations for the reported 30% of feline LSAs that are negative for infectious FeLV or viral antigen therefore include: the presence of a latent FeLV infection, as well as, the presence of a recombinant replication-defective virus, the presence of only a portion of the FeLV genome

which also renders it replication defective, or, a "hit and run" phenomenon such that FeLV induces LSA but does not itself become integrated into the host cell genome (Hardy *et al.*, 1980).

In this study, 56 of the 70 cases (80%) were positive for FeLV using the PCR method. These included 17 of the 30 cases which were negative for FeLV by IHC. Seven of the 17 PCR-positive, IHC-negative cases had been tested by ELISA and were negative. These findings support the theory that some FeLV antigen-negative LSAs involve latent or replication-defective FeLV. Alternatively, the inherent amplification of target DNA in the PCR could facilitate detection of replication-competent FeLV present in low levels or intermittently producing viral protein, and therefore not identified by traditional methods.

Previous studies suggest that latent infections involve bone marrow cells and a minor subset of nodal lymphocytes but not necessarily tumor cells themselves (Rojko *et al.*, 1982). In this study, DNA from tumor tissue was tested for FeLV by PCR. Bone marrow contained tumor and was available for PCR in only a few cases. Therefore, if latent FeLV infection was being detected by PCR, tumor tissue was a source of the virus in this study.

Although PCR detected FeLV in cases that were antigen-negative by IHC and/or ELISA, there remained 20% of cases that were negative or indeterminate for FeLV by PCR. Possible explanations for this include: not all LSAs in

cats are FeLV-related, the "hit and run" phenomenon may be occurring in a portion of LSAs, the FeLV infection was present/latent in tissues other than those tested (such as bone marrow), or FeLV DNA may have been undetectable by PCR due to interference, poor quality or otherwise inadequate template DNA.

The possible role of the feline immunodeficiency virus (FIV) in the LSA cases examined here, was not investigated. The relative risks of developing LSA are reported to be 5.6, 62.1, and 77.3 times greater in cats infected with FIV, FeLV, and FeLV with FIV, respectively, than in uninfected cats (Shelton *et al.*, 1990). Recently, a cat experimentally infected with FIV but FeLV antigen-negative developed LSA (Callanan *et al.*, 1992). The PCR for FeLV proviral detection was not applied in either of these two studies so that infection with replication-defective FeLV cannot be ruled out. To our knowledge, there is no conclusive evidence that FIV alone causes lymphosarcoma in cats. Nevertheless, since the presence of FIV antibody and/or DNA was not determined in this study, the possible role of FIV in the FeLV-negative LSAs is not known.

The distribution of LSAs according to anatomic site was similar to previous reports when lymphocytic leukemia had been included with the multicentric form (Hardy and MacEwen, 1989; Hardy *et al.*, 1980; Shelton *et al.*, 1990). In a study involving LSAs in 507 cats, 80.3% of the multicentric tumors, 77.0 % of the thymic (mediastinal)

tumors, and 23.2% of the alimentary (intestinal) tumors were positive for FeLV antigens (Hardy *et al.*, 1980). The PCR results of the present study were similar for the multicentric tumors (81% PCR positive) and the mediastinal tumors (82% PCR positive), however, 5 of the 8 intestinal tumors (63%) were FeLV-positive by PCR. The number of intestinal tumors available in the current study was small, however, the results suggest that previous findings that alimentary LSAs are usually FeLV negative (Hardy and MacEwen, 1989; Hardy *et al.*, 1977; Hardy, 1981; Hardy *et al.*, 1980), may relate more to the method of testing rather than the true presence or absence of the viral genome.

Lymphosarcoma in older cats is not usually associated with the presence of FeLV antigen or infectious virus (Hardy *et al.*, 1980; Niman *et al.*, 1977; Hardy and MacEwen, 1989). An interesting finding in the current study was that 18 of 26 (69%) cats $\geq$7 yr were FeLV-positive by PCR compared to 5 of 26 (19%) that were FeLV-positive by IHC. While there was no significant difference in PCR results according to age group, there was a significant difference in IHC results according to age group. Lymphosarcoma tissues from young cats (<7 yr) were several fold more likely to be FeLV-positive by IHC than were tumors from older cats ($\geq$7 yr). In general, older cats have probably been infected with FeLV for a longer period and there may be more opportunity for the development of recombinant forms of FeLV. Immune responses may effectively eliminate

replication-competent forms and inadvertently select for replication-defective strains of FeLV which are still capable of inducing transformation by insertional mutagenesis, transduction of cellular oncogenes, or some other unknown mechanism.

There was one PCR-negative, IHC-positive result in an older cat with an intestinal tumor. Since viral antigen was demonstrated within tumor cells, viral DNA would also be expected to be present locally within the tumor. Perhaps the DNA template was degraded or inadequate, or there was some chemical or physical interference prohibiting amplification. The simultaneous inclusion of a feline genomic positive control may have been of value to examine this discrepancy; however, the nonspecific 100 bp product was seen on AGE, indicating that amplifiable DNA was present.

The frequent finding of both 166 and approximate 210 bp PCR products that hybridized to the FeLV probe, is interesting. The region of the FeLV LTR being amplified in the PCR contains several enhancer binding consensus sequences, and it has been reported that duplications of a 50-90 bp segment of the enhancer relates to leukemogenic potential in FeLV-related neoplastic disease (Fulton *et al.*, 1990; Matsumoto *et al.*, 1992; Miura *et al.*, 1989). In this study, enhancer duplication was considered to explain the apparent 210 bp band, however, the sequence determination showed that the 166 and 210 bp bands

represented the same DNA with no repeats. The difference in migration on AGE might relate to secondary structure formation in some of the product retarding movement through the gel. Heating the product after adding the stop solution and before loading the gel may remove any alterations in configuration that have occurred during cooling of the amplified product.

The 100 bp product that was commonly seen showed no homology to known sequences. This likely represents a feline genomic sequence present in some but not necessarily all cats, and with some homology to the primers. When ample target FeLV was present, the primers appeared to preferentially bind to the exogenous virus. The 100 bp product was most evident when little or no target virus was amplified.

Contamination, particularly from amplified PCR product, is a major problem that commonly occurs in laboratories performing PCR regularly (Arnheim and Erlich, 1992; Marx, 1988; Persing, 1991; Lo *et al.*, 1988; Sarkar and Sommer, 1991; Ou *et al.*, 1991; Wright and Wynford-Thomas, 1990; Kumar, 1989; Defer *et al.*, 1992; Kwok and Higuchi, 1989). Occasionally in this study, even though there were no visible bands on stained agarose gels, signals were present on the Southern hybridizations of PCR products. For this reason, some samples were tested repeatedly by PCR and hybridization, and a system of interpreting results was devised (Materials and Methods).

The weak, equivocal, and discordant results sometimes obtained on the repeated Southern hybridizations, may have been due to a low level of contamination which some report to be unavoidable during the various steps involved in the PCR (The Scandinavian Multicenter Study Group, 1992).

The IHC and ELISA results agreed in those 30 cases for which FeLV ELISAs had been done, supporting the application of enzyme-based IHC for FeLV studies using paraffin-embedded, formalin-fixed specimens. The advantages of enzyme-based IHC over most antigen-detection methods include the ability to localize the FeLV antigen to specific tissues and to maintain a permanent record of the results simply by storing the stained slides.

Feline leukemia virus DNA was frequently amplified from tumor tissues of older cats that were IHC-negative, thus supporting the hypothesis that latent or replication-defective FeLV may be responsible for some cases of "virus-negative" LSA. Increased sensitivity of the PCR compared to antigen detection would not likely explain the higher level of detection by PCR in the select group of tumors - those from older cats. More study is warranted in applying the PCR technology to help elucidate the pathogenesis of FeLV antigen-negative LSA in cats.

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5. IMMUNOHISTOCHEMICAL IDENTIFICATION OF B AND T LYMPHOCYTES IN FORMALIN-FIXED, PARAFFIN-EMBEDDED FELINE LYMPHOSARCOMAS: RELATIONSHIP TO FELINE LEUKEMIA VIRUS STATUS, TUMOR SITE, AND PATIENT AGE

5.1. Abstract

The lymphocyte phenotype of 70 formalin-fixed, paraffin-embedded feline lymphosarcomas (LSAs) was determined immunohistochemically using a T cell polyclonal antibody, and a B cell monoclonal antibody. Forty-seven of 70 (67%) tumors were T cell, 19/70 (27%) were B cell, and 4/70 (6%) did not stain with either marker. Thirty-eight of 70 (54%) tumors were positive for feline leukemia virus (FeLV) antigen by immunohistochemistry (IHC), and 52/70 (74%) tumors were positive for FeLV DNA using the polymerase chain reaction (PCR). B cell tumors were as frequently FeLV-positive as T cell tumors using either IHC or PCR. Intestinal tumors were more likely to be B cell than T. The incidence of B and T cell tumors was not different among young (≤3yr), middle-aged (>3yr to ≤8yr), and old (>8yr) cats. Both B and T cell tumors from old cats were FeLV-positive more often by PCR than by IHC. Feline leukemia virus DNA but not antigen, was detected in B cell tumors and intestinal tumors from cats >8yr as often as it was detected in B cell tumors and intestinal tumors

from cats ≤8yr. Previously, most B cell and intestinal tumors from old cats were considered to be negative for FeLV. Here, the results suggest involvement of latent or replication-defective forms of the virus in such tumors from old cats. This study supports a role for FeLV in feline B cell as well as T cell tumorigenesis.

5.2. Introduction

Lymphosarcoma accounts for about 30% of all feline tumors, which is the highest rate of LSA in any outbred animal species (Hardy *et al.*, 1980a; Hardy, 1981b; Crighton, 1969; Dorn *et al.*, 1967; Dorn *et al.*, 1968; Hardy, 1978). About 70% of feline LSAs are FeLV-related using antigen detection or virus isolation (Hardy *et al.*, 1980b; Francis *et al.*, 1979; Hardy and MacEwen, 1989). However, we have reported (Jackson *et al.*, 1993 [Section 4]) that FeLV DNA may be detected using PCR in additional LSAs particularly from older cats, suggesting that the virus may be associated with a larger proportion of LSAs than previously determined.

There are several reports characterizing the phenotype of feline LSAs in relation to anatomic distribution of the tumor, patient age, and/or FeLV status (Holmberg *et al.*, 1976; Rojko *et al.*, 1989; Cockerell *et al.*, 1976; Hardy *et al.*, 1977; Mackey *et al.*, 1975; Hardy, 1978). Early studies related the anatomic distribution of neoplastic cells to specific compartments in lymphoid tissues, so that in alimentary LSA, the main tumor was described in Peyer's patches and germinal centers of mesenteric lymph nodes, suggesting B cell transformation. Whereas in thymic and multicentric LSA, the neoplastic cells occupied the paracortical or thymus-dependent areas, suggesting T cell transformation (Mackey and Jarrett, 1972). Later studies involved T cell identification by non-immune rosette

formation of guinea pig erythrocytes (GPE), and B cell identification by demonstration of surface immunoglobulin (Ig) and/or demonstration of complement receptors by erythrocyte, antibody, complement (EAC) rosettes (Holmberg *et al.*, 1976; Cockerell *et al.*, 1976; Hardy *et al.*, 1977; Mackey *et al.*, 1975; Mackey *et al.*, 1975). In one report of 14 LSAs, thymic tumors were T cell, alimentary tumors were B cell, and multicentric tumors lacked B or T cell surface markers (Holmberg *et al.*, 1976). In another study of 36 LSAs, all the thymic and just over half of the multicentric tumors were T cell, and most of the alimentary tumors were B cell (Hardy *et al.*, 1977). More recently, lymphocyte subtypes in 5 primary feline LSAs and 6 feline LSA cell lines were characterized using monoclonal and polyclonal antibodies, immunofluorescence, rosette-forming assays, and cytochemistry (Rojko *et al.*, 1989). Conclusions were that FeLV does not transform only mature T cells but also immature or prothymocytes, null cells, and possibly helper T cells or monocytes. Transformation of mature B cells was excluded by lack of surface Ig expression in the feline LSA cell lines and primary tumors that were examined (Rojko *et al.*, 1989). Most reports indicate that multicentric and mediastinal LSAs are T cell and FeLV-positive, while alimentary LSAs are B cell and mostly FeLV-negative (Neil *et al.*, 1984; Francis *et al.*, 1979; Hardy *et al.*, 1977; Hardy, 1981a). Non-producer LSA (FeLV antigen-negative) occurs most often in older cats

and/or in cats with alimentary LSA (Hardy *et al.*, 1980b; Hardy and MacEwen, 1989), however, there is evidence to suggest FeLV involvement in at least a portion of non-producer LSA (Hardy *et al.*, 1980b; Francis *et al.*, 1981; Jackson *et al.*, 1993 [Section 4]).

