ENDOGENOUS FELINE LEUKAEMIA VIRUSES:

THEIR ROLE IN LEUKAEMIA AND RESISTANCE TO INFECTION

ANN STEPHANIE McDOUGALL B.Sc.

A thesis submitted for the degree of

Doctor of Philosophy

in the University of Glasgow

The Beatson Institute for Cancer Research, Glasgow.

April, 1992

For Mum and Dad

CONTENTS Page.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr James Neil who, over the past three years, has been encouraging, critical, enthusiastic (and infuriating!), all to my benefit. Thanks must also go to the members of R8, Dr Ruth Fulton, Dr Monica Stewart, Mrs Anne Terry and Mr Robert McFarlane, for patiently passing on their skills in the laboratory, for stimulating discussions and some good advice. I am also grateful to Robert McFarlane for the photography contained within this thesis. I am grateful to many of the staff and students of the Beatson Institute, both past and present, who have helped make my time here both enjoyable and profitable. In particular, I thank Dr Ken Smith and Dr Brad Ozanne for their interest in this project, and for helpful discussions and advice. I would also like to thank Dr Mark Rigby, Dr Norman Spibey and Dr George Reid of the MRC Retrovirus Laboratory, University of Glasgow, for their help in the production of GST fusion proteins, and Professor Jennifer Rojko, Dept. of Veterinary Pathobiology, Ohio State University, who has taken an active interest in this work, and shared the results of her work on cell resistance with us. I am financially indebted to the Medical Research Council AIDS Directed Programme and the Cancer Research Campaign for funding this research and a scientifically rewarding trip to a meeting in the United States.

I would like to take this opportunity to thank my Mum and Dad for all the love and constant support that they have given me in the many years since I embarked on my "education", and my fiance, Roddy, who always believed I could succeed even when I did not, and gave me strength and faith to carry on.

Declaration

The work contained in this thesis was performed by myself with the following exceptions; the expression of subgenomic transcripts in 3201B cells (Chapter 4) by Robert McFarlane, the initial immune-precipitation studies (Chapter 4) by Anne Terry and the computer densitometry analysis (Chapter 3) by Lynn McGarry.

ABBREVIATIONS

A	adenine
ALV	avian leukosis virus
amp	ampicillin
BUdR	5-bromo-2' deoxyuridine
bp	base pairs
BPB	bromophenol blue
BSA	bovine serum albumin
C	cytosine
CA	capsid protein
cDNA	complementary DNA
chf	chick helper factor
Ci	curies
CIAP	calf intestinal alkaline phosphatase
cpm	counts per minute
cys	cysteine
dH_20/ ddH_2O	distilled/ double distilled water
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme linked immune-sorbent assay
enFeLV	endogenous FeLV
EPO-R	erythropoetin receptor
ER	endoplasmic reticulum
ERV	endogenous retrovirus
FeLV	feline leukaemia virus
fig.	figure
G	guanine
g	relative centrifugal force

GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
gp	glycoprotein
GST	glutathione-S-transferase
HIV	human immunodeficiency virus
HTLV	human T-cell leukaemia virus
IL-2-R	interleukin-2 receptor
IN	integrase
IPTG	isopropyl β-D-thiogalactopyranoside
λ	lambda
LCMV	lymphocytic choriomeningitis virus
LTR	long terminal repeat
kb	kilobase
kD	kilodalton
M	molar
mA	milliamps
MA	matrix protein
MCF	mink cell focus forming
met	methionine
mins	minutes
mmol	millimolar
MOPS	sodium morpholinopropane sulphonic acid
mRNA	messenger RNA
MuLV	murine leukaemia virus
NC	nucleocapsid protein
nt	nucleotide
o/n	overnight
oligo	oligonucleotide
orf	open reading frame
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
pbs	primer binding site
PCR	polymerase chain reaction
PEG	polyethylene glycol
pers.commun.	personal communication
PMSF	phenylmethylsulphonyl fluoride
ppt	polypurine tract
PR	protease
RAV	Rous-associated Virus
RNA	ribonucleic acid
RNase	ribonuclease
RSV	Rous sarcoma virus
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SFFV	spleen focus-forming virus
SU	surface envelope protein
T	thymine
TM	transmembrane envelope protein
UV	ultra violet light
V	volts
Vr	variable region
v/v	volume to volume
w/v	weight to volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

Fig.5a Oligonucleotide sequencing primers

LIST OF FIGURES

SUMMARY

This investigation was designed to characterise the expression of enFeLV *in vitro* and *in vivo* at both RNA and protein levels in feline cells and tissues because of the potential importance of endogenous virus expression in mediating resistance to infection and participating in genetic recombination. A new enFeLV specific probe was generated which enabled the examination of endogenous expression on a background of exogenous virus expression. EnFeLV expression was shown to be more widespread than had been previously been reported. The expressed transcripts contained an *env* open reading frame which was highly conserved in length and sequence content. Sequence analysis of these highly expressed enFeLV *env* genes suggested that they are not the primary source of recombinant viruses, although a novel recombinant virus, FeLV-B/GM1, may be an exception to this rule. The heterogeneity and conservation of individual loci were examined, and it was established that the expressed loci showed no more genetic polymorphism than their apparently non-expressed relatives. A C-terminal fragment of the enFeLV *env*-orf was expressed in bacteria and the purified protein product used to generate an enFeLV *env*-specific polyclonal antiserum. This serum detected a candidate protein product from enFeLV loci, and the expression of this protein was found to correlate with resistance to FeLV-B infection in feline cells. These studies provide new insights into the significance of enFeLV expression in resistance to infection and have major implications on the immune responses to enFeLV and exogenous FeLV.

SECTION 1 THE RETROVIRAL FAMILY.

1.1 Introduction.

Retroviruses are a highly specialised group of viruses whose members are closely related in genetic organisation, virion structure, and mode of replication. Viruses within this group display an exceptional diversity of biological effects on their hosts and have evolved a large variety of distinct lifestyles ranging from apparently benign transposable elements co-existing in the host germ line over the millenia, to horizontally infectious viruses causing the death of the host via the induction of neoplastic, lytic or immunological disease. The retroviruses are classified by a number of criteria, reviewed by Teich (1982, 1985). The taxonomic family Retroviridae is divided into three major sub-families based on the consequences of infection by well characterised representatives of each group. The Oncovirinae (oncoviruses) usually cause malignancies but some have no known pathogenicity. The Lentivirinae (lentiviruses) are "slow" viruses that induce various diseases (including Acquired Immune Deficiency Syndrome- AIDS), and the Spumavirinae (spumaviruses) cause vacuolation of cells in tissue culture but no apparent disease *in vivo*. Retroviruses are also classified according to morphology and subcellular location as viewed by electron microscopy. Most oncoviruses fall into the type-C virus group, which do not form intracytoplasmic core particles, and have mature virions with a dense, centrally located core. Of the exceptions, mouse mammary tumour virus (MMTV) is a type-B virus which can be distinguished by the doughnut shaped cores of budding viruses, the dense, eccentrically located cores of the mature particles, and the prominent envelope spikes on the virion surface. Some primate oncoviruses are type-D viruses, which are found as both intracellular ring shaped particles and mature, extracellular particles which have a core structure similar to the type-B viruses but without the prominent surface spikes. Type-A particles are only found intracellularly. The type-C oncoviruses, of which this study is concerned, are further classified according to whether they are passed on in the

germ line of vertebrate species (endogenous) or propagate by infectious virus spread (exogenous). (Coffin, 1982, Stoye & Coffin, 1985)

1.2. Type-C avian and mammalian oncoviruses.

Although the individual families designated as type-C oncoviruses are not closely related to one another, they are all quite similar in virion structure, genomic organisation and mode of replication. Most of the structural and functional data concerning both exogenous and endogenous viruses come from studies of the avian leukosis-sarcoma viruses and murine leukaemia viruses. Therefore at this stage, these viruses will be briefly described and feline leukaemia virus, with which this study is concerned, will be introduced.

1.2.1. The avian leukosis-sarcoma virus group.

This group consists of exogenous and closely related endogenous viruses of birds. The sarcoma viruses are modified by the presence of cellular oncogenes (such as *src* in Rous sarcoma virus), all except RSV are replication defective, requiring a "helper" virus for viral spread. They are further subdivided according to their cellular receptor utilisation (host range) into at least seven subgroups denoted A to G. The first four are characteristic of exogenous viruses of chickens. Subgroup E viruses are endogenous in chickens, and subgroups F and G are endogenous viruses in pheasants. The avian leukosis viruses (ALV) or Rous associated viruses (RAV) do not contain cellular oncogenes, and typically cause B-cell lymphomas and leukaemias. Other diseases associated with individual strains of ALV include erythroid or myeloid leukaemia, osteoporosis and fibrosarcomas. (Teich, 1982)

1.2.2. The murine leukaemia-sarcoma virus group.

The murine leukaemia and sarcoma viruses (MuLV and MSV) are a complex group of viruses found in both inbred and feral mice. Like the avian sarcoma viruses, murine sarcoma viruses (MSV) are replication defective, because

a portion of the viral genome has been substituted for cellular oncogene sequences. (Teich, 1982)

These viruses are further subdivided according to the species distribution of their specific receptors. (Coffin, 1990) Ecotropic viruses replicate only in mouse cells, xenotropic viruses replicate in many species except mice, and polytropic and amphotropic viruses replicate in many species, including mice. Ecotropic viruses can be found both as exogenous and endogenous viruses, xenotropic and polytropic viruses are endogenous, and amphotropic viruses are exogenous. (Kozak *et al.*1989) MuLVs are associated with the induction of a number of diseases including T-cell lymphomas. An interesting variant of MuLV is the Friend virus complex which is associated with the induction of erythroleukaemia. There are two viruses in this complex; a replication competent, ecotropic helper virus, Friend-MuLV, and a defective virus, spleen focus forming virus (SFFV). The Friend-MuLV is only acutely pathogenic in newborn mice, but SFFV is acutely pathogenic in adult mice. The LTR of Friend-MuLV encodes a strong determinant for erythroleukemogenesis in young mice presumably by promoting virus replication in erythroid cells. (Chatis *et al.*1984) The erythroleukemic ability of SFFV has been linked to its truncated recombinant envelope protein. (Berger *et al.*1985, Linemeyer *et al.*1981, Li *et al.*1986, Li *et al.*1987) The pathogenicity of many MuLV infections are further complicated by the frequent recombinations with endogenous viruses that occur to create the highly pathogenic MCF viruses. (See Section 4.2.3.)

1.2.3. Feline leukaemia virus.

Feline leukaemia virus (FeLV) is an important pathogen of the domestic cat, and is probably the major non-traumatic cause of death in this species. FeLV was first identified by Jarrett *et al.* (1964a, 1964b). Leukaemias, lymphomas and other tumours of mesodermal origin that are caused directly by FeLV account for more than a third of cancer deaths among cats. In addition, persistent infection establishes an immune-suppressed state that predisposes the animal to lethal infections by a variety of pathogens.

FeLV biology, and the molecular aspects of pathogenesis have been reviewed by Jarrett (1984) and Neil and Onions (1985). FeLV induced leukaemias are commonly T-cell in origin, although isolates from other tumour types, including a myeloid tumour, have been characterised. (Tzavaras *et al*, 1990) The mechanism of leukaemia induction by FeLV has been reviewed by Neil (1984) and Neil & Forrest, (1987). Capture of the *myc* oncogene is commonly found in cases of T-cell lymphoma, (Mullins *et al.*1984, Neil *et al.*1984, Levy *et al.*1984) and in feline fibrosarcomas, a variety of cellular oncogenes have been transduced. (Besmer, 1983)

There are three subgroups of FeLV which are distinguished by their host range and interference properties. (Sarma & Log, 1973, Sarma *et al.*1975) FeLV-A can be classed as an ecotropic virus having a host range that is restricted to replication in feline cells. FeLV-B can infect feline, human and canine cells, while FeLV-C can infect feline, human and guinea-pig cells and so both may be described as amphotropic. The frequency of occurrence of each subgroup is unusual in that FeLV-A is present in every natural isolate, with FeLV-B also present in 40-60% and FeLV-C in 1% of natural isolates. (Jarrett *et al.*1978a, Jarrett *et al.*1978b) This restriction is thought not to involve a genetic defect in these viruses, because each has been shown to replicate successfully on their own, in vitro. (Sarma & Log, 1971) There is an age related restriction on the replication of FeLV in cats. (Jarrett & Russell, 1978, Jarrett *et al.*1978b) Experimental inoculation of FeLV-A into neonatal cats results in a 100% induction of persistent viremia. Older cats have an increased ability to clear the virus from their system. By contrast, inoculation with FeLV-B results in a low (15-20%) rate of establishment of viraemia, even in neonatal cats, and no establishment of infection in weaned cats. In cats infected with both A and B, initially only FeLV-A can be detected and only a proportion of cats will later produce detectable FeLV-B.

1.3. Genomic organisation.

All retroviruses share the same basic genome structure. (Coffin, 1990) Figure 1 shows the structure of the RNA genome of FeLV and the viral products it

encodes. (Throughout this thesis, the nomenclature suggested by Leis *et al* (1988), will be used.

Short terminal redundancies (R) of 10-100nt flank the unique region of the RNA genome. At the 5' and 3' ends of the genome are unique sequences (U5 and U3) which, during reverse transcription, generate the long terminal repeats (LTRs). Within the U3 region are the promoters and enhancers which direct viral transcription. Between these sequences, from 5' to 3' are, a packaging signal required for the efficient incorporation of the RNA genome into the virion, and the three essential retroviral genes, *gag*, *pol* and *env*. Although the "complex" retroviruses, such as HIV and HTLV, have other open reading frames encoding regulatory proteins (Varmus, 1988) these have not been found in the basic type-C oncoviruses and so will not be considered here.

The *gag* and *pol* genes appear to be translated from RNA that is identical to the genomic virion RNA, whilst the *env* gene products are translated from a subgenomic spliced envelope mRNA. In MuLV and FeLV, the splice donor site is positioned upstream of the packaging signal and consequently, the spliced *env* message does not have the packaging signal, preventing its incorporation into virions. The *gag* gene, so named because the proteins encoded by it were first identified as group specific antigens, is translated to produce a precursor polyprotein that is subsequently cleaved to yield three to five capsid proteins (depending on the virus). The three invariant proteins are; the matrix (MA) protein, the capsid (CA) protein and the nucleic-acid binding nucleocapsid (NC) protein. Avian and mammalian C-type viruses have an additional cleavage product, translated from a region between the MA and CA coding regions (p10 in ALV and p12 in both MuLV and FeLV). This protein is found in the virion but as yet no function has been identified.