Much of the earlier work has been hindered by the fact that GPE rosette formation is not specific for T cells in the cat (Wellman *et al.*, 1986), limited numbers of spontaneous tumors were evaluated, and immunological markers recognizing T and B cells over a broad range of developmental stages and applicable to archival material, were not available. The development of the polymerase chain reaction (PCR) and immunohistochemistry (IHC) for detection of FeLV DNA (Fulton *et al.*, 1990; Matsumoto *et al.*, 1992; Jackson *et al.*, 1993 [Section 4]) and antigen (Jackson *et al.*, 1993 [Section 4]; Reinacher, 1989; Reinacher and Theilen, 1987) respectively, in paraffin-embedded, formalin-fixed tissue, has facilitated retrospective study of FeLV-negative versus FeLV-positive but non-producer LSAs. Recently, a polyclonal rabbit anti-human CD3 antibody (Dako Corp., Carpinteria, California) has been shown to detect T cells in formalin-fixed, paraffin-embedded feline lymphoid tissues (Beebe *et al.*, 1994). Also, a monoclonal antibody, RA3-6B2 (Cedarlane Laboratories Ltd., Hornby, Ontario) which recognizes only B lymphocytes, has been applied to formalin-fixed, paraffin-embedded feline lymphoid tissues and appears to stain B

cells over a broad range of differentiation (Newlands *et al.*,accepted). The purpose of this study was to determine the phenotype of 70 formalin-fixed, paraffin-embedded feline LSAs using T and B cell-specific antibodies, and to relate lymphocyte type to FeLV status by IHC for antigen and PCR for viral DNA, anatomic form of the tumor, and patient age.

5.3. Materials and Methods

5.3.1. Case Selection and Grouping

Seventy LSA cases from the archives of the Pathology Department at the Western College of Veterinary Medicine, were investigated. Cats were grouped by age as follows, ≤3yr, >3yr to ≤8yr, and >8yr to represent young, middle-aged, and old cats. Lymphosarcomas were classified according to anatomic distribution of the major tumor lesions on gross necropsy (Crighton, 1969; Hardy, 1978). Multicentric LSA involved tumor in lymph nodes, spleen, liver, and kidneys, alone or in any combination; lymphocytic leukemia was considered a manifestation of multicentric LSA (Hardy, 1981b; Shelton *et al.*, 1990). Mediastinal LSA included tumors of the thymus and/or mediastinal lymph nodes. Alimentary LSA involved tumors at any site in the gastrointestinal tract and/or mesenteric lymph nodes (Hardy, 1981b).

5.3.2. Polymerase Chain Reaction

The PCR targeted a 166 base pair segment of the FeLV U3 LTR (Fulton *et al.*, 1990; Matsumoto *et al.*, 1992), and was carried out as previously described (Jackson *et al.*, 1993 [Section 4.3.4]).

5.3.3. Immunohistochemistry

The ABC method for automated IHC staining of formalin-fixed, paraffin-embedded tissues (Haines and Chelack, 1991), was used for detection of FeLV, B cell, and T cell antigens. For immunohistochemical evaluation, duplicate

sections of each block were tested for staining with each antigen. The FeLV envelope glycoprotein, gp70, was detected using 1:400 and 1:800 dilutions of a goat anti-FeLV gp70 primary antiserum (National Cancer Institute, Bethesda, Maryland) with modifications as previously described (Jackson *et al.*, 1993 [Section 4.3.2]). For B cell identification, RA3-6B2, a rat monoclonal antibody to mouse B cells (B220) (Cedarlane Laboratories Ltd, Hornby, Ontario), was used as described for application to formalin-fixed, paraffin-embedded feline tissues (Newlands *et al.*, accepted), at dilutions of 1:50 and 1:100. Antigen retrieval was enhanced using Target Unmasking Fluid (TUF) (Monosan, Uden, The Netherlands) and incubation in a microwave oven according to the manufacturer's instructions. T cells were identified using 1:400 and 1:800 dilutions of a polyclonal rabbit anti-human CD3 antibody (Dako corp., Carpinteria, California). Negative controls were sections from each case in which the primary monoclonal antibody or polyclonal antiserum was sustituted with a similarly diluted species and isotype-matched irrelevant antibody or antiserum.

5.3.4. Statistical Analysis

Tumor phenotype was compared among cats using the following characteristics: FeLV status, anatomic site, and patient age group. Also, FeLV infection rates, assessed using IHC and PCR, were compared using the following characteristics: location of B cell tumors (intestinal v.

non-intestinal), age among cats with B cell LSAs, and age among cats with intestinal LSAs. These relationships were assessed using Pearson's chi-square test, or Fisher's exact test when appropriate (Daniel, 1990). The odds ratios and 95% confidence intervals were assessed when the relationships were statistically significant ($p \leq 0.05$) (Martin *et al.*, 1987). Within tumor phenotype groups in cats ≥8 yr, differences in FeLV detection rates using IHC and PCR were assessed using McNemar's chi-square test for two related samples (Martin *et al.*, 1987). Tumors that were indeterminate for PCR results as previously defined (Jackson *et al.*, 1993 [Section 4.3.8]), were included as PCR negative for the statistical calculations.

5.4. Results

Figure 5.1 demonstrates positive staining of LSA tissues using the T cell, B cell, and FeLV antibodies. Of 70 LSAs, 47 (67%) were T cell tumors, 19 (27%) were B cell tumors, and 4 (6%) did not stain with either T or B cell marker (Table 5.1). Thirty-eight (54%) of 70 tumors were positive for FeLV antigen by IHC, and 52 (74%) were positive for FeLV DNA by PCR. The distribution of IHC-positive and PCR-positive tumors according to lymphocyte type is given in Table 5.1. There was no difference in the ratio of T cell LSAs that were FeLV-positive compared to the ratio of B cell LSAs that were FeLV-positive using either IHC or PCR. Table 5.2 shows the distribution of lymphocyte type and FeLV status according to anatomic site of the LSA. No mediastinal or cutaneous tumors were stained by the B cell antibody. Intestinal tumors were almost 4 times more likely to be B cell than T cell. Using either IHC or PCR, the ratio of FeLV-positive intestinal B cell tumors was not different from the ratio of FeLV-positive non-intestinal B cell tumors. Table 5.3 shows the distribution of lymphocyte type and FeLV status of the LSAs according to patient age group. The proportion of T to B cell tumors did not differ according to age group. The PCR detected FeLV DNA more often than IHC detected FeLV antigen in both B and T cell tumors from cats >8yr. The distribution of lymphocyte type and FeLV status according to anatomic site of the LSA and patient age group is shown

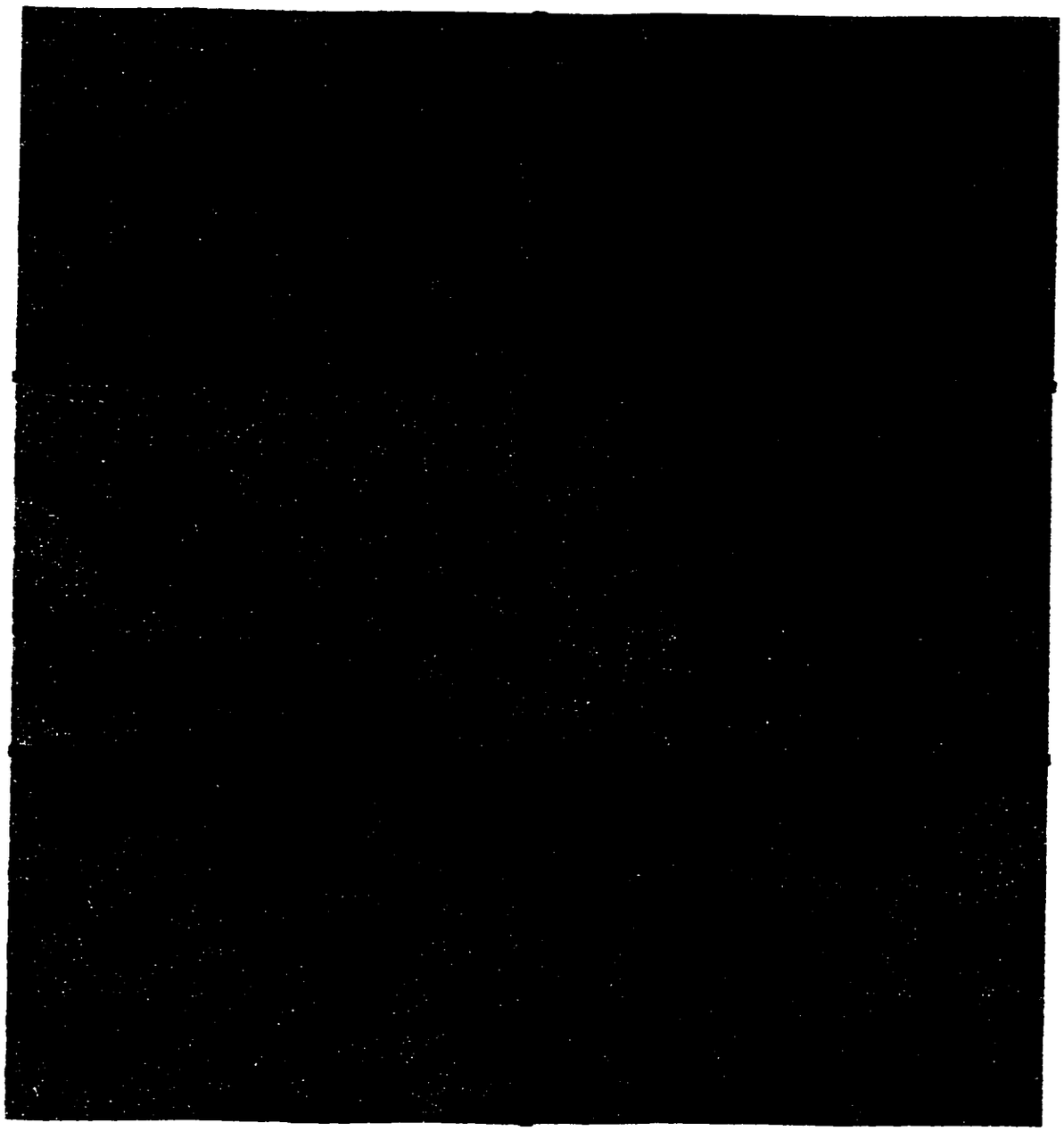

Figure 5.1. Formalin-fixed, paraffin-embedded kidney sections from 2 cats with lymphosarcoma. A, B, and C are from one cat, and D, E, and F from the other. Tissues have been stained with the T cell antibody in A and D, the B cell antibody in B and E, and the FeLV antibody in C and F. Dark brown staining indicates positivity for the T cell phenotype in A, the B cell phenotype in E, and FeLV antigen in C and F. Avidin-biotin complex immunoperoxidase with hematoxylin counterstain. Bar = 100 um.

Table 5.1. Lymphocyte type and FeLV status of 70 feline lymphosarcomas

FeLV status	T cell (47)	B cell (19)	Not T or B (4)
IHC+ (38)	24	10	4
PCR+ (52)	34	14	4

No significant difference in the ratio of T cell tumors that were FeLV positive compared to the ratio of B cell tumors that were FeLV positive by either IHC ($p = 0.91$) or PCR ($p = 0.91$).

Table 5.2. Lymphocyte type and FeLV status of 70 feline lymphosarcomas according to anatomic site

Site	T cell (47)		B cell (19)		Not T or B (4)	
	n	IHC+/PCR+	n	IHC+/PCR+	n	IHC+/PCR+
Mul[a](42)	27	17/23	12	7/9	3	3/3
Med[b](10)	9	4/7	0		1	1/1
Int[c](13)	6	2/2	7	3/5	0	
Cut[d](5)	5	1/2	0		0	

[a] = multicentric
[b] = mediastinal
[c] = intestinal
[d] = cutaneous

Significant difference in the ratio of B to T cell intestinal tumors compared to the ratio of B to T cell non-intestinal tumors (Fisher's exact 2-tailed p = 0.04, odds ratio = 3.99, test-based 95% confidence interval = 0.96-17.07).
No significant difference in FeLV detection by IHC between the B cell intestinal tumors and the B cell non-intestinal tumors (Fisher's exact 2-tailed p = 0.53).
No significant difference in FeLV detection by PCR between the B cell intestinal tumors and the B cell non-intestinal tumors (Fisher's exact 2-tailed p = 0.65).