The *pol* gene of MuLV and FeLV encodes three proteins with important enzyme activities. Reverse transcriptase (RT) and integrase (IN) are responsible for the

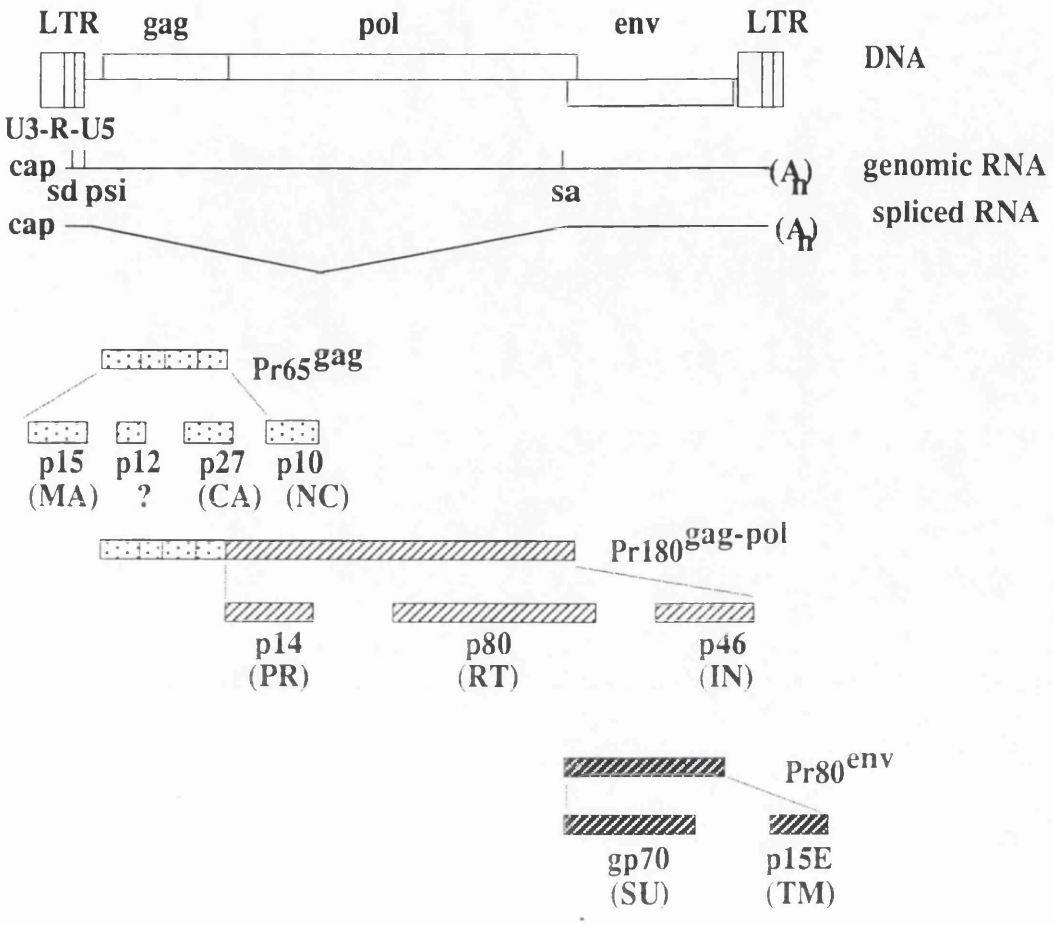


Fig.1. The genome of feline leukemia virus and its protein products.

This figure depicts the proviral DNA, the genomic RNA from which the DNA is transcribed, and the spliced mRNA which is the second transcriptional product of the provirus. The sites of functional signals are also shown. (**sd** splice donor, **sa** splice acceptor, **psi** packaging signal) Both the polyprotein precursors and final protein products translated from the two RNA species are shown below.

synthesis of viral DNA and its subsequent integration into cellular DNA. The protease (PR) is responsible for the cleavage of *gag* and *pol* polyproteins (and found at the 3' end of *gag* in ALV). The translation of the *pol* gene products occurs by a slip in the translation machinery, misreading the termination codon at the end of *gag*, and enabling translation to continue through the *pol* reading frame.

The *env* gene encodes the two envelope proteins that are themselves cleaved from a large precursor. The surface (SU) glycoprotein is responsible for recognition of cell surface receptors, and the transmembrane (TM) protein anchors the complex to the virion envelope.

1.4. The retroviral life cycle.

The unique retroviral life cycle is accomplished by three different enzyme systems, each responsible for a different phase. The first phase involves the synthesis of double stranded viral DNA and integration into the host genome and is accomplished by virus-coded enzymes carried in the infecting virion core. The second phase, replication of the integrated DNA during normal cell division, uses the usual cellular DNA polymerases. The third phase is the synthesis of progeny genomes, which depends both on the cells transcription machinery and virally encoded enzymes. The following synopsis is based on a number of reviews covering various aspects of the retroviral life cycle, including Coffin, (1990), Katz and Skalka (1990) and Hunter & Swanstrom, (1990).

1.4.1. Infection.

Retroviruses are composed of a ribonucleoprotein core surrounded by a lipid membrane which is derived from the host cell and is embedded with viral envelope proteins. The first stage of the retroviral life cycle initiates with virus infection, or entry of the virion core into the host cell. The SU protein on the surface of the virion is responsible for the recognition and binding of the virus to a specific host cellular receptor. (Hunter & Swanstrom, 1990) The viral membrane then fuses with the cellular membrane in a process that is not fully understood, but that appears to

involve exposure of a fusion domain on the TM protein. (White *et al.*1983, Gallaher, 1987) The virion core which enters the cell contains two identical, non-covalently linked RNA genomes, a number of virally encoded enzymes required for both production of the viral DNA and its integration into the host cell chromosomes, and a cellular tRNA which acts as the initial primer for reverse transcriptase. As the core disassembles, transcription of the RNA genome into a DNA form commences.

1.4.2. Synthesis of viral DNA.

The process of reverse transcription as it is currently understood is represented in Figure 2. (Hu & Temin, 1990b) 1. The tRNA binds to the primer binding site located near the 5' end of the genome, and is used as a primer for the synthesis of minus strand DNA. 2. Reverse transcription first copies U5 and R sequences forming minus strand strong stop DNA. 3. A ribonuclease (RNase) H activity of RT degrades the U5 and R of the template RNA exposing the now single stranded minus strand strong stop DNA. 4. This DNA region now transfers to the 3' end of the genome, presumably through interaction with the complementary R sequences also present at this end of the genome. 5. At this point, the enzyme jumps from one template to another and transcription of the minus strand continues. 6. RNase H makes a specific nick just 5' of the U3 sequences, at the polypurine tract (ppt). The nicked viral RNA acts as a primer for plus strand synthesis. 7. The minus strand U3-R-U5 DNA (the long terminal repeat-LTR), as well as a portion of the tRNA that is homologous to the pbs, is copied and forms the plus strand strong stop DNA. 9. The plus strand strong stop DNA transfers to the almost completed minus strand DNA, presumably through complementation of the R-U5-PBS sequences. DNA synthesis of the plus strand DNA involves a second "jump" for the RT enzyme. The second strand synthesis is sometimes discontinuous. Internally RNase H nicked viral RNA can act as transcription primers for RT; this discontinuous synthesis leaves gaps that are filled in by either displacement of the previously synthesised fragments by a 5' growing DNA strand or by ligation of the existing products. 10. DNA synthesis of both minus and plus strands proceeds to form a complete copy of

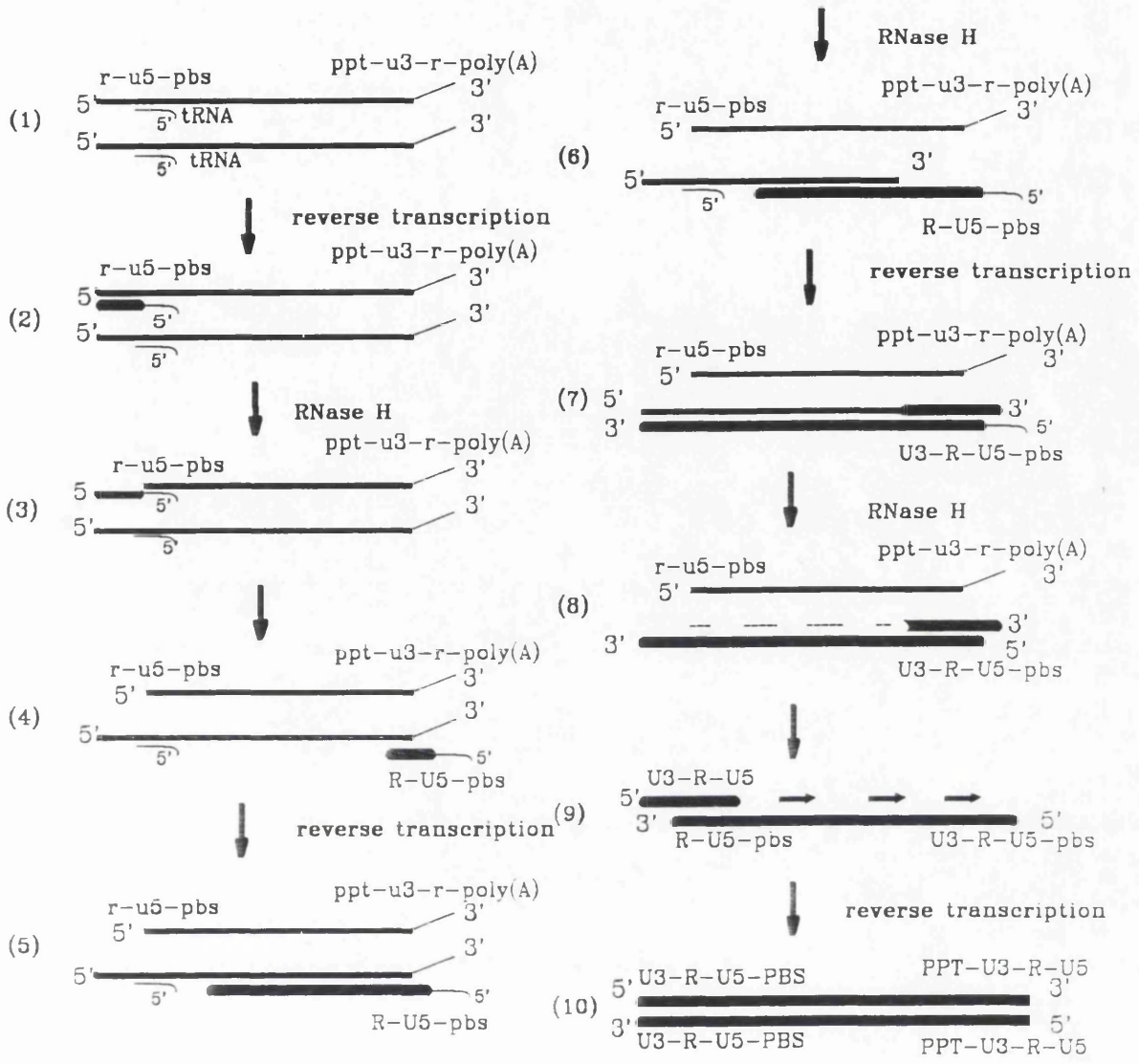


Fig.2. Reverse transcription of viral RNA into DNA by reverse transcriptase.

Represented above is the accepted rational for the process of reverse transcription. 1. The binding of primer tRNA. 2. Initiation of reverse transcription (minus strand synthesis). 3. Degradation of RNA base paired to DNA. 4. Translocation of primer and jump of RT from one template to another. 5. Continuation of reverse transcription. 6. Degradation of RNA base paired to DNA to form primer for plus strand synthesis. 7. Initiation of plus strand synthesis. 8. Degradation of RNA base paired to DNA to leave small primers for further transcription initiation. 9. Translocation of primer and jump of RT from one end of the template to the other. 10. Continuation of plus strand synthesis from both this primer and intermediate ones to produce double stranded DNA provirus.

the retroviral RNA genome flanked by two LTRs, the products of the reduplication of the U5 and U3 regions during the procedure. The ability of reverse transcriptase to transfer from one template to the next is one possible explanation for the frequent generation of retroviral recombinants.

1.4.3. Integration.

The viral DNA is integrated into the genome of the host under the control of the viral integrase enzyme (IN) and the core proteins, whose involvement is necessary but whose functions are as yet unknown. Retroviral integration shows no strict sequence requirement and can occur at many sites, although there have been reports of preferred sites (Shih *et al.*1988) and a general preference for regions of open chromatin. (Vijaya *et al.*1986, Rohdewohlde *et al.*1987) The integration of the DNA provirus, as it is now referred to, can be considered a mutagenic event of potential importance to the host. Integration of proviruses can lead both to the activation of neighbouring proto-oncogenes by the strong promoter and enhancer elements contained within the viral LTR, (Bishop & Varmus, 1985) and the inactivation of cellular genes by integration within a gene coding sequence, or in an intron, disrupting normal transcription.

1.4.4. Synthesis of progeny virions.

After integration, proviral transcription is accomplished by cellular enzymes. (Varmus & Swanstrom, 1982, 1985) The provirus is transcribed as a single species that initiates in the 5' LTR and undergoes a polyadenylation at the 3' end of the genome. A portion of this RNA is spliced to form the subgenomic *env* mRNA. The full length RNA functions both as the mRNA for *gag* and *pol* products, which are translated on membrane free ribosomes, and as the genomic RNA to be packaged into the new virions. The *env* mRNA is translated on membrane bound ribosomes and is co-translationally translocated into the endoplasmic reticulum (ER) via an N-terminal signal peptide. A hydrophobic stretch in the C-terminal half of TM anchors the polyprotein in the membrane. The *gag-pol* viral polyproteins are cleaved into

individual proteins by PR, and the *env* polyproteins by cellular proteases within the ER. The *env* proteins remain associated after cleavage and during transport to the plasma membrane. Studies on Rous sarcoma virus suggest that the envelope proteins associate as trimers and that this oligomerisation is necessary for translocation to the cell membrane. (Einfeld & Hunter, 1988) The envelope proteins of HIV are thought to associate as dimers or tetramers. (Thomas *et al.*1991) The SU protein (and in some viruses, TM) is heavily glycosylated on its transfer from the ER to the cell membrane via the Golgi. The *env* gene products reach the outer cell membrane, the TM protein anchoring the extracellular SU protein to the cell/virion surface. In the cytoplasm, the virion core assembles and buds out from the cell, surrounded by host cell membrane which is embedded with the envelope proteins. And so the cycle begins again.

SECTION 2. FACTORS INVOLVED IN THE HOST RANGE SPECIFICITY OF ONCOVIRUSES.

2.1. The surface envelope protein.

The ability to bind to cellular receptors is a major determinant of the host range of a virus and is a function of the SU protein of the *env* gene.

2.1.1. Sequence requirements for receptor recognition.

Comparisons of the *env* gene sequences encoding SU of related avian and mammalian type-C viruses which display different host ranges show that there are clustered amino acid changes (variable regions) within SU that are implicated in the recognition of different receptors. The construction of viruses with chimeric envelope genes has been particularly useful in localising the regions determining receptor specificity. (Dorner & Coffin, 1986, Battini *et al.*1992)

The avian leukosis viruses of chickens can be divided into 5 host range subgroups, A-E. The domain of the *env* gene product responsible for subgroup specificity has been mapped to the middle third of SU. Sequence analysis of three of

the subgroups, B, C and E, identified two major variable regions of low sequence homology (hr1 and hr2) and three minor ones (vr1 and vr2, displaying subgroup B variation and vr3). (Dorner *et al.*1985, Dorner & Coffin, 1986, Bova *et al.*1988) Construction of viruses with recombinant SU molecules containing different combinations of these variable regions showed that receptor binding specificity was determined by the two major regions, hr1 and hr2, and one minor region, vr3. These regions are presumably on the surface of the folded SU protein and interact directly with the cell receptor binding sites.

The SU proteins encoded by MuLVs and generally by all mammalian type-C retroviruses are composed of three structurally distinct domains. (Battini *et al.*1992) The N-terminal two thirds of the SU domain of MuLV contains the greatest sequence variation, consisting of 200-230 amino terminal residues with important sequence variations among viruses of different subgroups, and a proline-rich region of 50-60 amino acids. These regions presumably carry the determinants of specific receptor interaction. The last third of SU and the whole of TM are much more highly conserved. (Stoye & Coffin, 1987) The N-terminal regions of ecotropic viral envelopes are most dissimilar from the other MuLVs, with only about 40% sequence homology with xeno- or polytropic viruses in this region. This dissimilarity is emphasised by the presence of several insertions of 10-34 amino acids in the ecotropic *env* gene compared to other viruses. The xeno- and polytropic viruses are much more similar, with greater than 80% sequence homology.

The SU coding sequence of the three different subgroups of FeLV have been shown to display a number of variable regions relative to endogenous SU sequences, which have been designated variable regions (Vr) I-VII. (Kumar *et al.*1989) Figure 3 shows the amino acid sequences of the gp70 SU of different isolates of FeLV-A, B and endogenous FeLV (enFeLV), compared to FeLV-A/Glasgow1. A number of insertions and deletions in the sequences of the subgroup B viruses had to be made in order to get the best alignment of sequences. The variable regions are underlined. This comparison has identified a further variable region, which has been designated Vr Ia. The proline rich region is located just upstream of Vr V. There

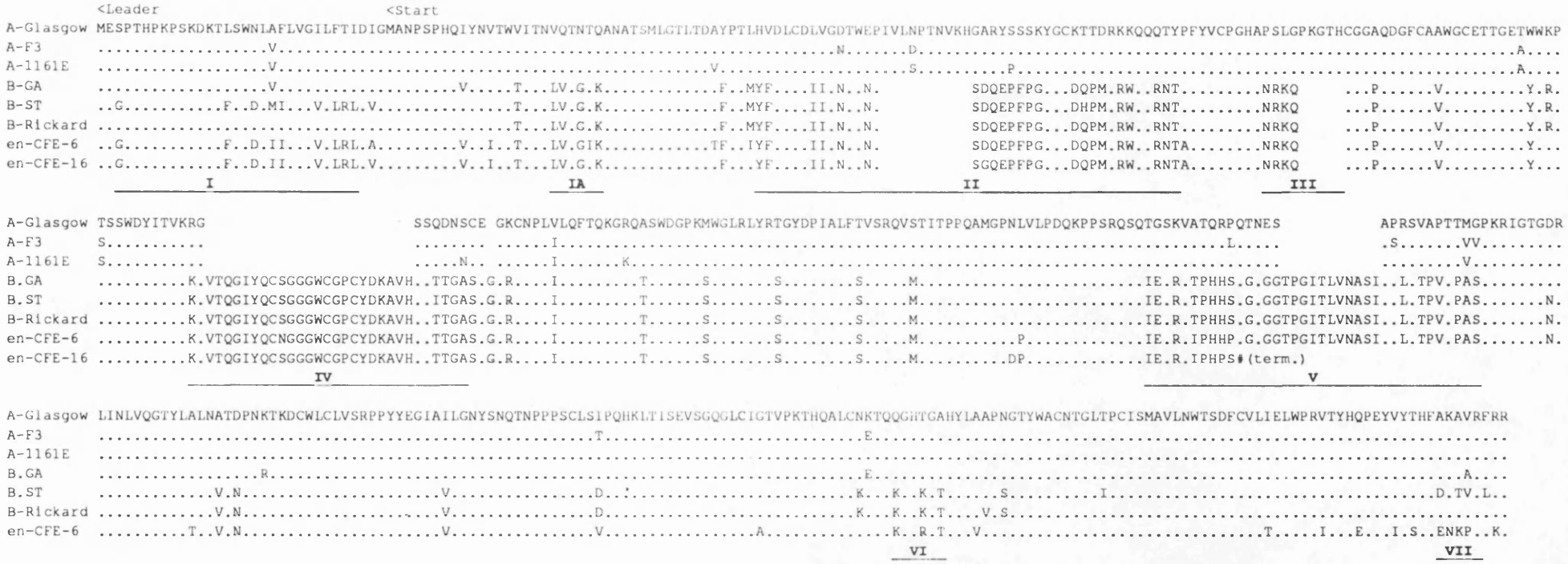


Fig.3. Alignment of the SU amino acid sequences of FeLV-A, FeLV-B and enFeLV.

The figure compares the amino acid homologies between different isolates of FeLV compared to the sequence of FeLV-A/Glasgow1. Dots represent sequence identity, spaces represent gaps inserted to maintain the homology. The regions of variation between endogenous sequences and FeLV-A are underlined and designated Vr.I to VII. The termination of the open reading frame of *env* in enFeLV CFE-16 is marked.(#) The sources of the sequence data are as follows; FeLV-A/Glasgow (Stewart *et al.*1986), FeLV-A/F3 and FeLV-A/1161E (Donahue *et al.*1988), FeLV-B/GA (Elder & Mullins, 1983,Nunberg *et al.*1984), FeLV-B/ST (Nunberg *et al.*1984), FeLV-B/Rickard (Elder & Mullins, 1985), en-CFE-6 and enCFE-16 (Kumar *et al.*1989).

are many amino acid changes in the sequence of FeLV-B SU relative to those of subgroup A SU. Those that are found in all FeLV-B SU sequences may be presumed to be responsible for the subgroup B specificity of these proteins.

Hybridisation studies of FeLV-B *env* sequences to feline DNA (Stewart *et al.*1986) and the sequence analysis of two endogenous FeLV *env* genes (Kumar *et al.*1989) have suggested that FeLV-B viruses are natural recombinants between FeLV-A and endogenous envelope sequences. This proposal has been supported by the production of FeLV-B-like viruses in feline cells that had been transfected with FeLV-A DNA. (Overbaugh *et al.*1988)

2.1.2. Viral interference.

Viruses can be shown to belong to the same or to different subgroups by their ability to overcome viral interference. Viral interference is established in productively infected cells presumably by the binding of newly synthesised SU to their specific cellular receptors. (Weiss,1982, Buller *et al.*1987,1989) Consequently, there is a significant reduction in free cellular receptors available for new infecting virus and as a result, virally infected cells cannot be reinfected by viruses of the same subgroup. Viral interference can be overcome by viruses of another subgroup, however, as they will use a different cellular receptor. Viral interference may benefit both the virus and host, as unrestricted viral entry and proviral integration would increase the chance of disruption or activation of important cellular genes, and lead to host cell death.

2.2. Cell receptors.

The host range of a virus depends both on the ability of the particular virus SU to bind to a specific cellular receptor and the distribution of that receptor. Therefore the identification and characterisation of these receptors is an important aspect of the study of retrovirus host range.

2.2.1. Avian virus receptors.

Genetic studies have shown the susceptibility of chicken cells to ALV is controlled by three genetic loci, *tv-a*, *tv-b* and *tv-c* (Weiss, 1982), and these are thought to encode receptors. *tv-a* controls susceptibility to subgroup A viruses and *tv-c* to subgroup C viruses. *tv-b* has a number of alleles and these are thought to control the susceptibility to infection by subgroups B, D and E, although it has been suggested that another locus, *tv-e* encodes the subgroup E receptor. (Pani, 1977). Subgroup B and D viruses can use the same receptors, but subgroup D viruses show only incomplete interference to B infection and, unlike subgroup B viruses, can infect mammalian cells. Subgroup E viruses also use the same receptors as B but are unable to interfere with B infection, presumably due to the subgroup E-SU having a decreased affinity for the receptor compared to subgroup-B viruses. (Weiss, 1982)

2.2.2. Murine virus receptors.

MuLVs with divergent host ranges and antigenicity, have also been shown to use different cellular receptors. The ecotropic receptor, *Rec-1*, mapped to chromosome 5, (Gazdar *et al.*1977, Kozak *et al.*1990) has been cloned and partially characterised. (Albritton *et al.*1989) Although the sequence of *Rec-1* has not been linked to the sequence of any known gene, there are a number of motifs typical of a transmembrane transport protein. Functional studies have suggested that *Rec-1* is a basic amino acid transporter. (Kim *et al.*1990, Wang *et al.*1991) The murine cell receptors for polytropic viruses, *Rmc-1* (Rein,1982, Kozak,1983) and amphotropic viruses, *Ram-1* (Gazdar *et al.*1977, Garcia *et al.*1991) have been mapped to chromosomes 1 and 8 respectively. Although xenotropic viruses cannot infect laboratory strains of mice, wild mice do possess a cell receptor, *Sxv-1*, which allows xenotropic virus infection. (Copeland *et al.*1983) As this genetic locus maps close to *Rmc-1* on chromosome 1, it suggests that an allele of *Rmc-1* may serve as a receptor for xenotropic viruses. It is likely that this locus has been lost during the inbreeding of laboratory mice.

2.2.3. Feline virus receptors.

The cellular receptors involved in the FeLV infection are less well characterised but it has been recently shown that FeLV-B can use the receptor for gibbon ape leukaemia virus in human cells. (Takeiuchi *et al.*1992) This human receptor shows between 31-38% homology to pho-4$^+$, a phosphate permease of *Neurospora crassa.* (Johann *et al.*1992) The receptor shows no significant homology to other known genes found in the GenBank or NBRF data bases. It has yet to be shown if this represents the FeLV-B receptor found in feline cells.

SECTION 3. ENDOGENOUS RETROVIRAL ELEMENTS.

3.1. Introduction.

As a consequence of the normal retroviral life cycle and the repeated integration of DNA proviruses into the host genome over a long period of time, the genomes of many vertebrates have accumulated a large number of retrovirus related sequences (endogenous retroviral elements-ERVs). (Coffin, 1982, Stoye & Coffin, 1985) In fact, all ERVs resemble integrated proviruses in both their gross gene structure (LTR-*gag-pol-env*-LTR) and at the cell-virus junction, with a deletion of LTR sequence and a duplication of cellular DNA at the site of integration, characteristic of retroviral integration. (Cullen *et al.*1983, Stoye & Coffin, 1985) In the relatively small time scale involved in genetic experiments, the endogenous loci behave as stable genetic elements and are inherited as Mendelian genes. However, over the longer period, these loci have proved to be genetically mobile. (Coffin *et al.*1991) In inbred mice, each strain contains a unique pattern of proviral DNA incorporated into the genome at unique chromosomal positions. (Stoye & Coffin, 1988) These can be viewed as virus-cell junction fragment polymorphisms, and the term "locus" will be used in this thesis to describe a provirus of unique structure and/or cellular location. In inbred mouse strains, some loci may be shared among related strains, but no two strains appear to have the same ERV pattern (or "fingerprint"). In fact only one locus has been identified as common to all inbred

mice tested. (Coffin *et al.*1991) In much less inbred chicken flocks, individuals possess an assortment of loci from a species pool. (Rovigatti & Astrin, 1983) The genetic heterogeneity of ERVs appears to be related to the length of time that the endogenous viruses have been integrated into the gene pool of the species. Compared to other vertebrates, the inbred mice have acquired many of their endogenous viruses relatively recently, and some within the last century of inbreeding. This has been complicated by the gain and loss of proviral sequences. The host genome is always subject to new proviral integrations of infectious sequences; if this occurs in germ line cells it will be inherited as a host gene. The homology of the LTRs flanking the provirus allows for rare homologous recombination resulting in the loss of the genome and leaving a single LTR in the host DNA. A number of single LTRs have been cloned from the mouse genome (Stoye & Coffin, 1987), and feline genomes are "littered" with single LTR sequences. (O'Brien, 1986)

Endogenous retroviruses are considered to be non-essential to the host, and so deletions and mutations, occurring at integration or as a consequence of cellular mutations, can be tolerated. Many ERVs have gross gene deletions and others, point mutational defects. (Hayward *et al.*1980, Hughes *et al.*1981, Soe *et al.*1983, 1985, Ikeda *et al.*1985) The flanking DNA of a number of loci have been shown, by restriction enzyme mapping, to be similar (Soe *et al.*1983) and the mapping of 6 ERV loci to chromosome 1 in chickens suggests that the process of gene duplication may have been involved in the generation of these ERVs (Tereba, 1981, Tereba & Astrin, 1982). After the duplication event each new locus would be subject to independent genetic drift. Other loci, however are clearly the result of unique integrations. (Soe *et al.*1985)

Endogenous proviral loci are less prone to individual sequence mutations than exogenous infectious virus since they are replicated by host DNA polymerases whilst the infectious viruses are replicated (more frequently) by the viral enzyme reverse transcriptase, an enzyme with lower fidelity, and no proof reading capacity. (Roberts *et al.*1989)

3.2. Characterisation of endogenous retroviral elements.

Endogenous (and exogenous) viruses of different species are sub-classified by a number of criteria but the most common distinguishing features are the polymorphic envelope genes and specific envelope antigens.