Table 5.3. Lymphocyte type and FeLV status of 70 feline lymphosarcomas according to age group

Age Group	T cell (47)		B cell (19)		Not T or B (4)	
	n	IHC+/PCR+	n	IHC+/PCR+	n	IHC+/PCR+
≤3yr (26)	18	13/15	5	4/4	3	3/3
>3-≤8yr (20)	13	9/13	6	5/6	1	1/1
>8yr (24)	16	2/6	8	1/5	0	

No significant difference in proportion of T to B cell tumors according to age group ($p = 0.65$).
Significant difference in FeLV detection by PCR compared to IHC in B cell tumors from cats >8yr ($p = 0.045$), and in T cell tumors from cats >8yr ($p = 0.02$).

in Table 5.4. Using IHC, B cell tumors from cats ≤8yr were more likely to be FeLV-positive than B cell tumors from cats >8yr. However using PCR, B cell tumors from cats ≤8yr were not more likely to be FeLV-positive than those from cats >8yr. Similarly using IHC, intestinal tumors from cats ≤8yr were more likely to be FeLV-positive than intestinal tumors from cats >8yr. Whereas using PCR, intestinal tumors from cats ≤8yr were not more likely to be FeLV-positive than those from cats >8yr.

Table 5.4. Lymphocyte type and FeLV status of 70 feline lymphosarcomas according to anatomic site and age

Site and Age Group	T cell (47)		B cell (19)		Not T or B (4)	
	n	IHC+/PCR+	n	IHC+/PCR+	n	IHC+/PCR+
Mul[a](42)						
≤8yr	21	17/20	7	5/6	3	3/3
>8yr	6	0/3	5	1/3	0	
Med[b](10)						
≤8yr	8	4/6	0		1	1/1
>8yr	1	0/1	0		0	
Int[c](13)						
≤8yr	1	1/1	4	3/3	0	
>8yr	5	1/1	3	0/2	0	
Cut[d](5)						
≤8yr	1	0/1	0		0	
>8yr	4	1/1	0		0	

[a] = multicentric
[b] = mediastinal
[c] = intestinal
[d] = cutaneous

Significant difference in FeLV detection by IHC between B cell tumors from cats ≤8yr compared to B cell tumors from cats >8yr (Fisher's exact 2-tailed p = 0.005, odds ratio = 31, test-based 95% confidence interval = 1.72-1363.94).
No significant difference in FeLV detection by PCR between B cell tumors from cats ≤8yr compared to B cell tumors from cats >8yr (Fisher's exact 2-tailed p = 0.26).
Significant difference in FeLV detection by IHC between intestinal tumors from cats ≤8yr compared to intestinal tumors from cats >8yr (Fisher's exact 2-tailed p = 0.03, odds ratio = 28, test-based 95% confidence interval = 0.88-4883.46).
No significant difference in FeLV detection by PCR between intestinal tumors from cats ≤8yr compared to intestinal tumors from cats >8yr (Fisher's exact 2-tailed p = 0.27).

5.5. Discussion

In this study of 70 feline LSAs, the T cell phenotype predominated (67%). This agrees with the previous largest study which found that 23 of 36 (64%) spontaneous feline LSAs were T cell (Hardy *et al.*, 1977). However, we found that 19 of 70 (27%) of the tumors were B cell, compared to 4 of 36 (11%) in the same report (Hardy *et al.*, 1977). Previously, B cells were identified by surface Ig which would not allow detection of immature B cells (Abbas *et al.*, 1991), whereas the B cell marker used here facilitated identification of B cells over a broad developmental range (Newlands *et al.*, submitted). This suggests that immature B cells are targets for transformation by FeLV. Here, B cell tumors were as frequently FeLV-positive as T cell tumors using both IHC for FeLV antigen and PCR for FeLV DNA. B cell tumors are generally considered to be FeLV-negative (Hardy and MacEwen, 1989; Rezanka *et al.*, 1992; Rojko *et al.*, 1989), however, the lack of a B cell marker reactive over a broad developmental range, and a bias toward study of T cell tumors (Rojko *et al.*, 1989; Cockerell *et al.*, 1976; Onions *et al.*, 1987; Fulton *et al.*, 1987; Mullins *et al.*, 1984; Neil *et al.*, 1984; Forrest *et al.*, 1987; Miura *et al.*, 1987), may have precluded adequate investigation of feline B cell LSAs. One report describes several methods to characterize the transformed cell type in FeLV-related LSAs (Rojko *et al.*, 1989). Although the markers for T cell characterization were for immature and

mature cell stages, B cells were identified by demonstrating surface Ig, a mature B cell characteristic (Abbas *et al.*, 1991). There was no evidence for transformation of mature B cells in the same study, however the 5 spontaneous tumors and 6 cell lines characterized were thymus and/or multicentric derived (Rojko *et al.*, 1989). In the present study, no mediastinal tumors and only 12 of 42 multicentric tumors were B cell so that only a subset of multicentric and intestinal tumors would demonstrate evidence of B cell transformation and these would not necessarily be mature B cells. Evidence for B cell transformation may not have been demonstrated in the previous study (Rojko *et al.*, 1989) because of the nature of the B cell markers used, or because of the select group of spontaneous tumors and cell lines investigated.

In the current study, there were no mediastinal or cutaneous B cell tumors. Although there were more multicentric B cell tumors than intestinal B cell tumors, intestinal tumors were 4 times more likely to be B cell than T cell. This supports previous studies which indicate a preponderance of B cell tumors among those involving the intestinal tract (Hardy, 1981a; Hardy *et al.*, 1977; Hardy, 1981b; Cotter, 1992; Holmberg *et al.*, 1976; Neil *et al.*, 1984), however, intestinal tumors were not exclusively B cell, nor were B cell tumors exclusively the intestinal form, in the present study. Intestinal B cell tumors were as likely to be FeLV-positive as non-intestinal B cell

tumors (all non-intestinal tumors were multicentric tumors in this study) by both IHC and PCR. Also, there was no increased preference for B cell tumors in old cats. Previous findings that intestinal and/or B cell tumors are negative for FeLV (Hardy *et al.*, 1980b; Francis *et al.*, 1979; Neil *et al.*, 1984; Rojko and Olsen, 1984), may relate more to the age of cat from which the tumor was derived than to lymphocyte type or anatomic form. Also, viral antigen rather than DNA was detected in most of the previous studies and we have demonstrated that FeLV DNA can be detected more often than FeLV antigen, particularly in older cats (Jackson *et al.*, 1993 [Section 4]). In the current study, both B and T cell tumors from old cats were FeLV-positive by PCR more often than by IHC. Virus DNA-positive, antigen-negative tumors suggest infection with latent or replication-defective FeLV (Jackson *et al.*, 1993 [Section 4]).

Here, FeLV DNA but not antigen, was detected in B cell and intestinal tumors from old cats as often as it was from young and middle-aged cats. However, DNA and antigen detection were not different when tumors were investigated by lymphocyte type and anatomic site. These findings provide additional support for viral latency or replication-defective forms in older cats rather than a lack of FeLV involvement in B cell and/or intestinal tumors.

This study supports a role for FeLV in feline B cell

as well as T cell tumorigenesis. However, no attempt was made to determine if viral integration was clonal. In natural LSAs, non-clonal integration of FeLV suggests superinfection following clonal expansion, so the presence of FeLV would be considered coincidental to tumorigenesis (Neil *et al.*, 1984). However, the likelihood of superinfection occurring in those B cell tumors from old cats with viral DNA but no viral antigen, is remote.

The lymphocyte type of the 4 tumors that did not stain with either the B or the T cell marker, is not known. There may be subsets of B and/or T cells which are not detected by the antibodies used here, or these may represent null cell tumors. Monoclonal antibodies recognizing feline T cell subsets have been developed to study the distribution of lymphocyte subsets in normal feline tissues (Tompkins *et al.*, 1990; Dean *et al.*, 1991; Klotz and Cooper, 1986; Ackley *et al.*, 1990), and to examine lymphocyte subset alterations associated with FeLV and feline immunodeficiency virus (FIV) infections (Tompkins *et al.*, 1991; English *et al.*, 1993; Quackenbush *et al.*, 1990; Abbas *et al.*, 1991). However, these monoclonal antibodies have been applied mainly to flow cytometric analysis. Panels of markers for both B and T cell subsets and applicable to formalin-fixed, paraffin-embedded feline tissues would allow more detailed phenotyping of archival lymphoid tumors. As has been found with human lymphoid tumors (Lippman *et al.*, 1988; Foon and

Todd, 1986), B and T cell subset identification of feline LSAs would allow evaluation of treatment protocols and prognostic factors in relation to specific tumor cell subtype. Determining the transformed B and T cell subsets in abundant archival feline LSAs and relating this to FeLV status, would contribute to our understanding of the pathogenesis of FeLV-induced versus FeLV-negative LSAs.

5.6. References

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6. FELINE LEUKEMIA VIRUS DETECTION BY ENZYME-LINKED IMMUNOSORBENT ASSAY AND POLYMERASE CHAIN REACTION IN PERIPHERAL BLOOD FROM 68 CATS WITH HIGH, MODERATE, OR LOW SUSPICION OF HAVING FeLV-RELATED DISEASE

6.1. Abstract

Clinicopathologic criteria were used to group 68 cats according to high, moderate, or low suspicion of having feline leukemia virus (FeLV)-related disease. Peripheral blood samples were tested for FeLV antigen by enzyme-linked immunosorbent assay (ELISA) and for FeLV DNA by polymerase chain reaction (PCR). There was no significant difference between ELISA and PCR results in the 68 cats. Within the high suspicion group, 46% (11/24) of cytopenic cats were test-positive (ELISA and PCR-positive), and 87% (13/15) with hemopoietic neoplasms were test-positive. Also within the high suspicion group, test-positive cats were 2.5 times more likely to die within the 1 year follow-up period than were test-negative (ELISA and PCR-negative) cats. Among cats in the moderate suspicion group, 15% (2/13) were test-positive, and none (0/16) of the cats in the low suspicion group was test-positive. The relative risk of a positive test (ELISA and PCR-positive) in the high suspicion group was 3.7 times that for the moderate suspicion group, and 22.8 times that for the low suspicion group. There was no

significant difference in the relative risk of a positive test between the moderate and low suspicion groups. The results indicate that FeLV detection by PCR can be adapted for diagnostic purposes using peripheral blood samples, however, results do not differ significantly from FeLV ELISA results. Also, a proportion of cats with a high suspicion of having FeLV-related cytopenia(s) and hemopoietic tumors, are negative for both circulating FeLV antigen and DNA. These cats may not have FeLV-related disease, or perhaps FeLV exists in a disease-producing but non-replicating form ultimately detectable by PCR of tissues other than peripheral blood.