3.2.1. Endogenous retroviruses related to ALV.

The genome of the domesticated chicken contains multiple ERVs related to ALV. (Rovigatti & Astrin, 1983) Using Southern blotting and genetic studies, more than 22 individual loci have been identified and characterised to varying degrees. (Astrin *et al.*1980b, Hughes *et al.*1981, Zemiecki *et al.*1988) These are known as the *ev-* loci. Some, like *ev-1, 2* and *7* represent complete proviruses, while others, like ev-*3, 6* and *9* contain gross structural deletions. The *env* genes of the *ev-* loci are classed as subgroup E, (Vogt, 1969) and the *ev-2* locus is responsible for producing the endogenous infectious virus, RAV-0. (Astrin *et al.*1980a) Most of the structurally complete loci are not expressed but a few truncated loci do express one or more viral gene products at high levels. (Hayward *et al.*1980, Rovigatti & Astrin, 1983)

3.2.2. Endogenous retroviruses related to MuLV.

In the mouse, the situation is more complicated, because there are 3 different classes of ERVs related to MuLV in mice. These are ecotropic, xenotropic and polytropic. Ecotropic loci occasionally can produce an infectious virus, whose replication is restricted to murine cells. Xenotropic loci can occasionally produce infectious virus, which is restricted to replicate in non murine cells only. The polytropic endogenous viral sequences never produce infectious virus in their own right but the *env* genes confer a new host range to ecotropic viruses via recombination. (Section 4.2.) The polytropic host range allows the virus to infect both murine and non murine cells. The polytropic loci can be sub-divided into polytropic and modified polytropic loci (the modifications being the acquisition of a HindIII site at the 3' end of *pol*, and a 27bp deletion in the SU coding region of the

env gene. (Stoye & Coffin, 1987) There is extreme genetic variation between strains of mice regarding the possession of endogenous loci. (Stoye & Coffin, 1988, Frankel *et al.*1989a, 1989b, 1990) A number of high leukaemia incidence strains of mice possess 2-3 ecotropic loci capable of producing virus. (Chattopadhyay *et al.*1980) Those in the high leukaemic AKR mouse strain are active from an early stage in development. (Rowe & Pincus, 1972, Kawashima *et al.*1976) Low leukaemia incidence strains such as BALB/c possess only one ecotropic locus, and many strains have none. (Chattopadhyay *et al.*1980, Kozak & Rowe, 1982) Mouse strains are also heterogeneous in the possession and production of xenotropic virus. (Hoggan *et al.*1986)

A laboratory mouse may contain from 0-6 ecotropic loci, 5-16 xenotropic loci, and from 30-50 poly- and modified polytropic loci. (Stoye & Coffin, 1988) Analysis of the ERV content of inbred mice is further complicated because the different classes of ERV related to MuLV are closely related, with differences limited to small regions of the *env* gene The development of oligonucleotide probes that distinguish between the individual classes of ERV (Stoye & Coffin, 1988) has resulted in the identification of 40 individual xenotropic loci, 47 polytropic loci, and 30 modified polytropic loci in the inbred mouse gene pool. (Frankel *et al.*1989a, 1989b, 1990)

3.2.3. Endogenous retroviruses related to FeLV.

The genome of the domestic cat contains approximately 8-12 integrated copies of endogenous proviral elements related to FeLV. (Koshy *et al.*1980) The loci are heterogeneous in size and chromosomal position. This can be shown, as in the other systems, as a length polymorphism of virus-cell junction restriction fragments. As the domestic cat is a diverse, outbred population, each animal has a unique set of loci which are inherited as Mendelian genes. Close relatives would be expected to have a greater proportion of common loci. EnFeLV elements are found in the domestic cat and its five closest relatives in the genus Felis. (O'Brien, 1986)(Fig.4) These are thought to have diverged from a common ancestor species

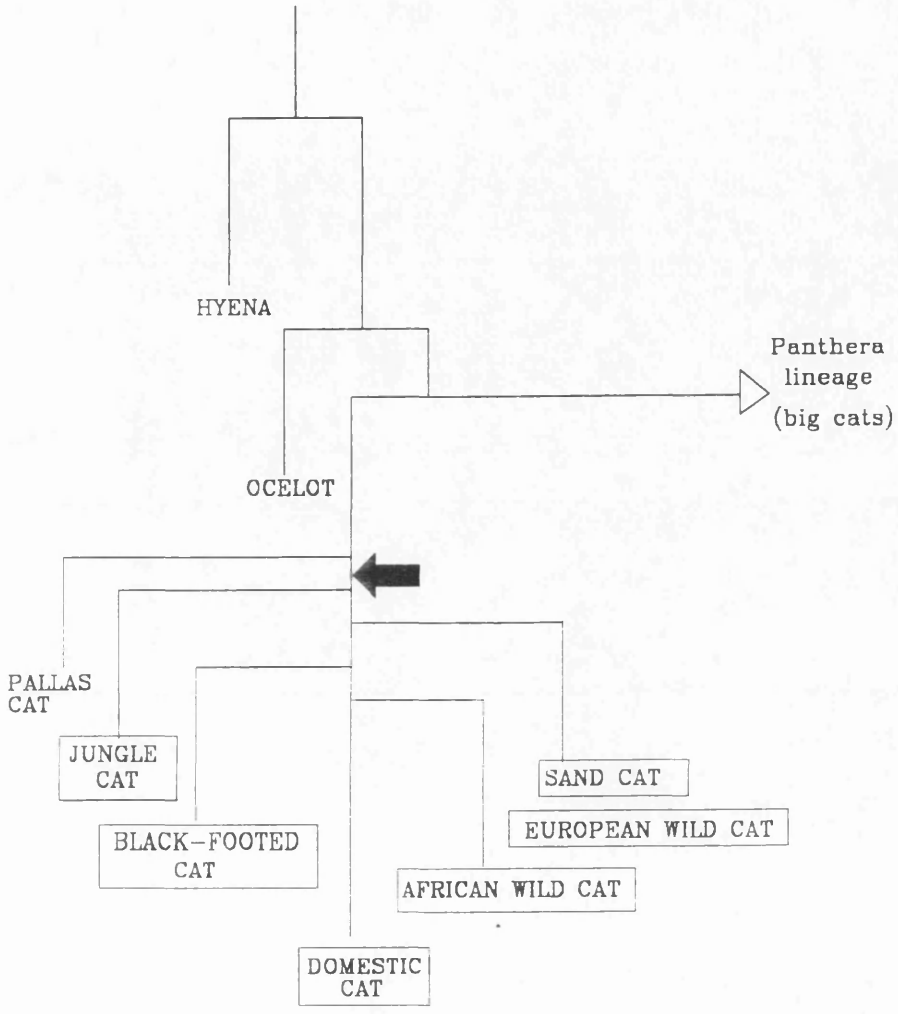


Fig.4. Integration of enFeLV into the feline genome.

The figure represents a portion of the phylogenic evolutionary tree of the genus Felis as calculated by the analysis of reciprocal immunological differences between species, using serum albumin-specific antiserum prepared in different species. (O'Brien, 1986) Boxed are species that contain FeLV-related sequences in their genomes. The arrow indicates the suggested point of integration of these sequences into a common ancestor of these species.

which was found in the Mediterranean basin (Southern Europe, North Africa, Asia Minor) (Benveniste & Todaro,1974) about 6-8 million years ago. (O'Brien, 1986) The incorporation of the retroviral elements into the genome of this species was presumably the result of a widespread epidemic resulting in the incorporation of DNA proviruses into germ line cells. EnFeLV elements presumably segregated as independent Mendelian elements in the subsequent evolution and divergence of the six species named. It is interesting to consider the origin of the enFeLV genetic information. The *env* gene of enFeLV displays an unexpectedly high sequence homology to the *env* genes of murine polytropic MCF viruses. (Elder & Mullins, 1983, Wunsch *et al.*1983) Recombinant *env* genes containing enFeLV sequences also confer a polytropic host range. (Jarrett *et al.*1978b, Stewart *et al.*1986) The MCF-related endogenous elements are found in wild mice in limited geographical areas, including the Mediterranean area. (Kozak & O'Neill, 1987) Although it cannot be said that these geographical limitations have been constant over 6 million years, it is an interesting coincidence. It is therefore conceivable that the enFeLV could have originated from a polytropic mouse virus. However, transmission in the opposite (cat to mouse) direction cannot be excluded. Another retrovirus family endogenous to the feline genome is the xenotropic virus, RD114. (McAllister *et al.*1972, Baluda & Roy-Burman, 1973, Livingston & Todaro, 1973) Although this virus is unrelated to FeLV, (Livingston & Todaro, 1973) it is found in the same six feline species that enFeLV loci are found. (Benveniste & Todaro, 1974, O'Brien, 1986) In fact, the sequence homology of RD114 suggests an origin from a baboon type C virus. (Hu *et al.*1977) A study of the segregation of RD114 and enFeLV related proviruses in feline DNA in experimental crosses of the domestic cat and a species negative for both endogenous families, indicated that in the backcross of the F1 progeny with the negative species, the two endogenous families segregated as if they were closely linked. (Benveniste & Todaro, 1975) However only two progeny were studied, and there is too little evidence to conclude that the two unrelated retroviral families are genetically linked in the complex feline genome.

Early Southern blot analysis data using FeLV-B proviral DNA as a probe showed that using appropriate restriction enzymes, individual endogenous loci could be separated and identified in virus negative tissue. (Koshy *et al.*1980) In this study, the most satisfactory results were obtained using an EcoRI digest which was thought to cut in the cellular flanking DNA and not within the provirus itself. Assuming that proviral DNA was of constant size, the differences in location of restriction enzyme sites in the flanking DNA would result in an unique fragment for each locus. Heterogeneity was observed between the proviral loci of two different cats. It was also confirmed that the proviral pattern was the same in different tissues of the same individual.

The cloning and characterisation of a number of enFeLV loci from a placental DNA library of a specific pathogen free cat showed that there was a great deal of heterogeneity in the overall size and structure of the enFeLV loci present in one individual. (Soe *et al.*1983, Soe *et al.*1985) Overall the enFeLV proviruses could be divided into two categories; those with essentially full length genomes, and those which were truncated, with gross deletions involving *gag*, *pol* or *env* genes. The clones were all analysed by restriction enzyme analysis. There appeared to be a conservation among all the clones of the *pol* to *env* boundary and the SU region of *env* gene itself (when these regions were present in the deleted clones). A much greater degree of heterogeneity was observed in the *gag* and TM-*env* regions. A proportion of truncated proviruses had similar restriction enzyme sites in the flanking DNA, suggesting that these loci may have been the result of gene duplication. (Soe *et al.*1983) However, the essentially full length isolates demonstrated unique flanking restriction maps suggesting that they are the result of independent insertions. (Soe *et al.*1985)

Nucleotide sequence analysis of the *env* genes of two of the enFeLV clones was performed. Of these, one represented a complete *env* gene and the other a substantially truncated gene, the deletion encompassing the 3' half of SU and the whole of TM. (Kumar *et al.* 1989) (See Fig.3)

3.3. Control of cellular expression of ERVs.

The expression of endogenous related genomes can, as discussed, result in the spontaneous production of infectious virus. However expression of ERVs can vary, ranging from spontaneous or induced virus production, to expression of single gene products to transcriptional silence (or transcription at levels that cannot be detected). (Coffin, 1982, Stoye & Coffin, 1985) Therefore expression varies widely and the controlling mechanisms are poorly understood.

The first requirement for expression is the inheritance of active loci. Many loci are transcriptionally repressed or silent. (Hayward *et al.*1980) This may represent a defect in the viral promoter and initiation sequences, but the expression of many structurally intact loci are also repressed.

3.1.1. Methylation.

One factor that has been shown to correlate with transcription of endogenous proviruses is DNA methylation. The *ev-1* locus is transcriptionally silent but contains the gross gene structure required for virus production. (Hayward *et al.*1980) A comparison of the relative levels of methylation of the relatively silent *ev-1* locus, and the transcriptionally active *ev-3* locus, showed that levels of methylation were inversely proportional to the rate of transcription. The transcription of *ev-1* is induced in vitro by the treatment of fibroblast cells with 5-azacytidine, an inhibitor of cellular DNA methylation. (Groudine *et al.*1981) However, the virus induced from this locus has been found to be non infectious, lacking reverse transcriptase and envelope surface antigens. (Conklin *et al.*1982) These are probably the result of small mutational defects. The action of methylation inhibitors has also been shown to induce the increased transcription of *ev-2*, the ERV locus capable of the production of subgroup E infectious virus, RAV-0. (Astrin *et al.*1980a) The observation that cloned viral DNA from the *Mov-3* substrain of mice was infectious after transfection, while genomic viral DNA was not infectious, was interpreted by Harbers *et al* (1981) to demonstrate the action of methylation of genomic DNA in repressing proviral expression. (However, the insertion of the provirus into a novel

chromosomal location could affect the ERV expression. (See 3.3.3.)) Further, transient exposure of murine fibroblasts to 5-azacytidine resulted in the expression of endogenous type-C and type-A viruses, and also to transformation of the cells. (Hsiao *et al.*1986)

It is possible that during viral infection a number of proviruses could be integrated into heavily methylated regions of DNA and that their further transcriptional activity would be repressed.

3.3.2. The long terminal repeat.

The study of the LTRs of *ev-1* and *ev-2* indicated that differences relative to the exogenous RAV-2 (subgroup A) LTR accounted for the relatively low level of virus production despite induction of transcription. The endogenous LTR was shown to be ten fold less active than the exogenous LTR. (Cullen *et al.*1983) This difference was linked to the divergent U3 sequences of exogenous and endogenous viruses. In particular, the endogenous LTRs lack the enhancer element present in the exogenous virus LTR. (Hughes, 1982, Schwartz *et al.*1983)

The U3 region of the FeLV LTR has been shown to be significantly different from endogenous LTR sequences. (Casey *et al.*1981) Transient expression studies have indicated a differential ability of enFeLV LTRs to drive transcription, but this was linked primarily to sequences in the flanking DNA. (See 3.3.3.)

3.3.3. Chromosomal position.

Another form of control was indicated by the observation that the *ev-2* locus was transcribed at low levels in *situ* but that the resulting virus was capable of high transcription after integration into a new cellular location. (Jenkins & Cooper, 1980, Humphries *et al.*1979, 1981) Aside from the possibility that the new location is in a different methylation state, these results suggested that cellular flanking sequences may have an effect the transcription of proviral DNA. This may reflect integration into a region of chromosome that is transcriptionally active or the actions of tissue specific cellular enhancers on the viral LTR.

EnFeLV have never been found to produce infectious virus. (Quintrell *et al.*1974, Benveniste *et al.*1975) EnFeLV *gag* sequences have been shown to contain extensive nonsense mutations that would prohibit the production of virus. (Berry *et al.*1988) The LTR of a cloned full length enFeLV locus has been shown to be potentially active but is strongly repressed by *cis* acting sequences within the 5' flanking DNA. (Berry *et al.*1988)

The importance of the influence of flanking DNA sequences for some ERV loci is demonstrated by the avian *ev-6* locus and the murine *Fv-4* locus. Both loci lack 5' LTRs and transcription is presumed to be from cellular promoters. (Hayward *et al.*1980, Hughes *et al.*1981, Ikeda *et al.*1985, Ikeda & Sugimura, 1989)

3.3.4. Cell differentiation and development.

The chromosomal locations of many ERVs have been mapped close to lymphoid and differentiation specific antigen genes, (Blatt *et al.*1983, Meruelo *et al.*1983, Wejman *et al.*1984) and the expression of some ERVs has been linked to differentiation. Expression of a xenotropic ERV (*Bxv*) in B-cells has also been induced by B-cell mitogens such as lipo-polysaccharide (LPS) (Moroni *et al.*1975, Moroni & Schumann, 1975), and amplified by chemicals such as BUdR. (Stoye & Moroni, 1983) An apparently defective virus (*Bdv*) is also induced by LPS stimulation of B-cells, but is unaffected by BUdR treatment. (Stoye & Moroni, 1983, Stoye & Moroni, 1984) Further studies have shown that the production of these endogenous viruses requires DNA synthesis, cell proliferation, and for *Bxv*, at least, B-cell differentiation. (Stoye & Moroni, 1985) Expression of some ERVs therefore may be linked to the multi-stage process of B-cell differentiation. An example of the control of ERV expression being linked to development is the *Akv-1* locus of AKR mice which is activated to produce ecotropic virus at around 18 days gestation. (Rowe & Pincus, 1972)

3.3.5. Tissue specific factors.

Tissue specific control of ERV expression may be affected by *trans* activator proteins expressed only in certain tissue types. The LTRs may also be influenced in *cis* by both cellular enhancers or silencers. The G_{IX} differentiation antigen, found on the surface of thymocytes in strain 129 mice (Stockert *et al.*1971) has been shown to be a polytropic *env* gene SU (Obata *et al.*1975, Levy *et al.*1985a) and its expression is controlled in *trans* by two distinct loci, *Gv-1* and *Gv-2*. (Stockert *et al.*1971, Levy *et al.*1985b) It has been demonstrated that a null allele at *Gv-1* correlated with a reduction in the steady state levels of retroviral RNA and a reduction in the transcription of the ERVs. (Levy *et al.*1982) Multiple sized mRNAs were linked to *Gv-1* action, and it may be that the product of *Gv-1* is a diffusible protein which interacts with DNA sequences associated with a number of ERV transcription units including the G_{IX} locus. G_{IX} levels are also hormonally regulated. The increased levels of expression found in male mice compared to female mice have been shown to be the result of the action of the steroid hormone, testosterone on an unidentified tissue common to both sexes. (Obata *et al.*1978) It is possible that the action of testosterone is linked with the action of *Gv-1* or *2*.

In summary, a number of ERV loci are transcriptionally inactive due to factors including methylation, *trans*-acting factors and *cis*-acting factors in the flanking DNA. Some ERV loci may be induced by activation of the transcription of flanking genes, by tissue specific factors or by other gene products acting in *trans*. In natural populations there appears to be a selection against the expression of ERV loci encoding fully infectious virus. ERV loci with the full genetic information required to produce virus are not translated to high levels and ERV loci which are transcribed represent substantially deleted proviruses and they express only a subset of viral gene products.

SECTION 4. BIOLOGICAL EFFECTS OF ERVS

Endogenous retroviral elements are considered non essential to the host. This has been formally shown for the *ev* loci in chickens where the polymorphic loci have

been deliberately bred out, with no ill effects. (Astrin *et al.*1979) (It has since been shown that these birds do contain reverse transcriptase related gene sequences. (Dunwiddie & Faras, 1985)) However, there is a growing body of evidence to suggest that ERVs should not be regarded as simply selfish DNA, but that they have significant effects to both disease potential and host susceptibility to infection.

4.1. Germ line insertion of retroviruses.

The possibility that endogenous retroviral elements are genetically mobile is predicted from the retroviral lifestyle and suggested further by the heterogeneity of loci present in different individuals or sub-strains of a species. Studies have shown that incorporation of a new ecotropic locus into the genome of the high leukaemogenic AKR mouse occurs on average once every 12 generations, (Buckler *et al.*1982) or once every 15-30 years. (Steffen *et al.*1982) This high rate of viral insertion may reflect the high selection placed on the development of leukaemia in the genetic inbreeding to produce the AKR strain. It is much more likely that in an unselected system, the acquisition of ecotropic viral loci capable of producing infectious virus would have been selected against. A much lower, but still detectable, frequency of novel integrations of the non-ecotropic ERVs has been measured. These are presumably under different selective pressures. The observations of both loss and gain of proviruses in the study of the linkage patterns of the three classes of non-ecotropic ERVs in recombinant inbred mice strains (Frankel *et al.*1989a, 1989b, 1990) led to the calculation that a novel provirus was gained every 3,500 generations, and the rate of virus loss was $4x10^{-6}$ per provirus per generation. (Coffin *et al.*1991)

The observed frequency of insertions and deletions of ERV loci is therefore very small, but a number of cellular gene mutations have been linked with endogenous proviruses, and two have been directly shown to be caused by virus insertions. The dilute (*d*) coat colour mutation in DBA mice is caused by the integration of an ecotropic provirus, *emv-3* (Jenkins *et al.*1981) The genetic markers for *d* and *em-3* were shown to be very closely linked, and spontaneous revertants

were found to have lost the proviral genomic sequences, presumably through homologous recombination of the LTRs. (Copeland *et al.*1983) The hairless mutation (*hr*) of HRS/J mice is autosomal, recessive and maps to chromosome 14. (Meier *et al.*1969) Again this locus was seen to display very close linkage to an endogenous retroviral locus, in this case a polytropic locus *pmv-43*, and again the isolation of a spontaneous revertant of the phenotype lacking the linked proviral DNA genome sequence established causality. (Stoye *et al.*1988) In both cases, therefore, it has been shown that the presence of a proviral genome integrated at the *hr* or *d* loci cause the defect in the phenotype coded by these genes. Although it is suspected that the proviruses may be integrated in a non-coding region (because the presence of the single LTR has no observed disruptive effect) the precise mechanism involved in gene disruption has not been established. (Coffin *et al.*1991)

4.2. Generation of viral diversity by recombination with ERVs.

ERVs have been shown to participate in recombination with infectious exogenous virus with a variety of consequences for both virus and host. The phenomenon of high frequency recombination and the natural forces of selection are largely responsible for the genetic variation of retroviruses. Retroviral recombination is a consequence both of the virus packaging two genomes in each virion, and the characteristics of the enzyme reverse transcriptase. (Coffin, 1990, Katz & Skalka, 1990)

4.2.1.Retroviral recombination.

Most evidence indicates that recombination of two different viral genomes requires the formation of heterozygous particles, with recombinants being observed in the second round of infection. This therefore requires the expression of both progenitor genomes in the cells in the first round infection (Weiss *et al.*1973) and the co-packaging of both genomes in the emerging virus particles. (Hunter, 1978, Hu & Temin, 1990a) Several mechanisms of recombination have been proposed but the evidence points to recombination being a function of the viral enzyme reverse

transcriptase and its ability to move from one template to another during DNA synthesis. (See section 1.4.2.) (Panganiban & Fiore, 1988) Within this model there are a number of different methods proposed as to when and how the reverse transcriptase transfers from one template to another, but there is evidence to indicate that recombination occurs both during minus (Coffin, 1979, Goodrich & Duesberg, 1990) and plus strand (Junghans *et al.*1982, Hu & Temin, 1990a) synthesis. Retroviral recombination has been shown to repair defective viruses, with endogenous sequences providing functional DNA sequences (Schwartzberg *et al.*1985, Martinelli & Goff, 1990) and the recombination of two retroviral genomes mediated by RT has been shown to produce a broad spectrum of frameshifts, substitutions and other mutations (Pathak & Temin, 1990a), deletions and deletions with insertions. (Pathak & Temin, 1990b)

4.2.2. Avian leukosis virus recombinants.

Recombination involving the *env* genes of endogenous loci has been identified in chickens. This has a number of consequences for both virus and host. The chicken *ev-2* locus harbours a complete provirus (Hayward *et al.*1980, Hughes *et al.*1981) capable of producing infectious virus of subgroup E. (Astrin *et al.*1980) However the expression of this locus and the corresponding virus production is low and the basis of this has been discussed previously. (See section 3.3)

A number of truncated endogenous avian proviral loci express large amounts of RNA including the loci *ev-3, -6* and *9*, which all express a subgroup E *env* gene. (Astrin, 1978, Astrin & Robinson, 1979, Hayward *et al.*1980) The protein product of this gene has been shown to be involved in phenotypic mixing with infectious virus, the resulting "pseudotype" having a mixture of both exogenous and endogenous SU, or in combination with envelope defective viruses such as the Bryan high strain of RSV, subgroup E alone. (Weiss, 1969, Weiss *et al.*1973) It was this ability to "rescue" envelope defective viruses that led to the coining of the term, chick helper factor (chf) to describe the product of the endogenous *env* gene. (Shields *et al.*1978, Hanafusa *et al.*1970a)

ERVs are also involved in genetic recombination to produce replication competent viruses of subgroup E host range. (Hanafusa *et al.*1970b, Weiss *et al.*1973) These viruses, collectively termed RAV-60 viruses, can be isolated by recombination with ALV of any subgroup, and from all chicken cells although the efficiency of isolation from cells not expressing endogenous *env* genes (chf-) is low. (Coffin *et al.*1983) Recombination has also been reported involving non-defective transforming RSV and again efficiency of expression was shown to be dependent on the expression of chf. (Weiss *et al.*1973) These recombinants generally involved only the endogenous *env* gene, which is sufficient to determine the subgroup properties of the virus. (Tsichlis & Coffin, 1980)

Other recombinants have been described in which exogenous subgroup B or D viruses acquired a small portion of an endogenous *env* gene, leading to new viruses with a "dual"-tropic host range, being able to infect both chicken cells which were permissive for subgroup B and D but not E, and turkey cells permissive for subgroup E but not B or D. (Tsichlis *et al.*1980) The sequence differences responsible for this expanded host range are more limited than those involved in subgroup specificity of the avian *env* gene, and is further complicated by the genetic diversity of the cellular receptors for these subgroups in chicken and turkey cells. (Vogt, 1977)

Avian endogenous viruses are generally non pathogenic in chickens, (Motta *et al.*1975, Robinson *et al.*1982) but the acquisition of a new subgroup would allow virus spread to new hosts, or the re-infection of cells already infected by the parental exogenous virus, by-passing receptor interference, through the use of a subgroup E receptor. However most chicken cells cannot support the replication of subgroup E virus, which has a xenotropic host range, and so the significance of the viral acquisition of an endogenously encoded host range in chicken infections may be limited.

4.2.3. Murine leukaemia virus recombinants.

In the inbred mouse, recombinants between exogenous and endogenous viral sequences are often complex, due in part to the large number and heterogeneity of the endogenous population of the mouse genome, and many of these recombinant viruses play an important role in MuLV induced leukemogenesis.

4.2.3.1. MCF viruses.

The MCF viruses are associated with the development of spontaneous leukaemias in a number of mouse strains. (Hartley *et al.*1977, Green *et al.*1980) These viruses were first described as novel agents associated with the development of thymoma in the AKR mouse strain. (Hartley *et al.*1977) AKR mice harbour ecotropic viral loci (Chattopadhyay *et al.*1980) which express large amounts of virus from about 18 days gestation until death (Rowe & Pincus, 1972), and the presence of this virus is a prerequisite for leukaemia development. (Lilly *et al.*1975) MCF viruses are detected in pre-leukemic tissues and appear to be the proximal leukemic agent in these mice. (Kawashima *et al.*1976, Hartley *et al.*1977) MCF viruses represent recombinants between the ecotropic virus and endogenous viral sequences. (Chattopadhyay *et al.*1982) The acquisition of some or all of the *env* gene of endogenous polytropic loci (Khan, 1984) results in an expanded host range with the viruses being able to infect both murine and non murine cells (Hartley *et al.*1977, Fischinger *et al.*1975). The name MCF (Mink Cell Focus-forming) comes from the observation that these viruses can induce cytopathic foci on mink lung cells (initially used as a diagnostic test for MCF viruses.) (Hartley *et al.*1977)

MCF related viruses have also been linked to leukemic induction by a number of exogenous MuLVs, including Moloney and Friend MuLV. (Bosselman *et al.*1982, Evans & Cloyd, 1985) Indeed it is now thought that a portion of the Moloney-MuLV genome itself is derived from endogenous envelope sequences. (Khan *et al.*1982) and the erythroleukaemia inducing virus, SFFV, possesses a truncated *env* gene that also is derived from endogenous sequences. (Troxler *et al.*1977, Ruscetti *et al.*1979, Amanuma *et al.*1983, Clark & Mak, 1983, Wolff *et al.*1983, Bestwick *et al.*1984)

The MCF viruses differ in detailed structure and points of recombination but they can be sub-divided into two groups on the basis of the extent of sequence substitution and leukemogenic potential (Lung *et al.*1980, 1983, Evans & Morrey, 1987). Class I MCF viruses represent the classic leukemogenic recombinants, with the ability to induce cytopathic foci on mink lung cells. They are composed of an ecotropic backbone, with a large region of the *env* gene substituted with endogenous polytropic *env* sequences. At least two thirds of the SU protein, including both the amino-terminal and the proline rich regions are involved. A second substitution involves the replacement of the C-terminal region of TM and U3 of the ecotropic virus with those from an endogenous xenotropic virus. Class II viruses are non-leukemogenic but do have the expanded host range, and contain only the envelope substitution, which usually involves the entire *env* gene. The mouse genome contains a large number (25-50) of endogenous loci related to the polytropic envelope genes. (Jenkins *et al.*1981, Stoye & Coffin, 1988, Frankel *et al.*1989a) It is probable that many do not commonly donate their *env* gene sequences to recombinants, and in fact some of those characterised display disabling deletions and mutations. (Stoye & Coffin, 1987) A comparison of the nucleotide sequence analysis of the *env* genes of a number of different MCF isolates suggested that those polytropic ERV loci participating in the generation of MCF viruses were highly related as regards to *env* gene sequences. (Khan, 1984) Studies of recombinant inbred mice have failed to link one single polytropic provirus to the generation of MCF related disease in mice. One locus, *pmv-25* was found in many, but not all, mice with a predominance of thymomas, (Frankel *et al.*1989a) and this may represent a preferred locus. In contrast, a single endogenous xenotropic locus capable of producing virus has been shown to be the most likely source of the MCF virus TM/U3 sequences. (Quint *et al.*1984, Hoggan *et al.*1986) This locus, *Bxv-1*, has been mapped to chromosome 1 and is found in most inbred strains of mice. (Kozak & Rowe, 1980, Morse *et al.*1982) In Class I MCF viruses, the U3 LTR has further undergone a somatic duplication of an approximately 70bp enhancer region (Holland *et al.*1989, Coffin *et al.*1991) containing the binding sites for at least six

transcriptional factors. (Speck & Baltimore, 1987) These LTR sequences have been shown to be responsible for the targeting and efficient replication of Class I MCF viruses in the thymus (Holland *et al.*1989) as compared to the non-thymotropic Class II MCF viruses containing ecotropic LTR elements. (Rosen *et al.*1985, Cloyd & Chattopadhyay, 1986)

4.2.3.2. Generation of MCF viruses.

It is generally considered that the generation of MCF viruses involves a series of steps initiated by the ecotropic virus infecting cells expressing the individual non-ecotropic progenitors, followed by a series of recombinations involving the polytropic *env* gene and the xenotropic TM/U3, and finally the duplication of the enhancer region of the U3 LTR. Recombinants appear to be produced in a remarkably consistent manner. Whether this is due to particular recombinations conferring a selective growth advantage to the virus over others, or a tissue tropism encouraging the infection of a tissue where further recombinations are favoured is a matter for speculation. A number of different, if related theories have been proposed regarding the locations and mechanisms involved in the generation of MCF virus. (Evans & Malik, 1987, Laigret *et al.*1988, Stoye *et al.*1991) and are based on the known locations of endogenous provirus expression, and the structures of potential intermediates in the recombination process that have been isolated. Overall however, it may be the ability of the final viral recombinant to replicate in its target organ that leads to its outgrowth above the background of the many random recombinants that are presumably formed. In general the genetic variability of retroviruses relies not only on the high mutation frequency, but also on the selective pressures which decide whether a particular mutant persists. MCF Class I viruses have been shown to selectively infect and replicate in immature lymphocytes found only in the thymic cortex. (Cloyd, 1983) Massive viral infection and viral integration of this cellular subset would lead to the activation of cellular oncogene(s) by insertional activation.