6.2. Introduction

Feline leukemia virus (FeLV) is a horizontally transmitted oncogenic, myelosuppressive, and immunosuppressive retrovirus, and represents the most important pathogen of domestic cats (Essex, 1980; Reinacher, 1989; Hardy *et al.*, 1980a; Hardy, 1981c; Hardy, 1981b). Both degenerative and neoplastic conditions of the hemopoietic system can be directly related to naturally occurring FeLV infection (Neil *et al.*, 1987; Rojko and Olsen, 1984; Reinacher, 1989; Hardy *et al.*, 1980a; Reinacher and Theilen, 1987; Sarin and Gallo, 1988; Neil, 1985; Jarrett, 1984; Hoover and Mullins, 1991; Hoover *et al.*, 1981; Hardy, 1981c; Hardy, 1981a). A proportion of cats have clinicopathologic findings consistent with FeLV infection, but are negative for FeLV using tests which rely on viral replication by detecting viral antigen. Circulating FeLV antigen is present in about 70% of cats with non-regenerative anemia, 88% with myeloproliferative disease (excluding lymphosarcoma), 70% with lymphosarcoma, and 80% with myelodegenerative disease (Cotter *et al.*, 1975; Hardy, 1981a). However, there is evidence that incorporation of FeLV within the DNA of host cells without accompanying viral replication, causes disease in some cats (Overbaugh *et al.*, 1988; Lafrado *et al.*, 1989; Sarin and Gallo, 1988; Tzavaras *et al.*, 1990; Hardy *et al.*, 1980b; Madewell and Jarrett, 1983; Rojko *et al.*, 1982; Jackson *et al.*, 1993 [Section 4]). Also, high levels of unintegrated

FeLV DNA have been associated with disease progression of the acquired immunodeficiency syndrome (AIDS) strain of FeLV (Mullins *et al.*, 1986). Traditionally, FeLV infection has been diagnosed using the immunofluorescent antibody (IFA) test for cell associated viral antigens, or the enzyme-linked immunosorbent assay (ELISA) which detects soluble FeLV group specific antigens in the plasma or serum of FeLV infected cats (Hardy and Zuckerman, 1991a). The polymerase chain reaction (PCR) facilitates the detection of minute amounts of DNA in tissues, and is particularly useful in instances where infectious virus is non-replicating or is present in very low copy number (Sninsky, 1990; Innis *et al.*, 1990). The PCR has been successfully used to detect FeLV in fresh and formalin-fixed feline tissues (Fulton *et al.*, 1990; Matsumoto *et al.*, 1992; Jackson *et al.*, 1993 [Section 4]), and has been shown to detect FeLV DNA in lymphoid tumors negative for antigen expression (Jackson *et al.*, 1993 [Section 4]). In this study FeLV was detected by PCR in peripheral blood samples collected from cats with a variety of clinicopathologic problems associated to varying degrees with FeLV infection. These samples were submitted for routine testing to a diagnostic laboratory. The objective of the study was to investigate the prevalence of FeLV by ELISA compared to PCR to determine if some cats harboured FeLV DNA in peripheral blood cells without expressing viral antigen.

6.3. Materials and Methods

6.3.1. Case Selection

The study group comprised 68 cats presented to the Western College of Veterinary Medicine and private veterinary clinics, with variable clinicopathologic evidence of FeLV infection. All available clinical, hematologic, biochemical, and necropsy information was used to group cats according to degree of suspicion of FeLV-related disease. The high suspicion group was subdivided into cats with A. peripheral cytopenia(s) without evidence of hemopoietic neoplasia, defined as one or more of the following: severe non-regenerative anemia [packed cell volume (PCV) ≤0.15 L/L; reference range, 0.24-0.45 L/L], severe neutropenia (neutrophil count $\leq 1.5 \times 10^9$/L; reference range, 2.5-12.5$\times 10^9$/L), and/or severe thrombocytopenia (platelet count $\leq 50 \times 10^9$/L; reference range, 300-500$\times 10^9$/L), or B. hemopoietic neoplasia involving erythrocytes, lymphocytes, granulocytes, monocytes, and/or thrombocytes. The moderate suspicion group included cats with diseases sometimes associated with FeLV such as feline infectious peritonitis (FIP), hemobartonellosis accompanied by a regenerative anemia, and chronic, non-responsive inflammatory conditions, as well as unexplained hematologic abnormalities exclusive of those in the high suspicion group. These abnormalities included red cell macrocytosis unaccompanied by anemia [mean corpuscular volume (MCV) >55 fL; reference range, 39-55 fL], circulating atypical

lymphocytes, mild to moderate anemia (PCV 0.16-0.23 L/L), and/or mild to moderate neutropenia (neutrophil count 1.6-2.4 x 10^9/L). The low suspicion group included cats with diseases or conditions not generally associated with FeLV such as chronic renal failure, feline urologic syndrome, pancreatitis, non-hemopoietic tumors, and primary fatty liver syndrome, or cats with vague illness and normal hematologic findings. The follow-up period was a minimum of one year from initial testing. The follow-up included a review of the clinical record, consultation with the attending clinician, and/or direct contact with the owner. Death was natural or by euthanasia, and was distinguished as FeLV-related or -unrelated.

6.3.2. Laboratory Data

The minimum data collected for each case included a complete blood count (CBC), ELISA for FeLV antigen, and PCR for FeLV DNA. The CBCs were done using an electronic multi-channel cell counting analyzer (Coulter Model S-plus IV, Coulter Electronics Inc., Hialeah, Florida) and manual techniques as described (Jain, 1986). The ELISAs to detect FeLV p27 antigen were done using one of two commercial test kits according to the manufacturer's instructions (CITE Feline Leukemia Virus Antigen Test Kit or CITE Combo Feline Leukemia Virus Antigen/Feline Immunodeficiency Virus Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, Maine).

6.3.3. Polymerase Chain Reaction

Template DNA for the PCR was extracted from peripheral blood samples collected into EDTA vacuum tubes. The procedure for genomic DNA extraction has been previously described (Plante *et al.*, 1986). Briefly, the buffy coat was separated from 0.25 to 2.0 mL of anticoagulated whole blood and transferred to a 1.5 mL microfuge tube. Then 1 mL of double distilled water (ddH_20) was added, the sample was vortexed briefly and 250 uL of 5X standard saline citrate (SSC) was added and mixed. The lysate was microfuged for 3 min, the supernatant removed, and 1 mL of 1X SSC was added, vortexed briefly, and the sample spun for 2 min. The supernatant was removed and the buffy coat resuspended in 500 uL of 1X SSC; 35 uL of 20% sodium dodecyl sulfate (SDS) was added, the sample mixed, and left at room temperature for 5 min. Then 12 uL of Proteinase K (20 mg/mL) (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Maryland) was added, mixed, and the samples incubated at 65°C overnight. Following overnight digestion, samples were extracted once with phenol, twice with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1), then precipitated with ethanol according to standard technique (Sambrook *et al.*, 1989). The dried samples were resuspended in 50 uL of HPLC grade water (BDH Inc., Toronto, Ontario). A 1 to 10 uL sample volume was used as template for the PCR.

The protocol for amplification of a 166 base pair segment of the FeLV long terminal repeat (LTR) has been

previously described for application to formalin-fixed, paraffin-embedded feline lymphoid tumors (Jackson *et al.*, 1993 [Section 4.3.4]). No modifications were made for DNA template extracted from peripheral blood samples. Southern hybridization using a non-radiolabeled probe, positive and negative controls, and sequencing of amplified products were also as previously described (Jackson *et al.*, 1993 [Sections 4.3.5, 4.3.6, 4.3.7]). Results from clinical cases were accepted and interpreted only when controls produced the expected result on agarose gel electrophoresis (AGE) of a 10 uL aliquot of PCR product. A definite band of the appropriate size (166 bp) on AGE was interpreted as positive for FeLV provirus; Southern hybridizations were done randomly on such samples to confirm specificity of the PCR for exogenous FeLV LTR. The PCR was repeated at least once on samples yielding negative or equivocal results. Samples were considered negative if no 166 bp band was visualized on AGE of PCR products from two PCRs. Samples with equivocal results on AGE were accepted as either positive or negative based on Southern hybridization findings. If both AGE and Southern hybridization results were equivocal, the sample was interpreted to be negative for FeLV provirus.

6.3.4. Statistical Analysis

McNemar's chi-square test for two related samples (Martin *et al.*, 1987) was used to compare the FeLV infection rate diagnosed by ELISA with that of PCR. Within

the high suspicion group, the risk of death in test-positive cats was compared to the risk of death in test-negative cats by calculating relative risk and test-based 95% confidence intervals (Rothman, 1986). Cats with positive results in both ELISA and PCR were considered "test-positive", those with negative results in both ELISA and PCR were considered "test-negative", and the remainder were "test-discordant". Discordant results were excluded from the calculation of risk of death. For the purpose of estimating risk, cats were considered to have survived if the clinical outcome was unknown.

The association between test result and disease suspicion category was examined using chi-square analysis (Daniel, 1990). If the overall relationship was significant among the 3 disease suspicion categories, the relationship between categories (high v. moderate, high v. low, and moderate v. low) was examined. If any cell total was 0, then the quantity 0.5 was added to all cells for the calculations. Fisher's exact test was used when appropriate (Daniel, 1990).

6.4. Results

Table 6.1 shows the distribution of cases, FeLV test results, and clinical outcome in 68 cats with high, moderate, or low suspicion of having FeLV-related disease. There was no significant difference between FeLV ELISA and PCR results ($p = 0.5637$). In the high suspicion group 2 cases were weakly positive by ELISA (reported here as ELISA-positive), but unquestionably positive by PCR. Only 3 cats, all within the cytopenia subgroup, had discordant results. The 2 ELISA-positive/PCR-negative cases included a 6 yr old cat with severe non-regenerative macrocytic anemia which died 8 mo after diagnosis, and a 1 yr old cat with severe neutropenia that was well 2 yrs after diagnosis. The 1 ELISA-negative/PCR-positive cat was 14 yrs old with chronic neutropenia and was well 2 yrs after testing. Twenty-five cats died out of 39 (64%) in the high suspicion group, all directly due to FeLV-related disease. Within the high suspicion group, test-positive cats were 2.5 times more likely to die during the follow-up period than were test-negative cats (Fisher's exact 2-tailed $p = 0.0067$, test-based 95% confidence interval = 1.3-4.9).

Of 13 cats in the moderate suspicion group, 4 had hemobartonellosis, 3 had FIP, 2 had chronic non-responsive inflammatory disease, and 4 had various hematologic abnormalities as described in the materials and methods. One of 2 test-positive cats that died in the moderate suspicion group had hemobartonellosis, the other had

Table 6.1. FeLV status by ELISA and PCR, and clinical outcome in 68 cats with pre-test high, moderate, or low suspicion of having FeLV-related disease

Suspicion Group	Test result n	ELISA/PCR	Clinical outcome n	Dead/Alive/Unknown
1. High	39			
a. cytopenias	(24)	+ / +	11	7 / 0 / 4
		- / -	10	2 / 8 / 0
		+ / -	2	1 / 1 / 0
		- / +	1	0 / 1 / 0
b. hemopoietic neoplasia	(15)	+ / +	13	13 / 0 / 0
		- / -	2	2 / 0 / 0
2. Moderate	13	+ / +	2	2 / 0 / 0
		- / -	11	6 / 3 / 2
3. Low	16	- / -	16	2 / 13 / 1

No significant difference between FeLV ELISA and PCR results, McNemar's chi-square, p = 0.5637.
Test positive (ELISA and PCR positive) cats in the high suspicion group had a relative risk of death 2.5 times that for test negative (ELISA and PCR negative) cats in the high suspicion group (Fisher's exact 2-tailed p = 0.0067, test-based 95% confidence interval = 1.3-4.9)

chronic non-responsive diarrhea. Three of 6 test-negative cats that died in the moderate suspicion group had FIP, 1 had intravascular hemolysis and pyothorax, 1 had chronic intestinal and respiratory infections with terminal disseminated intravascular coagulation, and the last had erythrocyte macrocytosis but died 2 yrs later with diabetes mellitus. Two cats died in the low suspicion group, 1 with chronic renal failure, and 1 with hemangiosarcoma.

Thirty-three of 39 cats in the high suspicion group were ≤8 yrs, 11 of 13 in the moderate suspicion group were ≤8 yrs, and 12 of 16 in the low suspicion group were ≤8 yrs. The 2 test-negative cats in the high suspicion group with hemopoietic tumors were >8 yrs and had lymphosarcoma without evidence of leukemia.

Table 6.2 shows the likelihood of a positive test according to the pre-test suspicion group. There were significant differences in positive test rates between the high and moderate suspicion groups (chi-square = 10.10, p = 0.001), and between the high and low suspicion groups (chi-square = 19.81, p = < 0.0001). There was no significant difference in positive test rates between the moderate and low suspicion groups (Fisher's exact p = 0.19). Cats in the high diagnostic suspicion category were 3.7 times more likely to be test-positive compared to cats in the moderate diagnostic suspicion category (relative risk = 3.70, 95% confidence interval = 1.65-8.29). Similarly, cats in the high diagnostic suspicion category were about 23 times more

Table 6.2. Relationship between FeLV test results and pre-test level of suspicion of having FeLV-related disease

Suspicion	Test positive*	Test negative†	Rate %‡
1. High	24	12	66.7[a]
2. Moderate	2	11	15.4[b]
3. Low	0	16	0.0[b]

* Test positive = FeLV ELISA and PCR positive
† Test negative = FeLV ELISA and PCR negative
‡ Rate of a positive test result expressed as a percentage; rates with different superscripts are significantly different.
Cats in the high suspicion group had a relative risk of a positive test that was 3.70 times that of cats in the moderate suspicion group (test-based 95% confidence interval = 1.65-8.29). Cats in the high suspicion group had a relative risk of a positive test that was 22.8 times that of cats in the low suspicion group (test-based 95% confidence interval = 5.76-90.25).

likely to be test-positive than were cats in the low suspicion category (95% confidence interval = 5.76-90.25).