There is abundant evidence to suggest that the xenotropic LTR with its enhancer duplication is required for efficient replication in the target cells for MCF

leukemogenesis. The role of the polytropic *env* sequences in leukemogenesis are less well understood. The replication defective virus SFFV, which causes acute erythroleukaemia in adult mice, encodes a truncated envelope SU gp55 which has been directly implicated in leukemogenesis. (Li *et al.*1987) The observation of high sequence homology between gp55 and polytropic *env* sequences, have suggested that gp55 is derived from these MCF-like *env* genes via a recombination step and a major deletion and point mutations. (Troxler *et al.*1977, Ruscetti *et al.*1979, Amanuma *et al.*1983, Clark & Mak, 1983, Wolff *et al.*1983, Bestwick *et al.*1984) Recent work has established that gp55 binds the cellular receptor for erythropoetin (Epo-R) and that this interaction can activate cell growth. (Li *et al.*1990) Further to this theory, Tsichlis and Bear have demonstrated MCF SU binding to the IL-2 receptor (one of a family of related cellular receptors including Epo-R) of a rat T-cell lymphoma line resulting in IL-2 independent growth. (Tsichlis & Bear, 1991) These studies suggest that the role of the polytropic *env* gene sequences in MCF viruses may be to promote the proliferation of the target cell population thereby providing increased numbers of cells for virus infection, and so increasing the chance of an "oncogenic" integration.

4.2.3.3. MCF viruses and wild mice.

It is interesting to note that there are differences in the endogenous proviral content of different strains of wild (outbred) mice. The high conservation evident in the mitochondrial DNA of many inbred strains, suggests that they were all derived from a single female. (Ferris *et al.*1982) Studies of the endogenous *env* content of a variety of wild mouse species have shown that the two progenitors of Class I MCF viruses, the xenotropic and polytropic loci, are restricted to geographically separate populations, with xenotropic loci found in mice species from Japan (*Mus musculus molossinus*), Thailand (*Mus musculus castaneus*) and the south eastern regions of the Soviet Union (as was), China, and eastern Europe (*Mus musculus musculus*), and polytropic loci from mice species of Western Europe, the Mediterranean basin and North America (*Mus musculus domesticus*) (Kozak & O'Neill, 1987) The few exceptions are thought to be natural hybrids of *M.m. domesticus* and *M.m.musculus* (central Europe) or *M.m. castaneus* (Lake Casitas mice, California). The Lake

Casitas (LC) mice (Gardner *et al.*1991) have a high incidence of infectious MuLV and disease. Two viruses are commonly isolated from these mice. The first group are not found in the germline DNA of mice and have an amphotropic host range. (These viruses are distinct from inbred polytropic viruses and are found only in wild mice.) (Hartley & Rowe, 1976, Rasheed *et al.*1976) These have been shown to induce a low level of lymphoma in infected mice. The second group of viruses isolated from these mice have an ecotropic host range, but can be distinguished from inbred ecotropic viruses. There is about 70% sequence homology of the *env* genes of Akv-1 (from AKR mice) and Cas-Br-E from (LC mice) (Rassart *et al.*1986, Masuda & Yoshikura, 1990). These wild mouse ecotropic viruses have been implicated in lymphomagenesis but are also responsible for a low incidence of hind limb paralysis. MCF like viruses have been isolated from a species of wild mouse possessing polytropic endogenous loci, after infection by Moloney-MuLV. (Villar *et al.*1988) This mouse species does not contain any functional ecotropic viral loci and so recombination would require exogenous infection. Recombination occurs readily between amphotropic viruses and endogenous loci of inbred mice, but there is little information on the relevance of this in the natural mouse population. (Kozak *et al.*1989) The differences between pathogenic and non pathogenic ecotropic viruses may be due to more subtle genetic changes which do not alter viral host range as defined by receptor interference. This may become apparent with further studies of the natural viral isolates of the outbred species.

In the inbred mouse system therefore, endogenous retroviral elements play a significant role in the induction of leukaemia. Their effects in the outbred wild mouse population are less obvious.

4.2.4. FeLV recombinants.

EnFeLV proviruses of the cat appear to be highly related in their *env* sequences. (Stewart *et al.*1986, Kumar *et al.*1989) The recombination between exogenous FeLV-A (with an ecotropic host range) and endogenous *env* sequences, results in FeLV-B viruses which have a polytropic host range. (Stewart *et al.*1986,

Kumar *et al.*1989) As with MuLV recombinants, the replaced *env* gene sequences invariably include the amino-terminal and the proline-rich domains. (See Fig.3) The generation of FeLV-B *in vivo* has been much less closely studied than the MCF viruses. FeLV-B isolates were found in 40-60% of virus infected cats (in association with FeLV-A as always) (Jarrett *et al.*1978a) However these studies were carried out in multi cat households and it was not determined what percentage of isolates resulted from *de novo* recombination, as opposed to horizontal spread.

The leukemic potential of FeLV-B is less clear than that of MCF viruses. FeLV-B infection has not been linked to any particular disease state (unlike the rare FeLV-C viruses which induce fatal erythroplasia (Onions *et al.*1982)), but the frequency of isolation of FeLV-B is higher from leukemic cats than healthy cats (Jarrett *et al.*1978a). This may result from superinfection of exogenous virus infected cells and consequently, an increased chance of integration near cellular proto-oncogenes. Alternatively it may be a consequence of binding of FeLV-B to growth factor receptors or some more subtle effect of FeLV-B specific sequences on the feline haematopoetic system.

FeLV-B viruses can be more pathogenic than their ecotropic parents, but the difference is not generally as dramatic as that observed in the generation of murine MCF viruses. (Jarrett *et al.*1978a,b). This may reflect the importance of the LTR substitution in MCF viruses, as the FeLV-B viruses retain the ecotropic virus LTR (Casey *et al.*1981). However, it may also reflect the possible restriction of the replication of FeLV-B due to resistance to infection of important target cells.

4.3. Viral resistance.

The study of ERVs has suggested another role for endogenous retroviral products in the restriction of replication of infectious retroviruses. From the current information, it appears that the mediation of viral resistance by ERVs may be the most significant role of endogenous retroviral elements in outbred species, and may go some way to explain the persistence of ERVs at high copy number in the host genomes. The mechanism of resistance is thought to involve the blocking of specific

cellular receptors significantly reducing the rate of penetration of virus into cells. (Robinson *et al.*1981, Ikeda & Odaka, 1983, Delwart & Panganiban, 1989) Therefore, the expression of SU is inversely correlated with viral entry into cells.

4.3.1. Viral resistance in mice.

A number of genetic loci in the mouse have been linked with the restriction of viral replication in particular cell types. Of these, two have been directly linked with the expression of an endogenous SU protein.

4.3.1.1. The *Fv-4* resistance gene.

Fv-4 was first described as a gene mediating resistance to N- and NB-tropic Friend MuLV in the FRG strain of mice. (Kai *et al.*1976, Odaka *et al.*1978, 1981) $Fv\text{-}4^r$ expression was associated with the expression of a unique cell surface antigen related to ecotropic SU. (Ikeda & Odaka, 1983, 1984) A second locus, *Akvr-1* described in a number of species of feral mice (Gardner *et al.*1980) has shown 100% sequence homology to the proviral DNA found at the *Fv-4* locus. (Dandekar *et al.*1987) Resistance is dominant, and the gene frequency of the two alleles (r for resistant and s for sensitive) corresponded precisely for the observed viraemia and disease in LC feral mice. Both loci map to the same chromosome (Odaka *et al.*1981, O'Brien *et al.*1983) and the flanking DNA has the same restriction enzyme map.(Dandekar *et al.*1987) Both loci behave as alleles in cross breeding experiments (O'Brien *et al.*1983). The entire *Fv-4* proviral locus and approximately 13kb of upstream sequence has been cloned and analysed. It represents a truncated provirus consisting of the C-terminal 850bp of *pol*, an ecotropic related *env* gene, and 3' LTR. (Ikeda *et al.*1985) No 5' viral sequences were found in the 13kb of upstream flanking DNA and so this locus is the result of either a widespread deletion or the insertion of at least 12kb of cellular DNA. This upstream flanking DNA was shown to be important for the biological function of the provirus, and was considered to contain sequences capable of driving the transcription of the proviral DNA in the absence of the 5' LTR. (Ikeda & Sugimura, 1989) The molecular mechanisms controlling expression have not yet been described. Nucleotide sequence analysis of

the *env* gene of *Fv-4/Akvr-1* showed that it encodes complete SU and TM proteins. Comparison of the *Fv-4* sequence to other MuLV sequences showed only 70-75% sequence homology in the N-terminal region of *env* to inbred ecotropic viruses (Ikeda *et al.*1985) but 90% sequence homology to a cloned ecotropic virus isolated from a wild mouse (Cas-Br-E) (Masuda & Yoshikura, 1990) again indicating that the *Fv-4/Akvr-1* locus is more specific for wild mice and was incorporated into FRG mice by inbreeding with *Fv-4* positive wild mice. This gene therefore functions in a number of species of wild mice from south east Asia (or hybrids with other species) to restrict the replication of the wild mouse ecotropic virus by receptor interference. Although wild and inbred ecotropic virus SU share only about 70% homology, (Rassart *et al.*1986) this appears sufficient for the *Fv-4* gene product in FRG mice to bind the ecotropic receptor of inbred mice and establish viral interference.

4.3.1.2. The *Rmcf* resistance locus.

The *Rmcf* locus is another resistance gene that may exert its effects via the expression of an endogenous envelope glycoprotein. This locus was originally described in DBA/2 mice as a gene located on chromosome 5, mediating the resistance to MCF virus replication. (Hartley *et al.*1983) These mice also produce a non ecotropic SU protein related to MCF virus SU. (Bassin *et al.*1982, Ruscetti *et al.*1981) Other strains of mice permissive for MCF viral replication ($Rmcf^S$) express either no endogenous SU (IRW mice) or a xenotropic SU (C57BL/6). (Buller *et al.*1987) In back crosses, the two genes encoding the different endogenous SU proteins were found to be linked to *Rmcf* phenotype, and were allelic. (Buller *et* al.1987) However, one discordancy in co-segregation of these alleles in recombinant inbred mice suggested that *Rmcf* may not encode the non-ecotropic SU directly. (Frankel *et al.*1989a) The discordant *Rmcf* locus however, could lack sequence homologous to the oligonucleotide probe (28bp) used. The expression of $Rmcf^r$ linked SU has been shown to be restricted to erythroid precursors, BFU-E and CFU-E, as well as the myeloid progenitors, CFU-GM. (Buller *et al.*1989) A percentage of more primitive multipotential CFU-S also expressed *Rmcf*-related SU and these were considered to represent a sub-population that were about to differentiate. No expression of *Rmcf*-related SU was detected in

mature T or B lymphocytes or myeloid cells. *Rmcf* mediated resistance is thought to protect mice from early erythroleukaemia induced by Friend-MuLV by restricting the replication of recombinant MCF viruses in specific target cells. (Ruscetti *et al.*1981, Buller *et al.*1985, 1988, 1989) However there is no effect on the late myeloid, lymphoid or erythroid diseases induced by the ecotropic virus. (Ruscetti *et al.*1985) The restriction of MCF-related SU expression may reflect the target cells for the disease inducing Friend MCF virus. The *Rmcf* locus does not protect mice from disease induced by Moloney-MuLV MCF recombinants. (Brightman *et al.*1991) This difference has been mapped to the LTR of Moloney-MCF recombinants and possibly results from the targeting of the Moloney-MCF virus to a cell population which does not express *Rmcf.*

4.3.2. Viral resistance in chickens.

In the avian system, the expression of chf (ALV SU) has been shown to be responsible for the decrease in susceptibility in chf+ cells of ALV of subgroup E. (Robinson, 1976, Robinson *et al.*1981) The endogenous loci involved are *ev-3,-6* and *-9.* (Astrin, 1978, Astrin & Robinson, 1979, Astrin *et al.*1980b) *Ev-3* is also responsible for the expression of *gag* gene products identified as group specific antigens (gs). (Astrin & Robinson, 1979) The differences in susceptibility to infection of cells carrying one of the responsible endogenous loci was shown to be linked to the transcriptional activity of the loci and by inference the levels of SU production. (Robinson *et al.*1981, Shields *et al.*1978) Although the loci encoding subgroup E viruses are generally repressed (Rovigatti & Astrin, 1983), productive infections can be established (Vogt & Friis, 1971), and the endogenous *env* genes can rescue defective exogenous (Weiss, 1969) and endogenous viruses (Robinson *et al.*1979). It has also been suggested that the expression of enALV SU protects chickens from lethal inflammatory responses frequently seen in chf- chickens, by establishing a partial tolerance to ALV envelope antigens. (Halpern & Friis, 1978, Crittenden *et al.*1982) If enALV *env* is expressed during thymic education and is recognised as self, those antigens common to all ALV *env* proteins (group specific)

will also be regarded as self. Infection of exogenous ALV of subgroups A-D would therefore stimulate an immune response to only the proportion of antigens that are type specific, that is, specific for the particular subgroup or individual isolate. However the infection of an exogenous virus related to endogenous viral sequences has been shown, in some instances, to break tolerance, leading to attack of endogenous antigen-expressing cells by the immune system. (Ohashi *et al.*1991, Oldstone *et al.*1991)

Therefore there is a paradox, that expression of endogenous SU can lead to the rescue and outgrowth of viruses with altered receptor specificities, leading to infectious spread but that the same SU expression restricts the spread of these viruses.

SECTION 5. EXPRESSION OF FELINE ERVS.

The models of retroviral recombination with ERV sequences, and of resistance via receptor interference generally require the expression of the ERV involved. The expression of ERVs in chickens and mice has been extensively studied and some of the limiting factors have already been discused. (Section 3.3.)

The characterisation of the ERVs present in the domestic cat has only recently been studied in detail. The study of expression of endogenous FeLV elements (enFeLV) has long been hampered by the lack of highly specific DNA probes that were capable of high stringency identification of enFeLV sequences without being affected by the presence of exogenous FeLV.

S1 nuclease analysis of cellular RNA using labelled FeLV-B cDNA failed to detect enFeLV transcripts in the majority of virus free lymphomas, which generally occurs in older cats. (Niman *et al.*1977b) Further studies detected enFeLV related RNA in placental and embryonic thymus tissues of specific pathogen free animals. (Niman *et al.*1980, Busch *et al.*1983) Accompanying immune precipitations failed to detect any $p30^{gag}$ protein in any feline virus free tissues tested. Using a portion of FeLV-B *env* as a probe in Northern blot hybridisation analysis, Busch *et al* (1983) identified a number of *env* related RNA transcripts in the placental tissue of a virus

free cat, with sizes ranging from 4.8-1.8kb. In this study no *gag* or *pol* related transcripts were detected.

Of a number of enFeLV loci isolated all but one failed to display significant promoter activity, and in one case the lack of activity was shown to be due to repression by cis acting sequences in the flanking DNA. (Berry *et al.*1988)

CHAPTER 2 MATERIAL AND METHODS.

INDEX

2.1 MATERIALS.

2.1.1. Chemicals.

All chemicals were of "Analar" quality, and were obtained from the Sigma Chemical Company, BDH Chemicals, Boehringer Mannheim, Gibco BRL or Pharmacia Ltd, except:

Ampicillin "Penbritin" - Beecham Ltd.

Ethyl alcohol - James Burrows (FAD) Ltd.

Bacto Agar and Tryptone - Difco labs.

Yeast Extract - Beta Lab.

PBS tablets - Oxoid Ltd.

The sources of chemicals specific for particular procedures are given in the relevant methods section.

All enzymes were from Gibco BRL, NBL or Pharmacia Ltd. except where indicated.

2.1.2. Radiochemicals.

$\alpha[^{32}P]$ dCTP [3000 Ci/mmol]	Amersham International plc
$[^{35}S]$ dATP αS [600 Ci/mmol]	Amersham International plc
[35S]-Met/Cys Trans35S-Label [1000 Ci/mmol]	ICN Flow.

2.1.3. Equipment.

Particular pieces of equiptment are mentioned in the relevant section. The following are the manufacturers of the most general items:

Microcentrifuge tubes	Sarstedt	
Pipette tips	Sarstedt	
Tissue culture flasks	Nunclon	
Collodium dialysis bags	Sartorius	
Hybridisation membranes	Genescreen	Dupont NEN Research Products
	Nitrocellulose	Schleicher+Schuell
Film for autoradiography	Kodak	

pFGA-5/	Replication competent subgroup A virus clones (FeLV-A/Glasgow-1)
pFGA-22	(Stewart *et al.*1984).
pFGB	Replication competent subgroup B virus (FeLV-B/Gardner-Arnstein)
	(Mullins *et al.*1981.)

2.1.4. Cloning Vectors and recombinant DNA clones

pIC20H/ pIC20R	lac z^+/$ampR^+$ pBR322 derived plasmid vectors. (Marsh *et al.*1984)
pBluescript	lac z^+/$ampR^+$ pUC19 derived phagemid vector (Stratagene).
pGEX-2T	$ampR^+$ GST gene fusion vector (Pharmacia).
pLCM-1	full length endogenous provirus clone in pK125 (pBR322 with a HaeIII deletion) (from Dr J Mullins, Stanford University).
pLCM1/KO	a modified version of pLCM-1 containing a single LTR. (Robert McFarlane).
pBCM-3	full length endogenous provirus clone in pBR322 (from Dr J Mullins, Stanford University).
pGMB-3	Replication defective subgroup B component of the FeLV/GM1 complex in pUC18. (Tzavaras *et al* 1990)
pEPP-3	Subclone of pGMB-3 containing a 2.1kb PstI fragment containing the complete envelope gene in pIC20H (Dr A Tsujimoto, National Cancer Centre Research Institute, Tokyo.).

2.1.5. Cell lines.

AH927	Non-established primary feline fibroblasts. (Rasheed & Gardner, 1980) Maintained in Special Liquid Medium (Gibco).
FEA	Non-established primary feline fibroblasts. (Sarma *et al.*1975) Maintained in Special Liquid Medium (Gibco).
3201B	Virus negative T-cell tumour line established from a thymic lymphosarcoma. (Snyder *et al.*1978) Maintained in RPMI medium (Gibco)/15% FCS.
MCC	Virus negative lymphoid cell line derived from a large granular lymphoma. (Cheney *et al.*1990) Maintained in RPMI medium (Gibco)/15% FCS.

T3	Virus positive T-cell tumour line established from a thymic lymphosarcoma. (Neil, 1984) Maintained in RPMI medium (Gibco)/10% FCS.
F422	Virus positive T-cell tumour line established from a thymic tumour. (Rickard *et al.*1969) Release FeLV-A/Rickard. Maintained in RPMI medium (Gibco)/10% FCS.
FL74	Virus positive T-cell tumour line established from a thymic tumour. (Theilen *et al.*1970) Release FeLV-ABC/KT. Maintained in RPMI medium (Gibco)/10% FCS.
BHK21-pFGA-5	Suspension syrian hamster kidney fibroblast line derived at Wellcome Co. Maintained in modified Eagle's Medium (SMEM) (Gibco).

2.1.6. Experimental Animals.

All feline tissues came from the Feline Breeding Colony, Veterinary School, University of Glasgow.

2.1.7. Bacterial Strains.

E Coli JM101 is designed for use with lacZ-containing M13 phage vectors. The natural lac locus is deleted and that part of lacZ which can be complemented with the β-galactosidase gene contained in the vector sequences is incorporated into an F plasmid, along with the repressor sequence. This allows blue/white colony screening of colonies on bacterial plates containing X-gal.

E Coli DH5α, prepared by BRL is designed for the generation of cDNA libraries using pUC derived plasmid vectors. The lacZ marker provides α-complementation of the β-galactosidase gene contained in these vectors therefore can be used to produce blue/white colony screening of colonies on bacterial plates containing X-gal.

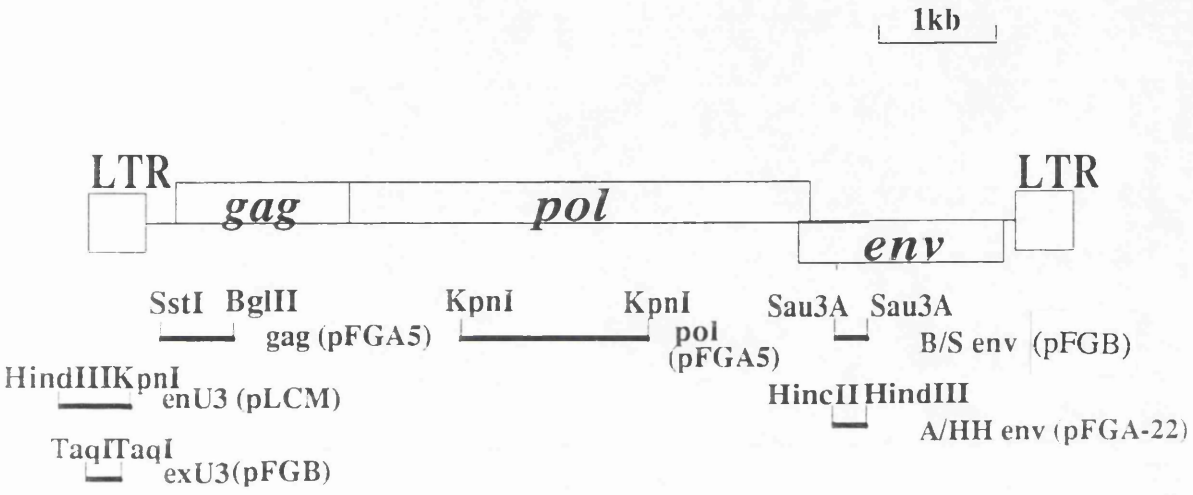


Fig.5. Origin of DNA probes specific for FeLV.

The FeLV-specific DNA restriction fragment probes used in this study are shown, and their locations shown relative to the virus genome. Indicated are the restriction sites used to generate the probes and the cloned proviruses from which they were derived.

2.1.8. DNA probes.

A number of DNA probes specific for particular regions of the FeLV genome were used in this work. The relative position to the genome, restriction enzymes used and the virus source of each probe is shown in Figure 5. The preparation and relative position to the genome of new, synthesised probes is reported in the results sections.

The presence of intact cellular RNA in Northern blots was tested by re-hybridisation of the blots with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe provided by Robert Hennigan (Beatson Institute for Cancer Research)

2.1.9. FeLV-specific sera.

Goat anti-F422 $gp70^{env}$ was raised by immunisation with lentil-lectin purified FeLV-A protein from F422 tumour cells. (V. Moennig, University of Giessen, Germany) Rabbit anti-FeLV $p15^{gag}$ was raised by immunisation with sepharose 6B chromatography purified FeLV-A protein (J.Neil)

2.1.10. Solutions and growth media

Acrylamide Elution Buffer: 500mM NH_4OAc, 10mM $Mg(OAc)_2$, 0.1mM EDTA, 0.1% SDS.

Acrylamide Gel (6% denaturing): 180ml acrylamide/bisacrylamide(19:1), 252g urea (42%), 60ml 10x TBE, 165ml dH2O.

Acrylamide Gel (13% protein gel): 17.3ml acrylamide/bisacrylamide (30:0.39), 15ml 1M Tris pH 8.8, 3.5ml glycerol, 400µl SDS (to 40ml total volume).

Acrylamide Gel (stacking gel): 4.5ml acrylamide/bisacrylamide (30:0.8) 5.75ml 0.5M Tris pH 6.8, 300µl SDS (to 30ml total volume).

Agar plates: 500ml L-broth, 1.5% bacto agar, 100µg/ml ampicillin.

Buffer A: 10mM EDTA, 10mM Tris, 10mM NaCl, 0.5% SDS, pH 8.0.

Coating buffer: 15mM Na_2CO_3, 33mM $NaHCO_3$, pH 9.6.

Denaturation Buffer: 1.5M NaCl, 0.5M NaOH.

Denhardt's Solution (50x): 1% bovine serum albumin, 1% Ficoll (Fraction V), 1% polyvinyl pyrollidone.

DNA running dye (10x): 30% glycerol, 0.2% bromophenol blue, 0.2% xylene cyanol green.

GTE buffer: 50mM glucose, 25mM Tris, 10mM EDTA, pH 8.0.

Guanidinium buffer: 8M Guanidinium chloride, 20mM NaOAc, 5mM EDTA, 0.05% β-mercaptoethanol, pH 7.0;

H plates: 1% bactotryptone, 0.8% NaCl, 1.2% agar, w/v.

H top agar: 1% bactotryptone, 0.8% NaCl, 0.8% agar, w/v.

L-broth: 1% Bacto tryptone, 0.5% Yeast extract, 1% NaCl, w/v.

Lysis solution for bacteria: 50mM Glucose, 25mM Tris, 10mM EDTA, pH 8.0.

Lysis solution for eukaryotic cells (5x): 0.5M Tris, 0.7M NaCL, 5mM EDTA, 5% Triton-X-100, 2.5% deoxycholate, 0.5% SDS, 5% aprotinin, w/v, pH 7.4.

Minimal medium: 34mM Na_2HPO_4, 22mM KH_2PO_4, 20mM NH_4Cl, 8mM NaCl, 1mM $MgSO_4$, 0.1mM $CaCl_2$, 0.1mM Thiamine hydrochloride, 0.2% D-glucose.

MOPS (10x): 200mM MOPS (morpholinopropane-sulphonic acid), 50mM NaOAc, 10mM EDTA, pH 7.0.

Neutralisation Buffer: 3M NaCl, 0.5M Tris pH 7.0.

Neutralisation solution for plasmids: 60% 5M potassium acetate, 11.5% acetic acid.

PEG/NaCl: 20% polyethylene glycol 6000, 2.5M NaCl.

Phosphate Buffer (20x): 0.5M Na_2HPO_4, 0.5M NaH_2PO_4. pH 6.5

Prehybridisation solution: 23ml 50% formamide, 10ml 20xSSC, 4ml 50x Denhardt's solution, 4ml 20x phosphate buffer, 8ml 50% dextran sulphate, 0.2ml 20% SDS.

Protein Gel Fix-Stain: 42.1% methanol, 5.26% Acetic acid, 0.0125% Coomassie Blue.

Protein Gel Destain: 5% Methanol, 7.5% Acetic acid v/v

Protein Sample buffer(2x): 0.0625M Tris, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.8.

RNA Loading Dye (10x): 50% glycerol, 1x MOPS buffer, 0.2% bromophenol blue.

RNA Loading Buffer: 50% formamide, 2.2M formaldehyde, 1x MOPS buffer.

RPMI medium: RPMI medium/ 10 or 15% FCS/ 2mM glutamine/ 0.15% $NaHCO_3$ w/v.

SLM medium: Special liquid medium/ 9% FCS/ 4mM glutamine.

SMEM: 9% Suspension Modified Eagle's Medium (Gibco), 9% Tryptose phosphate broth (Gibco), 9% FCS, 0.15% $NaHCO_3$, w/v, 4mM glutamine.

SSC (20x): 3M NaCl, 0.3M trisodium citrate.

STET: 5% Triton-X-100, 50mM EDTA, 50mM Tris, 8% sucrose, pH 8.0.

Substrate buffer: 0.1M Tris, 0.1M NaCl, 5mM MgCl2, pH 9.5.

TAE (1x): 40mM Tris, 20mM NaOAc, 20mM NaCl, 2mM EDTA, pH 8.15.

TBE (1x): 90mM Tris, 90mM boric acid, 2.5mM EDTA, pH 8.3.

TBS (5x): 20mM Tris, 500mM NaCl, pH 7.5.

TE: 10mM Tris, 1mM EDTA, pH as appropriate.

Towbin (Western Buffer): 25mM Tris, 200mM glycine, 20% methanol, v/v, pH8.3.

Tris-glycine buffer: 25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3.

2xTY medium: 1.6% bactotryptone, 1% yeast extract, 0.5% NaCl w/v.

X-gal top agar: 1.5% bacto agar, 100ug/ml ampicillin, 200ug/ml X-gal in L-broth.

2.2. METHODS.

2.2.1. Isolation of high molecular weight DNA.

High molecular weight DNA was prepared from 10^8 cells or 200-500mg of tissue.