6.5. Discussion

In this study, 13 of 39 cats (33%) with high clinicopathologic suspicion of having FeLV-related disease were ELISA-negative. Previous studies have also shown that only a proportion of cats with diseases often associated with FeLV, are positive for circulating FeLV antigen (Cotter *et al.*, 1975; Hardy, 1981a). If these antigen-negative cats have very low levels of infective virus or are infected with latent or defective forms of FeLV, then PCR might be expected to detect viral DNA in this group of cats. However, this study showed no difference between ELISA and PCR results in 68 cats grouped according to level of suspicion of FeLV-related disease.

The ELISA is a sensitive test for circulating FeLV antigen, requiring only 0.2 mL of serum, plasma, or anticoagulated whole blood, and being unaffected by peripheral cytopenia (Hawks *et al.*, 1991). The PCR is able to detect minute amounts of viral DNA in tissues (Sninsky, 1990; Innis *et al.*, 1990). In this study, PCR was performed on peripheral blood that remained after all other diagnostic tests were completed; in many cases there was only 0.25 mL of blood, often with a very low nucleated cell count. Despite the potential limitations of sample quantity and quality, PCR detected FeLV at the same rate as the ELISA.

We examined peripheral blood so that PCR could be evaluated in direct relation to ELISA; however, cats

harbouring latent or defective virus may do so only in certain tissues such as bone marrow, lymph nodes, or epithelial tissues (Lafrado *et al.*, 1989; Jarrett, 1984; Hoover and Mullins, 1991; Rojko and Kociba, 1991; Madewell and Jarrett, 1983; Rezanka *et al.*, 1992). Antigen could be released from such sites periodically without cell associated viremia, or antigen may never be released in the case of replication-defective but disease-producing FeLV. The high suspicion test-negative cats may have harboured FeLV in tissues other than peripheral blood cells, the target region for the PCR primers may have been unrecognizable due to mutation or deletion, sample limitations may have produced false-negative results, or the hematologic abnormalities in these cats may not have been FeLV-related.

Virus isolation and IFA usually correlate well (Lopez *et al.*, 1990; Hardy and MacEwen, 1989), whereas, a proportion of cats are ELISA-positive and IFA- or virus isolation-negative (Lutz *et al.*, 1983; Jarrett, 1991; Hardy and Zuckerman, 1991a; Jarrett *et al.*, 1991; Hoover and Mullins, 1991; Jarrett *et al.*, 1982; Hardy and Zuckerman, 1991b; Loar, 1987). Controversy exists as to whether ELISA-positive/IFA-negative cats are ELISA false-positive, IFA false-negative, in the primary stage of infection before development of cell associated viremia, in a transient phase of infection before recovery, or experiencing latent infection with periodic low level

antigenemia. We found no significant difference between ELISA and PCR results, implying that ELISA-positive cats in this group also had cell associated viral DNA and were likely true positives. Though the 3 discordant cases did not significantly affect the results and interpretation, possible explanations are: false-positive or false-negative ELISA or PCR results, antigenemia without cell associated viremia in the 2 ELISA-positive/PCR-negative cats, or defective or latent virus in peripheral blood cells with no or low level antigenemia in the ELISA-negative/PCR-positive cat. This latter case was a 14 yr old cat with chronic neutropenia and few clinical signs.

The two test-negative cats with hemopoietic neoplasia (lymphosarcoma) were >8 yrs and had solid tumors with no leukemia. We have previously reported that using archival lymphosarcoma tissue from old cats, FeLV DNA can be detected by PCR more often than FeLV antigen can be detected by immunohistochemistry (Jackson *et al.*, 1993 [Section 4]). In the present study, direct examination of tumor tissue for proviral DNA may have revealed FeLV in the 2 cases with negative peripheral blood results.

The rate of a positive test among high suspicion cases was 66.7%, but was only 6.9% among the combined moderate and low suspicion cases. Perhaps surprising was that the relative risk of being test-positive was not significantly different between moderate and low suspicion groups. The number of cases in the moderate suspicion group may have

been too low to detect a difference, or a relationship between FeLV and conditions such as hemobartonellosis, FIP, and chronic inflammatory diseases may be overemphasized. Some reports suggest that FeLV is involved in 30-50% of hemobartonellosis cases, 40-60% of FIP cases, and 52-60% of chronic non-responsive infections (Jarrett, 1983; Rojko and Olsen, 1984; Cotter *et al.*, 1975; Hardy, 1981a; VanSteenhouse *et al.*, 1993). In a large retrospective study by necropsy, only 19% of FIP cases were FeLV antigen-positive, and several mycotic, viral, and parasitic infections had no significant association with FeLV infection (Reinacher, 1989; Reinacher and Theilen, 1987). Results from our study support grouping cats by clinicopathologic findings according to only 2 levels of suspicion of FeLV-related disease.

Test-positive cats in the high suspicion group were 2.5 times more likely to die during the follow-up period than test-negative cats in the same group. The relative risk of death for these test-positive cats may be understated. Cats with unknown clinical outcomes were considered to be alive for the purpose of the calculation, and all 4 cats with unknown outcomes were test-positive. These 4 cats were very ill and in most cases were sent home to die, although at the time of follow-up, the owners could not be located.

Epidemiologic studies show that about 50% of viremic cats die within 6 months and 80-100% die within 3 years of

FeLV diagnosis (Jarrett, 1983; Mullins and Hoover, 1990). In this study, 8 of 12 test-negative cats in the high suspicion group were alive at least 1 year following testing. The better prognosis in these test-negative cats may indicate they were not FeLV-infected, or FeLV infection was transient, undetectable at the time of testing, and recovery occurred. Whether test-positive cats were euthanatized more readily than test-negative cats with the same clinicopathologic abnormalities, is not known. However, the survival rate at 1 year suggests that test-negative cats in this group had a better prognosis which is not explained solely by higher euthanasia rates in test-positive cats.

To our knowledge there are no previous reports comparing FeLV detection by ELISA to PCR in sick cats; however, conflicting results appear in the literature comparing PCR to serology for human immunodeficiency virus (HIV) diagnosis in high risk groups (Bruisten *et al.*, 1992; Petru *et al.*, 1992; He *et al.*, 1993; Farzadegan *et al.*, 1993; Jung *et al.*, 1992; Luce *et al.*, 1991; Horsburgh *et al.*, 1990; Sonnerborg *et al.*, 1990). Some describe a high percentage of PCR-positive/antibody-negative patients (Sonnerborg *et al.*, 1990; Luce *et al.*, 1991), whereas, others have found no difference between PCR and serology results and suggest that antigen and antibody tests are appropriate and conclusive most of the time (Bruisten *et al.*, 1992; Farzadegan *et al.*, 1993). Generally serologic

data including HIV-1 antigen tests, agree with nucleic acid detection data. However, PCR is useful for evaluating infants born to seropositive mothers, who have detectable HIV antibody that may persist for as long as 15 months (Petru *et al.*, 1992), patients prior to seroconversion, patients receiving anti-retroviral treatment, and patients with inconclusive serologic tests (Luce *et al.*, 1991; Petru *et al.*, 1992; He *et al.*, 1993). Many of these situations do not exist in the case of FeLV. Traditional FeLV tests detect viral antigen which appears early in infection and usually persists if an adequate immune response is not raised (Rojko and Olsen, 1984; Jarrett *et al.*, 1982; Hoover and Mullins, 1991; Rojko and Kociba, 1991; Neil and Onions, 1985). In this study, 2 samples were weakly positive by ELISA, but unquestionably positive by PCR; PCR may be useful in such cases of uncertainty. Many clinicians accept weakly positive ELISAs as positive, as we did for this study, while others recommend retesting in the near future and/or IFA testing.

We have previously reported the application of PCR for retrospective FeLV detection in formalin-fixed, paraffin-embedded lymphoid tumors from cats (Jackson *et al.*, 1993 [Section 4]). The PCR detected FeLV DNA in tumors from a significant proportion of older cats (>7 yrs) that were negative for viral antigen by immunohistochemistry. These results suggested defective or latent virus may be involved in tumor development in a subgroup of older cats. We were

unable to show a similar relationship here, using peripheral blood from cats with hematologic abnormalities often associated with FeLV. However, 33 of 39 cats in the high suspicion group were young to middle-aged (≤ 8 yr). Perhaps PCR only detects additional FeLV-positive cases in older cats which may be more likely to have latent or defective FeLV. A molecular technique such as PCR is ideally suited to explore the question of latent or replication-defective FeLV infection. Tissues other than peripheral blood may have to be examined by PCR to support or refute a role for FeLV in the pathogenesis of antigen-negative but FeLV-suspect disease.

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7. SEQUENCE ANALYSIS OF THE PUTATIVE VIRAL ENHANCER IN TISSUES FROM 33 CATS WITH VARIOUS FELINE LEUKEMIA VIRUS-RELATED DISEASES

7.1. Abstract

Diseases resulting from infection by feline leukemia virus (FeLV) and several other retroviruses relate in part, to non-coding regulatory sequences within the viral long terminal repeat (LTR). Both enhancer repeats and mutations within the LTR have been implicated in FeLV-related disease. In order to investigate the relationship between nucleotide sequence of the FeLV LTR and disease, tissues from 33 cats with 7 types of degenerative and proliferative FeLV-related disease were studied. Diseases included anemia (6 cases), leukopenia (5), erythrocytic sarcoma (5), granulocytic sarcoma (3), undetermined hemopoietic cell sarcoma (4), antigen-positive lymphosarcoma (5), and antigen-negative lymphosarcoma (5). An FeLV LTR region containing the putative transcriptional enhancer unit was amplified by polymerase chain reaction (PCR) from FeLV-infected tissues.

Phylogenetic analysis of FeLV U3 sequences revealed only 1 meaningful grouping which contained 4 of the 5 antigen-negative LSAs. No sequence duplications were found

in any of the 33 FeLV U3 regions. Point mutations relative to the corresponding region of FeLV-A/Glasgow, were identified at 102 positions; 68 of these were accounted for by mutations at 5 locations. Only 1 point mutation was found within the leukemia virus b - simian virus 40-like core (LVb-CORE) site. However the nuclear factor 1 (NF1) site contained 11 mutations, and the FeLV-specific (FLV-1) site contained 26 mutations. Most of the remaining mutations were upstream of the LVb site or between the glucocorticoid response element (GRE) and FLV-1. As a group, the 10 LSAs and particularly the 5 antigen-negative LSAs, had the fewest mutations compared to the corresponding sequence for FeLV-A/Glasgow.

Conclusions were that the spectrum of neoplastic and non-neoplastic FeLV-related diseases investigated in this study, developed in the presence of FeLVs containing the single enhancer unit. The significance of the point mutations is unknown however those occurring with high frequency and within nuclear protein binding sites should be first to be investigated in functional studies.

7.2. Introduction

Genetic differences which underlie disease manifestation by different FeLV isolates can be identified through nucleotide sequence analysis (Donahue *et al.*, 1988). Such analyses have focused primarily on long terminal repeat (LTR) and envelope (*env*) regions of the FeLV genome (Donahue *et al.*, 1988; Guilhot *et al.*, 1989; Stewart *et al.*, 1986; Berry *et al.*, 1988; Miura *et al.*, 1989; Golemis *et al.*, 1990; Casey *et al.*, 1981; Hampe *et al.*, 1983; Fulton *et al.*, 1990; Plumb *et al.*, 1991; Neil *et al.*, 1991; Matsumoto *et al.*, 1992; Rohn and Overbaugh, 1995; Athas *et al.*, 1995). Disease determinants of FeLV strains causing feline acquired immunodeficiency syndrome (FeLV-FAIDS) and erythroid hypoplasia (FeLV-C/Sarma) have been localized in part, to *env* sequences (Overbaugh *et al.*, 1988; Donahue *et al.*, 1991; Poss *et al.*, 1989; Poss *et al.*, 1990; Kizaki *et al.*, 1991; Quackenbush *et al.*, 1990; Reinhart *et al.*, 1993; Thomas and Overbaugh, 1993; Riedel *et al.*, 1986; Riedel *et al.*, 1988; Rigby *et al.*, 1992; Dean *et al.*, 1992). Whereas *env* sequences confer receptor binding properties for FeLV (Jarrett *et al.*, 1973; Sarma and Log, 1973), the LTR contains transcriptional promoter and enhancer sequences for viral gene expression (Luciw and Leung, 1992; Berry *et al.*, 1988; Hampe *et al.*, 1983).

With some murine leukemia viruses (MuLVs), the level of LTR-directed transcription within specific cell types correlates with tumorigenic potential (Speck *et al.*, 1990b;

Boral *et al.*, 1989). Both duplications and sequence variation within the enhancer framework of the LTR are implicated in oncogenicity through accelerated transcriptional activity (Clark *et al.*, 1985; Holland *et al.*, 1989). Also, using recombinant MuLVs, variations in LTR sequences have been shown to explain in part, the wide variety of diseases and latent periods resulting from viral infection (Golemis *et al.*, 1989). Levels and/or types of DNA-binding proteins may vary with cell type, therefore, *trans*-acting factors within infected tissues may directly influence viral transcription through interactions with LTR enhancer motifs (Boral *et al.*, 1989; Speck *et al.*, 1990b; Thornell *et al.*, 1988). In addition to enhancer sequence variation specifying disease phenotype in MuLVs (Clark *et al.*, 1985; Speck *et al.*, 1990a), highly variable sequences flanking enhancer motifs may also relate to disease manifestation (Golemis *et al.*, 1989).

Endogenous and exogenous FeLV LTRs diverge within segments of the LTR unique 3 (U3) region, indicating that replication efficiency and other selective advantages of the exogenous virus are likely to relate to these sequences (Berry *et al.*, 1988; Casey *et al.*, 1981). Alignment of U3 regions of several type C mammalian retroviruses reveals transcriptional enhancer consensus motifs as well as highly conserved promoter elements (Golemis *et al.*, 1990). In the FeLV U3, enhancer motifs include potential binding sites for leukemia virus factor b (LVb), simian virus (SV)40-like

core (CORE), nuclear factor 1 (NF1), glucocorticoid response element (GRE), and a FeLV-specific binding site (FLV-1) (Fulton *et al.*, 1990). If similar to other type C retroviral LTRs, the basic enhancer framework in FeLV would include LVb, CORE, NF1, and GRE sites.

Functional roles of putative enhancer elements in FeLV have not been thoroughly examined. Although several potential binding sites exist in the FeLV U3, protein binding only to the CORE, NF1, and FLV-1 sites has been demonstrated (Fulton *et al.*, 1990). Altered transcriptional activity related to mutation of the NF1-binding site in FeLV, varies with cell type. For instance, the degree of LTR impairment due to mutated NF1 is less in FeLV-induced T lymphosarcoma (LSA) cell lines which also exhibit decreased NF1 activity. The low NF1 binding in these immature cells is speculated to decrease responsiveness to growth limiting or differentiation-promoting signals (Plumb *et al.*, 1991).

The FeLV U3 region has been examined in experimentally-induced and naturally-occurring thymic LSAs (Fulton *et al.*, 1990; Plumb *et al.*, 1991; Matsumoto *et al.*, 1992; Rohn and Overbaugh, 1995), and a few hemopoietic tumors of uncertain phenotype (Athas *et al.*, 1995), non-lymphoid hemopoietic tumors (Matsumoto *et al.*, 1992), and non-neoplastic diseases (Matsumoto *et al.*, 1992). Duplicated enhancer sequences, containing at least the CORE but excluding the FLV-1 site, appear to be prevalent

particularly in FeLV-related thymic LSAs (Matsumoto *et al.*, 1992; Rohn and Overbaugh, 1995; Fulton *et al.*, 1990). Enhancer repeats have been shown to arise *de novo* following experimental infection of cats with molecularly cloned FeLVs containing the single enhancer unit (Rohn and Overbaugh, 1995).

The FLV-1 site is thought to be a negative regulatory element (Fulton *et al.*, 1990), and is invariably excluded from enhancer duplications found in several FeLV-induced thymic LSAs (Fulton *et al.*, 1990; Miura *et al.*, 1989; Matsumoto *et al.*, 1992; Rohn and Overbaugh, 1995). However, a 21 bp tandem triplication downstream from the FeLV enhancer framework and encompassing the FLV-1 site, has been implicated in the induction of 2 feline hemopoietic tumors of uncertain phenotype (Levesque *et al.*, 1990; Athas *et al.*, 1995). Whether the FLV-1 site plays a negative transcriptional role in certain cell types, but an enhancer role in others, requires further study.

Although FeLVs with enhancer repeats might be expected to have a growth advantage, functional assays of LTR-driven growth hormone expression in feline T and fibroblast cell lines reveal only modest increases in activity (Plumb *et al.*, 1991). However, FeLV LTR-directed chloramphenicol acetyltransferase (CAT) expression in feline embryonic fibroblasts, human malignant T cells, and human malignant hemopoietic progenitor cells, show significant differences between transcriptional activity of the single enhancer LTR

compared to LTRs with either a duplicated enhancer structure or with triplication of the 21 bp downstream sequence (Athas *et al.*, 1995). The triplication-containing LTR acts in a cell type specific manner with most marked effects in human malignant hemopoietic progenitor cells. This has lead to the hypothesis that the neoplastic feline cells containing this FeLV variant, are perhaps not non-T lymphocytes, but less differentiated feline hemopoietic cells (Athas *et al.*, 1995).

Nucleotide sequencing studies employing PCR to amplify the putative FeLV enhancer, have involved cloning of PCR products prior to sequencing (Matsumoto *et al.*, 1992; Fulton *et al.*, 1990; Rohn and Overbaugh, 1995). However, direct sequencing of PCR products may be more accurate than using traditional cloning methods (Cao and Brosius, 1993; Thomas and Kocher, 1993), which is important when single or subtle nucleotide changes may be responsible for altered disease phenotype.

The purpose of this study was to amplify and directly sequence the putative FeLV enhancer region from tissues of cats with a broad range of clinically important, naturally-occurring neoplastic and non-neoplastic FeLV-induced diseases. Nucleotide sequences were then analyzed to determine if genomic variation within this part of the LTR related to disease manifestation. Specific mutations were evaluated with respect to frequency and location relative to nuclear protein binding motifs.

7.3. Materials and Methods

7.3.1. Case Selection

Thirty-three cats with clinicopathologic and/or histopathologic evidence of FeLV-related disease, and with tissues containing FeLV DNA, comprised the study group. The 33 cases included: 5 cats with severe non-regenerative anemia and 1 with regenerative anemia and hemobartonellosis [in all cases packed cell volume (PCV) ≤0.15 L/L, reference range, 0.24-0.45 L/L]; 5 cats with panleukopenia [white blood cell (WBC) ≤2.0 x 10^9/L, reference range, 5.5-19.5 x 10^9/L] or neutropenia (neutrophils ≤1.0 x 10^9/L, reference range, 2.5-12.5 x 10^9/L); 5 cats with erythrocytic sarcomas; 3 cats with granulocytic sarcomas; 4 cats with hemopoietic tumors of undetermined cell type; and 10 cats with LSA. Morphologic detail evaluated by cytopathologic and/or histopathologic examination, was used to identify tumor cell types. Six of the LSAs were previously characterized as T (n=5) or B cell (n=1) phenotypes (Jackson *et al.*, 1995b [Section 5]).

7.3.2. Polymerase Chain Reaction

The presence of FeLV DNA was determined using PCR amplification of a 166 base pair (bp) segment encompassing the putative LTR enhancer (Fulton *et al.*, 1990; Matsumoto *et al.*, 1992). Sample sources for PCR included: fresh or frozen peripheral blood; fresh, frozen, or formalin-fixed bone marrow; and fresh, frozen, or formalin-fixed solid tumor tissue. The procedure for DNA extraction and

purification from fresh tissues has been previously described (Misra *et al.*, 1992; Plante *et al.*, 1986), with modifications (Jackson *et al.*, 1995a [Section 6.3.3]). Sample preparation from formalin-fixed, paraffin-embedded tissues has also been described (Innis *et al.*, 1990) with minor modifications (Jackson *et al.*, 1993 [Section 4.3.3]). Primer sequences for FeLV LTR amplification that have been successfully used in fresh (Fulton *et al.*, 1990) and formalin-fixed (Jackson *et al.*, 1993 [Section 4.3.4]) feline tissues, were synthesized in two different laboratories (University of British Columbia, Oligonucleotide Synthesis Laboratory, Department of Biochemistry, Vancouver, British Columbia, and DNA Technologies Unit, National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, Saskatchewan). Conditions and controls for PCR amplification of the FeLV U3 region are described in an earlier report (Jackson *et al.*, 1993 [Sections 4.3.4 and 4.3.6]).

7.3.3. FeLV Antigen Detection

FeLV antigen expression was not a requirement for case selection, but was evaluated using either enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (CITE Feline Leukemia Virus Antigen Test Kit or CITE Combo Feline Leukemia Virus Antigen/Feline Immunodeficiency Virus Antibody Test Kit, IDEXX Corp., Portland, Maine), or avidin-biotin complex immunohistochemistry (IHC) as described (Haines and

Chelack, 1991) with modifications for FeLV gp70 detection (Jackson *et al.*, 1993 [Section 4.3.2]).

7.3.4. DNA Template for Nucleotide Sequencing

Three 10 uL aliquots of PCR product were each mixed with 3 uL of stop buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and electrophoresed through a 2% agarose minigel (Low Melt Preparative Grade, Bio-Rad Laboratories, Richmond, California). Gels were stained with ethidium bromide (Sigma Chemical Company, St. Louis, Missouri) and visualized with ultraviolet transillumination (Spectroline Model TR 365, Spectronics Corporation, Westbury, New York).

For each case, the PCR product bands were excised from the gel, placed in sterile 1.5 mL microfuge tubes along with 500 uL of sterile HPLC grade water (BDH Inc., Toronto, Ontario), and heated to 68°C to melt the agarose. Samples were divided equally into 2 - 1.5 mL microfuge tubes, extracted once with equal volumes of phenol, and then extracted twice with equal volumes of phenol-chloroform-isoamyl (25:24:1). One-tenth volume of 7.5 M ammonium acetate was added to each sample, followed by precipitation with ethanol according to standard technique (Sambrook *et al.*, 1989). Dried samples were resuspended in 50 uL of HPLC grade water (BDH Inc., Toronto, Ontario).

DNA concentration was estimated by comparison to a DNA mass ladder according to the manufacturer's instructions (DNA Mass Ladder, GIBCO BRL, Life Technologies, Inc.,

Gaithersburg, Maryland).

7.3.5. Nucleotide Sequencing of PCR Products

Nucleotide sequencing of double stranded PCR products was performed in another laboratory (DNA Technologies Unit, National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, Saskatchewan). All samples were sequenced in forward and reverse directions using the same primers as for PCR. A sequencing kit was used according to the manufacturer's directions (*Taq* DyeDeoxy Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, California). Approximately 6 ng of DNA template in a volume of 8 uL was used in the sequencing reaction mixture, and temperature cycling was done with a thermal cycler (Perkin-Elmer Cetus Model 480, Applied Biosystems, Foster City, California). Spin columns were used to purify extension products of the cycling reaction (Centri-Sep columns, Princeton Separations, Adelphia, New Jersey). Samples were then prepared and loaded onto an automated sequencer according to instructions in the user's manual (Applied Biosystems 373A DNA Sequencer, Applied Biosystems, Foster City, California).

7.3.6. Nucleotide Sequence Analysis

Nucleotide sequences from the 33 cases were analyzed using the UPGMA (unweighted pair group method with arithmetic mean) Tree Window (GeneWorks, IntelliGenetics, Inc., Mountain View, California). This computer program displays a tree showing the calculated evolutionary

relationships of the aligned sequences. FeLV U3 sequence data were then combined according to disease so that descriptive comparisons could be made among disease categories and the minimally pathogenic FeLV-A/Glasgow. For reporting purposes, individual nucleotides deviating from those of FeLV-A/Glasgow were called "mutations". However, nucleotide differences may not have represented true mutations since the sequence of the infecting FeLV for each case was not known.

7.4. Results

Using IHC or ELISA for FeLV antigen, the 10 LSAs were further subdivided into 5 antigen-positive and 5 antigen-negative neoplasms. All 5 FeLV antigen-positive LSAs were lymphocytic leukemias and therefore characterized anatomically as multicentric (Hardy, 1981; Shelton *et al.*, 1990; Jackson *et al.*, 1993 [Section 4.3.1]). One of these was T cell origin; the other 4 were not phenotyped. Four of 5 antigen-negative LSAs were multicentric, including 3 T cell and 1 B cell phenotypes. The fifth antigen-negative LSA was a mediastinal (thymic) T cell tumor. All 23 non-LSA cases were positive for FeLV antigen by ELISA or IHC. In all 33 cases, only the predicted 166 bp PCR product was seen when ethidium bromide-stained gels were examined using ultraviolet transillumination.

Figure 7.1 shows nucleotide sequences of the amplified FeLV U3 region from tissues of 33 cats with FeLV-related diseases. Sequence data are grouped according to type of FeLV-related disease and aligned with the corresponding region for FeLV-A/Glasgow.

There were 102 mutations in total when comparing the 33 FeLV U3 sequences to FeLV-A/Glasgow sequence. There were 40 mutations in FeLVs from 11 cases of non-neoplastic disease compared to 62 mutations in 22 cases of neoplastic disease. Sixty-eight of the 102 mutations were due to point mutations at one of 5 locations. These included 13 - thymine (T) to adenine (A) mutations at position -232

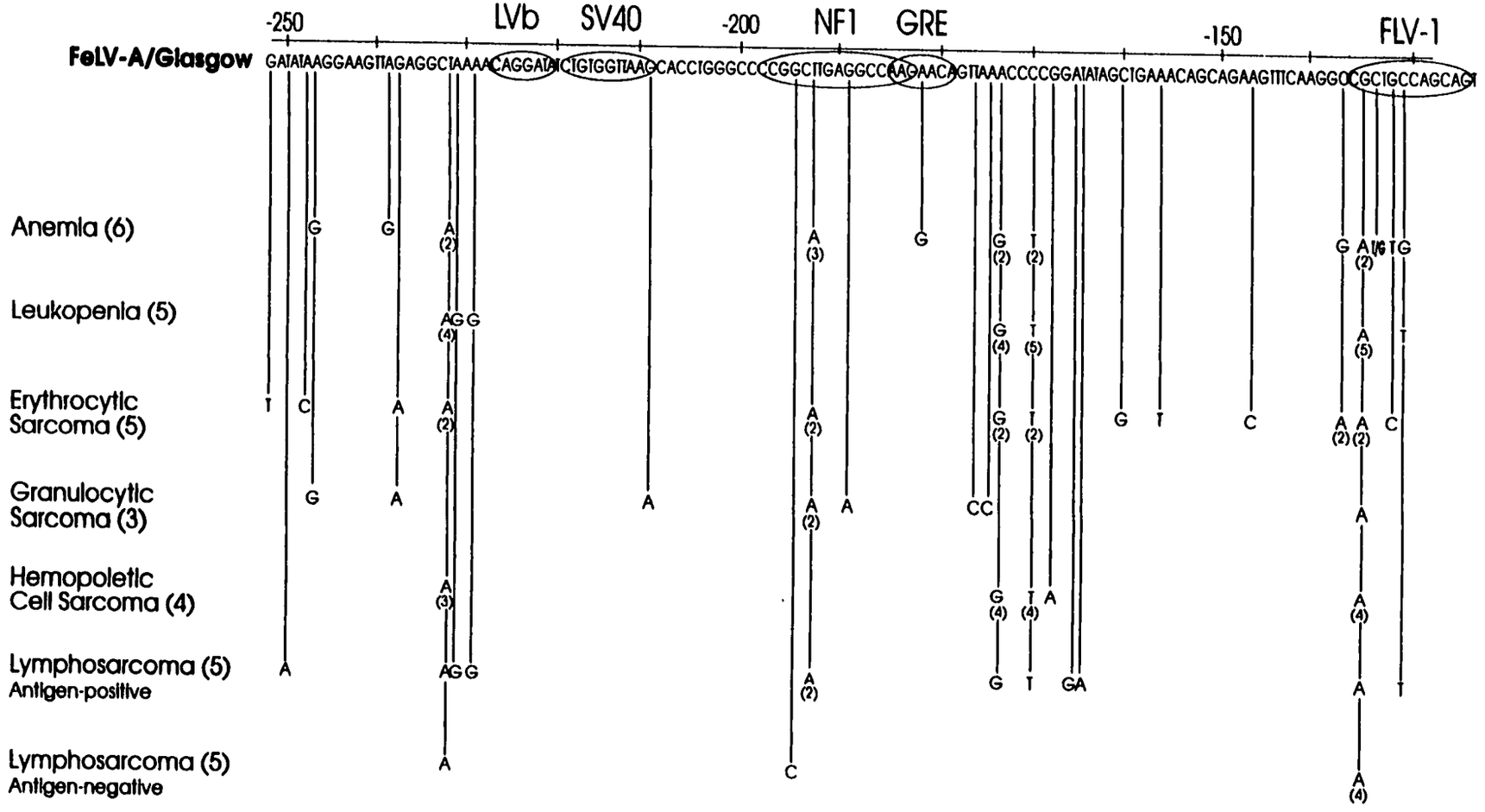

Figure 7.1. Nucleotide sequences of FeLV U3 regions grouped by disease. The number of cases in each group is shown in brackets after disease category. Only nucleotides differing from FeLV-A/Glasgow (Stewart *et al.*, 1986) are shown. The number of point mutations occurring ≥2 times is indicated in brackets below the nucleotide. Primer sequences for PCR (Fulton *et al.*, 1990; Matsumoto *et al.*, 1992) were at positions -274 to -254 inclusive, and -129 to -109 inclusive (sequences not shown).

(relative to the cap site shown in Figure 2.7), 9 - T to A mutations at position -193, 13 - A to guanine (G) mutations at position -173, 14 - cytosine (C) to T mutations at position -170, and 26 - G to A mutations at position -135, as shown in Figure 7.1.

There were no mutations within the LVb site, and only 1 mutation was present within the CORE region. The NF1 site was mutated 11 times, and 9 of these were at the same site. The GRE site was mutated in one case of non-regenerative anemia. The FLV-1 site contained 26 of the 102 mutations, and 19 of these were at the same site. Other clusters of mutations occurred immediately upstream of the LVb-CORE site, and immediately downstream of the GRE site.

There were 18 mutations in sequences from the 10 LSAs compared to 84 mutations in the 23 non-LSA cases. The 5 FeLV antigen-negative LSAs with 6 mutations, appeared to deviate least from the corresponding region of FeLV-A/Glasgow.

Although several groupings were generated by phylogenetic analysis using the alignment UPGMA tree, only one appeared to relate to disease manifestation. Four of the 5 FeLV LTR sequences from cats with antigen-negative LSA clustered within one subgroup (data not shown).

7.5. Discussion

There were no enhancer repeats within any of the 33 amplified FeLV U3 regions in this study. Data compiled from previous reports of 35 FeLVs (Rohn and Overbaugh, 1995; Miura *et al.*, 1989; Matsumoto *et al.*, 1992; Fulton *et al.*, 1990), indicate enhancer repeats in 16 of 18 thymic LSAs, 3 of 8 other anatomic forms of LSA, 2 of 3 myeloid tumors, and 1 of 6 non-neoplastic FeLV-related diseases. Nucleotide sequences in these studies were determined following cloning of FeLV proviruses, LTRs, U3 regions, or U3 region PCR products. Sources of FeLV-infected cells included experimentally-induced thymic LSAs, a tumor cell line established from a spontaneous T cell leukemia, naturally-occurring tumors, and tissues affected by FeLV-related non-neoplastic diseases.

Only 16 of the 35 FeLV enhancer regions were sequenced in these studies (Rohn and Overbaugh, 1995; Miura *et al.*, 1989; Matsumoto *et al.*, 1992; Fulton *et al.*, 1990); single or duplicated enhancers were predicted in the remaining 19 cases based upon sizes of hybridizing PCR products (Matsumoto *et al.*, 1992). Of the 16 FeLV enhancer regions that were sequenced, only the CORE site was common to the 14 which contained repeats (Rohn and Overbaugh, 1995; Miura *et al.*, 1989; Matsumoto *et al.*, 1992; Fulton *et al.*, 1990). Although in some cases several clones from a single tumor were sequenced (Matsumoto *et al.*, 1992; Rohn and Overbaugh, 1995; Fulton *et al.*, 1990), sequences derived by direct

analysis without cloning of FeLV U3 PCR products as in this study, may be more representative of the prevalent FeLV genotype.

Repeat structures within the amplified U3 region may have been present but undetected in at least some of our cases. Using a technique of competitive PCR, others have found that a double enhancer species representing 2% or more of the total LTR species can be detected by PCR, although whether detection was by gel electrophoresis alone or by hybridization, was not indicated (Rohn and Overbaugh, 1995). Regardless, we would not have detected duplicated enhancers representing less than at least 2% of the total PCR product.

Southern hybridization of 25 FeLV LTR PCR products from a heterogeneous group of FeLV-related diseases, indicated only 3 thymic LSAs had more intense bands representing the duplicated enhancer species compared to those with single enhancers (Matsumoto *et al.*, 1992). If the single enhancer-containing LTR is usually more abundant than the duplicated form, the relative importance of the duplicated form in tumorigenesis or non-neoplastic disease induction may be uncertain. However, if neoplasia results from upregulation of a host proto-oncogene such as c-*myc*, *flvi*-1, *flvi*-2, or *fit*-1 through insertional mutagenesis or transduction, the relative number of LTRs containing repeat structures may not be as important as specific interactions with the host genome during viral integration.

Although only 1 non-thymic LSA has been evaluated at the nucleotide level for FeLV enhancer repeats, duplicated enhancer motifs are thought to be particularly prevalent in thymic LSAs (Matsumoto *et al.*, 1992; Fulton *et al.*, 1990; Rohn and Overbaugh, 1995). Nine of 10 LSAs in our study were multicentric, and only 1 was a mediastinal (thymic) tumor. The 2 multicentric LSAs described previously had no evidence of duplicated FeLV enhancer regions based on Southern hybridization of PCR products {Matsumoto *et al.*, 1992}. The lack of repeat sequences in our cases may relate to the relative abundance of non-thymic tumors and non-neoplastic diseases investigated. Nevertheless, factors other than FeLV enhancer repeats must have accounted for tumorigenesis in the 22 FeLV-related neoplasms investigated in this report.

Few studies have been done to evaluate transcriptional activity in FeLV LTRs with single versus repeat enhancer motifs. LTR-driven growth hormone expression with the duplicated enhancer was between 1.4 and 1.9 times that for the single enhancer unit in the feline cell lines tested in one study (Plumb *et al.*, 1991). Another report of LTR-directed CAT expression showed significant differences between the single enhancer unit and both the duplicated enhancer and the triplicated downstream sequence containing the FLV-1 site. The increased activity with the duplicated and triplicated structures was cell type-specific. Although there was no difference between the duplicated

enhancer and triplicated sequence forms in feline fibroblasts and human T cells, CAT activity was significantly higher in human malignant hemopoietic progenitor cells with the triplicated sequence compared to the duplicated enhancer (Athas *et al.*, 1995).

Previously, the FLV-1 site was considered to be a negative regulatory element because it was always excluded from enhancer duplications, but its inclusion in the triplicated structure suggested either transcriptional enhancer activity or a spacer function relative to the FeLV promoter (Athas *et al.*, 1995). However, differences among cell types may more likely relate to varying levels and/or types of DNA-binding proteins interacting with enhancer motifs rather than a spacer function.

In this report, no repeats were found downstream of the traditional enhancer framework. Tandem triplications of the 21 bp sequence downstream of the traditional enhancer, have been reported in 2 of 4 feline splenic tumors of unusual phenotype (Athas *et al.*, 1995). These tumors were first characterized as non-T and possibly non-B cell LSAs (Levesque *et al.*, 1990), but later hypothesized to be of hemopoietic progenitor cell origin (Athas *et al.*, 1995). In our study the 4 hemopoietic sarcomas of undetermined cell type which contained only single enhancer units, could be of similar phenotype to the splenic tumors.

Three mutations were common in FeLV LTRs derived from the 4 splenic tumors, independent of presence or absence of

repeated sequences. Compared to FeLV-A/Glasgow, these differences included A versus G at position -173, C versus T at -170, and G versus A at -135. These 3 nucleotide differences are also present in the erythroid hypoplasia-inducing FeLV-C/Sarma, leading to speculation that the triplication-containing virus and FeLV-C are related, and share a common target cell such as a non-lymphoid hemopoietic progenitor (Athas *et al.*, 1995). The same 3 nucleotide changes common to FeLV-C/Sarma and the splenic tumor derived FeLVs, accounted for 46 of the 102 mutations in our 33 sequences. However, disease specificity related to these 3 mutations is unlikely since they were present in cases from most disease groups in our study. Functional studies involving these mutations would be required to determine pathogenicity. Differences related to *trans*-acting factors within different cell types are unlikely for the G and T mutations since they are not within known protein-binding motifs. However, in some MuLVs, variation within regions flanking enhancer motifs may relate to disease (Golemis *et al.*, 1989).

In this study, the LVb-CORE site was highly conserved which could indicate that mutations of this region are poorly tolerated due to an essential role in transcription. Previously the CORE sequence was thought to be a basic requirement for efficient enhancer function in T cells to allow viral establishment and persistence (Fulton *et al.*, 1990). However, conservation of the CORE and possibly the

LVb sites may both be required for efficient viral transcription in all FeLV-infected cell types.

Most MuLVs which induce erythroleukemias, have a CORE motif which is distinct from those inducing T cell LSA (Clark *et al.*, 1985). Also, mutation of the Moloney MuLV (MoMuLV) CORE motif can alter disease specificity from thymic LSA to erythroleukemia (Speck *et al.*, 1990a). Only 1 of the 33 LTRs contained a mutation in the CORE in this study, and it was from a granulocytic tumor. Although 5 T lymphocytic and 5 erythrocytic tumors were evaluated in this study, no relationship such as between MuLV CORE sequences and tumor phenotype, was found.

There were several mutations within the NF1-GRE site, but most involved a T to A change at position -193 of the NF1 binding site. A point mutation at position -196 within NF1 has been shown to impair transcription in a cell-type specific manner (Plumb *et al.*, 1991). Tissues containing low levels of NF1 protein, such as FeLV-positive feline T LSA cell lines, are less affected by NF1-binding site mutations. NF1-binding sites may be involved in responses to growth regulators and loss of this activity could decrease responses to normal growth and differentiation regulating factors (Plumb *et al.*, 1991). In our study, although the previously described mutation (Plumb *et al.*, 1991) was not identified, mutations in the NF1 motif were seen in both neoplastic and non-neoplastic FeLV-related diseases.

Previously the FLV-1 site was reported to be highly conserved in FeLVs (Fulton *et al.*, 1990), however, about 1/4 of all mutations in our study, were within this site. There is controversy over whether the FLV-1 site is a negative regulatory element (Fulton *et al.*, 1990), or part of a transcriptional enhancer (Athas *et al.*, 1995). The FLV-1 site may represent a weak NF1 binding motif (Fulton *et al.*, 1990), so that mutation of this region may alter transcription in a cell-specific manner similar to the traditional NF1 binding site.

Of the 5 most frequent mutations noted in this study, 2 were common in the FeLV LTRs sequenced from 5 LSAs and 1 myeloblastic leukemia (Matsumoto *et al.*, 1992). One mutation was at position -170, between the GRE and FLV-1 sites, and the other was at position -135, within the FLV-1 site. These 6 tumors contained an additional A at position -232 (Matsumoto *et al.*, 1992), whereas the T at position -232 was mutated to A in 13 of our 33 sequences. Whether these changes are important in disease development is not known.

In this study there was an apparent lower mutation rate in FeLV U3 regions from LSAs, particularly the antigen-negative LSAs, relative to FeLVs from other neoplastic and non-neoplastic diseases. Also with phylogenetic analysis, 4 of 5 antigen-negative LSAs formed 1 subgroup indicating close sequence homology among the 4 U3 regions. Perhaps oncogene transduction or insertional

activation is an early event in LSA, and tumorigenesis occurs before viral LTR mutations can accumulate. However, antigen-negative LSAs often occur in older cats (Jackson *et al.*, 1993 [Section 4]). Since FeLV infection peaks at 3-5 years of age in outdoor cat populations (Pedersen, 1990), these cats are probably infected as younger adults. Immunologic responses in cats that develop antigen-negative LSAs may suppress viral replication soon after infection and therefore decrease the likelihood of viral mutation. Whether these cats harbour latent replication-competent or replication defective FeLVs in not known. Whereas LTR mutations may be important in induction of FeLV-related non-LSA disease, the non- or minimally-mutated single unit FeLV U3 may be sufficient to induce LSA, particularly multicentric LSA, perhaps through altered expression of transduced or insertionally activated host oncogenes.

In this study we examined the putative FeLV enhancer region amplified from tissues of 33 cats with FeLV-related neoplastic and non-neoplastic disease. No duplicated enhancer regions were identified, indicating that FeLV-related diseases developed from viruses containing only the single enhancer unit in these 33 cats. Point mutations were present at 102 sites within the amplified U3 region, but 68 of these were accounted for by changes at 5 different sites. Two of these mutations were previously reported in FeLV LTRs derived from several tumors (Matsumoto *et al.*, 1992). Almost all mutations excluded

the LVb and CORE binding sites, however the NF1 and FLV-1 motifs contained 37 of the 102 changes. The significance of the point mutations may be discovered through *in vitro* studies of mutated LTR-driven transcription, and *in vivo* experimental infections using mutated LTR-containing viruses.

7.6. References

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8. SUMMARY AND CONCLUSIONS

The primary objective of this work was to apply the PCR technique for FeLV detection in both fresh and formalin-fixed, paraffin-embedded tissues of cats. The PCR adds a new dimension to FeLV diagnosis. Previously FeLV infection was diagnosed by antigen detection either in cells by IFA or in serum by ELISA. Virus isolation, although technically difficult and impractical for routine diagnosis, has been the traditional standard to which other diagnostic tests have been compared. However, virus isolation requires the presence of replication-competent virus. Results of these studies suggest that non-replicating (latent or replication-defective) FeLV provirus can be associated with LSA in the cat. Although latent infection may be identified using virus culturing techniques involving corticosteroid treatment, these manipulations do not detect replication-defective viruses.

Results of this work also support earlier epidemiologic evidence that non-producer FeLV may be associated with LSA in cats. The majority of antigen-negative, provirus-positive LSAs in this study were from older cats. One explanation for this finding is that older cats may have mounted an early immune response, sufficient to eliminate replicating virus but insufficient to

eliminate quiescent or defective virus which persists within their genome. In fact, immune responses may inadvertently select for non-replicating forms of the virus which through a multi-step process and over considerable time, induce neoplasia.

All LSAs but particularly antigen-negative tumors contained FeLV enhancer regions which were most similar to the common form of FeLV. This supports speculation that virus replication in antigen-negative LSA is suppressed early in infection before considerable mutational events occur. Also, lack of viral gene expression is unlikely to be related to enhancer sequences in antigen-negative tumors since these sequences were most similar to the enhancer of replication-competent, common form FeLV.

One might then expect antigen-positive LSAs to contain highly mutated LTRs if mutational events are directly proportional to rate of viral replication. However, these LTR enhancers may have been minimally mutated because LSA developed rapidly in this group of cats. Therefore, on one hand enhancers may not be mutated in old cats with long-standing infection, because the virus is not replicating (latent or defective), whereas antigen-positive tumors may develop rapidly leaving little opportunity for mutation of replication-competent FeLVs before death of the host cat occurs.

Results of the prospective study comparing the prevalence of FeLV DNA to antigen in peripheral blood from

cats with varying suspicion of FeLV-related disease, were initially surprising. A proportion of cats with high suspicion of FeLV-related disease based on clinical and hematologic findings, are negative for FeLV antigen. Detection of FeLV by PCR was predicted in these cases since PCR is a very sensitive test and detects DNA rather than antigen. The lack of difference between the two diagnostic tests indicates that even cats with strong clinicopathologic evidence of FeLV-related disease, may not be infected with the virus.

Forty-six percent of cytopenic cats were test-positive (PCR and ELISA-positive), and 87% of cats with hemopoietic tumors were test-positive. There were few older cats with LSA in the tumor group, however the 2 test-negative cats with hemopoietic tumors were >8 yrs and had solid tissue LSA without leukemia. However, as shown in the first study, direct evaluation of tumor tissue from these older cats may have revealed FeLV DNA.

In cytopenic cats, the possibility exists that virus was harboured in tissues other than peripheral blood cells, or that infection was transient, undetectable at the time of sampling and recovery occurred. Testing multiple sample sources and time points would be useful to investigate these possibilities. In any case, FeLV test-negative (ELISA and PCR-negative) cytopenic cats had better 1 yr survival rates suggesting that regardless of clinicopathologic findings, FeLV test result on peripheral

blood sampling has prognostic significance.

Immunohistochemical identification of B and T cell phenotypes in feline LSAs showed 67% of tumors were T cell and 27% were B cell. B cell tumors were FeLV positive as often as T cell tumors using either IHC or PCR. However, consistent with earlier results, tumors from older cats were positive for FeLV DNA more often than antigen.

Earlier reports that B cell tumors and/or intestinal tumors are FeLV negative, probably relate to the use of B cell markers which detected only mature B cells, and the fact that older cats which may have proportionately more B and/or intestinal LSAs, are often FeLV antigen-negative but provirus-positive.

This work clearly supports a role for FeLV in B cell oncogenesis. This is reasonable since the virus replicates in B cells and local oncogene activation resulting from viral replication and integration, is at least one mechanism of FeLV oncogenesis. It would be logical that the same cells that are infected with FeLV could also become neoplastic as a result of their infection.

The final study involved sequencing and analysis of a portion of the FeLV LTR containing the putative enhancer region. In contrast to previous reports, no enhancer repeats were identified in this work. Compiled data from previous studies indicate repeats in 22 of 35 FeLV LTRs investigated. However, enhancer repeats were determined by nucleotide sequencing in only 14 of these and predicted

in others based on sizes of PCR products. Perhaps enhancer repeats are not as prevalent as previously thought or they may be prevalent only in mediastinal (thymic) tumors.

If duplicated structures were undetected in this work because they were in low proportions relative to the single enhancer, then their importance may be questionable. If FeLV enhancer repeats play a role in development of mediastinal but not LSAs from other sites or non-lymphoid hemopoietic tumors, then other factors must be involved in tumors of other phenotypes.

Research involving FeLV-related neoplasia has focused on mediastinal LSAs, yet multicentric LSAs are most common, and hemopoietic tumors of several cell types also occur. Multiple factors are likely to contribute to oncogenesis including oncogenes, tumor suppressor genes, viral gene products, LTR sequences, and host cell receptors for FeLV. It will be interesting to study FeLV-induced B cell tumors and non-lymphoid hemopoietic tumors to determine if oncogene activation and transduction occur and if so, if the family of oncogenes differs from that in mediastinal LSAs.

Sequence variation within the FeLV enhancer region does not appear to correlate with disease manifestation with the possible exception of antigen-negative LSAs which showed minimal deviation from the common form virus. However, point mutations of 5 nucleotides were particularly prevalent and represented almost 70% of the total mutations

in this study. Further research needs to be done to determine if enhancer activity is affected by these mutations and if so, if alterations are cell-type specific.

Little is known about cellular receptors for FeLV. This information may be critical to elucidating the manner in which FeLV causes such a diversity of neoplastic and non-neoplastic diseases. FeLV-related diseases receiving the most attention have been mediastinal LSA, FeLV-C/Sarma-induced anemia, and immunodeficiency due to FeLV-FAIDS. Woefully lacking are studies of myeloid and erythroid tumors, non-mediastinal LSAs, megaloblastic anemias, and other cytopenias - conditions which are at least equally important in infected cats.

The FeLV was first identified in 1964 and despite more than 30 years of research, there is still much to be learned about the virus. The basic biology and epidemiology of FeLV are well documented, and study now is focused at the molecular level. The diversity of diseases caused by this single virus is fascinating. Genomic variance resulting from *in vivo* recombinational or mutational events, and host factors including receptor sites, proviral integration sites, and immune responses, are likely to affect the outcome of FeLV infection in individual cats. All aspects of FeLV disease pathogenesis need to adequately investigated, not only those with obvious application to human health. Ideally, FeLV research will be driven by its relevance to feline health

as it remains the scourge of pet cats.