Cells were washed in PBS before resuspending in 5ml guanidinium chloride buffer. Frozen tissues were ground in a mortar then disrupted in 5ml guanidinium chloride buffer, in a "Dounce" homogeniser with 8-10 strokes of a loose fitting pestle. An equal volume of isopropanol was added and the precipitated DNA spooled gently through the interface using a glass hook. The spooled DNA was washed sequentially with 3 washes of 70% then 3 of 100% ethanol. The dehydrated DNA was air dried for a few minutes before resuspending in an appropriate volume

of Buffer A (5ml) containing 50µg/ml Proteinase K. The solution was gently shaken o/n at 37°C. Three phenol extractions were performed to remove contaminating proteins, then the solution was dialysed against TE pH 8.0 until all phenol was removed. The solution was incubated with 50µg/ml RNase for 1 hour at 37°C, then repeated phenol extractions performed. The solutions were dialysed against TE pH 8.0 until all phenol was removed (OD_{270}<0.05). The yield of DNA was calculated on the basis that a 50µg/ml solution of double stranded DNA would have an OD_{260} measurement of 1

2.2.2. Isolation of plasmid DNA.

Recombinant plasmid DNA was isolated according to the alkaline lysis procedure introduced by Birnboim & Doly, (1979) involving the denaturation of E.coli DNA and the selective renaturation of covalently closed circle DNA.

(i) Small scale isolation.

A single colony was innoculated into 10ml of L-broth and incubated at 37°C o/n. 1.5ml of o/n culture was pelleted, then resuspended in 300µl STET and 50µl of fresh lysozyme solution added (10mg/ml in STET). The solution was boiled for 45 seconds, then the lysate cleared in a microcentrifuge (12,000xg). The pellet was removed and the DNA precipitated from the solution with 0.5 volumes 7.5M ammonium acetate and 2-3 volumes ethanol in dry ice for 30 minutes. The precipitate was pelleted in a microcentrifuge, washed in 70% ethanol, dried and resuspended in 20µl dH2O.

(ii) Large scale purification.

High quality plasmid DNA was purified from a 500ml exponentially growing o/n culture. Cells were pelleted at 4,420xg at 4°C, then resuspended by vigorous stirring in 20ml lysis solution containing 5mg/ml lysozyme. After 30 minutes on ice, 40ml of fresh 0.2M NaOH/1% SDS was added, and left on ice for a further 5 minutes. The lysates were neutralised with 30ml of neutralisation solution for 15 minutes on ice. The lysates were clarified by centrifugation at 11,300xg for 5 minutes and the supernatant filtered through gauze swabs (Vernon Carus Ltd.).

DNA was precipitated with the addition of 0.6 volumes of isopropanol, at room temperature for 30 minutes, or -20°C o/n, and pelleted by centrifugation at 11,300xg for 5 minutes. The pellet was drained thoroughly then resuspended in 6.7ml TE pH 8.0., then 7.2g caesium chloride and 700µl of a 3mg/ml solution of ethidium bromide added. The refractive index was adjusted to 1.390 with CsCl or TE and the plasmid DNA separated on a density gradient by centrifugation at 126,000xg for 40 hours at 20°C. Closed circular DNA (the lower band) was removed by syringe and needle and the ethidium bromide removed by repeated extraction with isopropanol until no trace of colour was left. The CsCl was removed by extensive dialysis in collodium bags against TE pH 8.0 and the yield of DNA calculated on the basis that a 50µg/ml solution of double stranded DNA would have an OD_{260} measurement of 1. The DNA was concentrated by extraction of the aqueous phase with butan-2-ol then precipitated with 0.1 volumes 3M NaOAc, and 2-3 volumes ethanol.

An intermediate scale preparation from 100ml of culture used the corresponding volumes of reagents and the density gradient produced on a Beckman TL-100 bench top ultracentrifuge at 346,000xg for 18-20 hours.

(iii) Small scale preparation of sequencing grade plasmid DNA.

1.5mls of o/n cultures were spun on a bench top microcentrifuge (12,000xg), and the pellets resuspended in 60µl GTE buffer and 40 µl of GTE with 10µg/ml lysozyme added. This was mixed gently, and 200µl 0.2M NaOH/1% SDS added and the solutions incubated on ice for 5 minutes. 150µl of plasmid neutralisation solution was added then mixed well and incubated on ice for 10 minutes. 450µl of 5M $LiCl_2$ was added and incubated on ice for 5 minutes. The lysates were clarified by centrifugation for 5 minutes at 12,000xg and the DNA precipitated from the supernatant with 0.6 volumes isopropanol. After 5 minutes on ice, the DNA was pelleted, then washed with 80% ethanol, and dried. The pellet was disolved in 50µl TE containing 0.01µg/ml RNase A and incubated at 37°C for 30 minutes. The DNA was precipitated with 0.6 volumes of 20% PEG/ NaCl at 0°C for 2-3 hours. The DNA was pelleted by a 5 minute spin, washed with 70% ethanol, dried and

resuspended in 20-50µl TE. A third of this DNA preparation was used per sequencing reaction.

2.2.3. Preparation of M13 phage replicative forms.

M13 vectors were prepared in double stranded replicative form for sub-cloning. Fresh plaque stabs or glycerol stocks were incubated in 5ml 2xTY medium for 3 hours. Concurrently, 5ml of a freshly grown o/n culture of E.Coli JM101 was diluted 1:100 with fresh medium and grown for 3 hours. The two cultures were mixed, and incubated for a further 5 hours, the bacteria pelleted and vector DNA extracted as in 2.2.2.(ii).

2.2.4. Preparation of single stranded M13 phage DNA.

Single stranded M13 DNA was prepared by a method based on that of Sanger *et al.* (1980), described in the Amersham "Cloning and Sequencing" handbook. Bacteria expressing recombinant M13 phage were inoculated into 1.5 ml of a 1:100 dilution of a freshly grown JM101 culture, in polycarbonate tubes, and shaken at 37°C for 5 hours. The cells were pelleted by microcentrifugation for 10 minutes and the virus precipitated from the supernatant with 200µl PEG/NaCl incubated at room temperature for 15 minutes. After the virus was pelleted by a 5 minute spin, all the liquid was carefully removed then the DNA purified from the virus by phenol extraction followed by ethanol precipitation. The DNA was dried and resuspended in 24µl of TE. 3µl was was used for analysis by agarose gel electrophoresis, and typically 7µl of DNA used per sequencing reaction.

2.2.5. Isolation of cellular RNA.

(i) Isolation of total cellular RNA.

Total cellular RNA was prepared by a Guanidinium thyocyanate-phenol chloroform extraction method (Chomczynski & Sacchi, 1987) marketed as RNasol B (Biogenesis Ltd.) All procedures were performed using diethyl pyrocarbonate treated plasticware. The concentration of RNA extracted was calculated on the basis

that a 40µg/ml solution of single stranded RNA would have an OD_{260} measurement of 1 RNA was stored in a small volume of diethyl pyrocarbonate treated dH2O at -70°C.

(ii) Isolation of poly-$(A)^+$ mRNA.

Poly-$(A)^+$ mRNA was separated from total cellular RNA using the Poly-ATtract mRNA Isolation System (Promega) which uses a biotinylated oligo d(T) primer to hybridise in solution to the 3' poly (A) region present in eukaryotic mRNA species. The hybrids are captured and washed using streptavidin coupled to magnetic particles and a magnet. The mRNA was eluted from the solid phase by the addition of ribonuclease free dH2O. The concentration of mRNA was determined by spectrophotometry, by the assumption that a 40µg/ml solution of mRNA has an adsorbance at 260nm of 1. For further use, the RNA was concentrated by alcohol precipitation using 0.1 volumes of 3M sodium acetate, and 1 volume of isopropanol, which was incubated at -20°C o/n. The RNA was pelleted, dried for 15 minutes in a freeze dryer, and resuspended in ribonuclease free dH2O at 1µg/µl at -70°C.

2.2.6. Southern blotting

DNA was prepared for hybridisation analysis by the transfer technique of Southern. (1975)

20µg high molecular weight DNA was digested with 100 units of the appropriate restriction enzyme overnight, precipitated and resuspended overnight in 45µl 0.1x TE. 3µl of running dye was added and the digested DNA separated by electrophoresis in a 250ml (20x14.5cm) 0.8% agarose/TAE gel o/n at 25V. λphage DNA, digested by HindIII was used as molecular weight markers (1µg of cold markers and $5x10^3$ counts per minute of end labelled DNA per lane). After electrophoresis, the gels were stained in buffer containing 3µg/ml ethidium bromide. The DNA was visualised using a 312nm transilluminator and the gel photographed using Polaroid Type 57 high speed film. The gel was trimmed and measured then washed for 30 minutes each in denaturation buffer then neutralisation buffer. Finally

the gel was washed in three changes of 1x phosphate buffer for a total of 90 minutes. The DNA was then transfered to a filter membrane by capillary blotting.

A glass plate just larger than the gel was placed in a dish half full of phosphate buffer, supported above the buffer by rubber bungs. Three thicknesses of Whatman 3MM paper were immersed in buffer and placed on the glass plate so as the ends of the paper were placed in the buffer reservoir and all air bubbles between the sheets carefully removed by rolling a glass tube over them. The gel was carefully placed on top of the paper, and a presoaked piece of GeneScreen Hybridisation Transfer Membrane (DuPont) cut to the same size as the gel was carefully layered on top. This was covered by two pieces of precut and presoaked 3MM paper. Again all air bubbles were carefully removed. The area around the gel was sealed using pieces of X-ray film to ensure that the capillary action was concentrated over the area of the gel. Several layers of paper towels placed on top provided the capillary action, and these were weighted down and the transfer allowed to proceed for 16-20 hours. The membrane was carefully removed, washed once in 1x phosphate buffer, and the DNA permanently bound to the membrane either by baking at 80°C for 2-6 hours, or UV cross-linking (UV Stratalinker 1800, Stratagene)

2.2.7. Northern blotting

20µg of total cellular RNA was lyophilised then resuspended in 20µl of RNA loading buffer. Samples were denatured at 65°C for 15 minutes and quenched on ice before 5µl RNA Running dye was added and the samples electrophoresed for 3-5 hours at 120V in 200ml 1% agarose gels containing 2.2M formaldehyde, and 1x MOPS, in 1x MOPS running buffer that was continually recirculated. RNA ladders (Gibco) were used as molecular weight markers. Commonly, 3-6µg were used per lane and prepared in the same way as the RNA samples.

The marker lanes were removed and separately stained in 3µg/ml ethidium bromide, and photographed. The gel was washed in three changes of distilled water to remove the formaldehyde before the RNA was transferred to a GeneScreen

membrane as described in 2.2.6. After blotting, the membrane was carefully removed and the RNA permanently bound to the membrane either by baking at 80°C for 2-6 hours, or UV cross-linking.

2.2.8. Radio-labelling of DNA probes.

(i) End labelling of λHindIII markers.

1μg of HindIII digested lambda (λ) phage DNA was added to 5μg of nick-translation solution 1 (Amersham Nick Translation Kit) which contained 100μM each of dATP, dGTP and dTTP. This was incubated with 5μl α[^{32}P] dCTP and 10 units of T4 polynucleotide kinase in a total aqueous volume of 50μl for 5 minutes at 37°C, then 100μM unlabelled dCTP added, and incubated for a further 25 minutes. The sample volume was increased to 200μl and phenol extracted. The sample was dialysed against TE pH 8.0 in microcollodion bags for at least 5 hours with more than one change of TE. The activity of 1μl was determined and the sample diluted appropriately.

(ii) Labelling of DNA probes by nick-translation.

PCR or restriction fragment DNA probes were radio-labelled using α[^{32}P] dCTP by a method of nick-translation (Rigby *et al.*1977) marketed as a Nick-Translation Kit (Amersham) and the products purified on Sephadex-G50 beads (Nick Columns, Pharmacia). Generally, incorporations of 10^7-10^8 cpm/μg were achieved.

2.2.9. Hybridisation of labelled probes to membrane bound nucleic acids.

The detection of specific nucleic acid species that had been immobilised onto membranes was achieved by the hybridisation of specific radiolabelled probes at high stringency. The membranes were pre-wetted with 1% Triton-X-100 before they were pre-incubated at 42°C in a sealed polythene bag containing 15ml prehybridisation solution that had been supplemented with freshly boiled salmon sperm DNA to a concentration of 0.4mg/ml. Freshly boiled DNA probe was added to the prehybridisation solution to a final concentration of 5ng/ml and incubated at 42°C overnight. After rinsing briefly in 2xSSC the membrane was washed in three

changes of 0.1xSSC/0.5%SDS at high stringency (65°C) for up to 60 minutes, then rinsed in 0.1x SCC prior to autoradiography. Blots were reprobed with GAPDH after the initial signal had decayed or after the probe had been stripped from the blot by washing at 70°C in 70% formamide for 30-60 minutes. Hybridisation with GAPDH was carried out under high stringency conditions, but washing stringency was reduced at 42°C for 20-40 minutes only.

2.2.10. Synthesis and purification of synthetic oligonucleotides.

Oligonucleotides were synthesised on an Applied Biosystems 392 DNA synthesiser. The 5' methoxy-trityl protecting group remained in place after incorporation of the final (5') nucleotide in order to ensure efficient purification of fully synthesised oligonucleotides from any aborted synthetic products. The oligonucleotides were eluted from the synthesis columns with 2ml concentrated ammonia, and the side chain protecting groups removed by overnight incubation at 55°C in air tight tubes. The trityl protected oligonucleotides were purified by their entrapment on a trityl affinity column. (Applied Biosystems) The column was prepared for trityl binding by the passage of 5ml of concentrated acetonitryl through the column followed by 5ml of 5M triethymamine. 1.5ml of the oligonucleotide/ammonia solution was further diluted with 500µl dH_2O before being passed through the column twice. The unbound oligonucleotides were removed from the column by subsequent passage through the column of 20% ammonia (15ml), dH_2O (10ml), 2% trifluoroacetic acid (10ml) and dH_2O (12ml). The purified oligonucleotides were released from the column by 2x1ml washes of 20% acetonitryl, the majority of the DNA being eluted in the first wash.

The DNA was lyophilised in a centrifugal evaporator system (Hetovac) and the resuspended in dH_2O. The concentration was determined by spectrophotometry, based on the assumption that a 35µg/ml solution of single stranded oligonucleotides has an absorbance at 260nm of 1. The oligonucleotides were resuspended at 1µg/µl for use as PCR primers, and at 30ng/µl for use as sequencing primers. Oligonucleotide solutions were stored at -20°C.

2.2.11. Amplification of DNA sequences by polymerase chain reaction.

Amplification of specific DNA sequences by polymerase chain reaction (PCR) (Saiki *et al.*1988, White *et al.*1989) was achieved using specific oligonucleotide primers (typically 20-22 bases long) and the Gene Amp DNA Amplification kit (Perkin Elmer Cetus) using AmpliTaq recombinant Taq DNA polymerase. All reagents except the template and enzyme were premixed to ensure standard conditions and to avoid contamination of the reactions with unwanted DNA. All the reagents were handled using Gilson positive displacement pipettes. 1μg of each oligonucleotide primer was used per reaction. Reaction mixes were placed in 0.5ml micro centrifuge tubes and the volumes adjusted to 95μl minus the volume of template to be used. 50μl of light liquid paraffin was layered on top to prevent the evaporation of substrates during the reaction. The template DNA (1μg of cDNA or 10ng of plasmid DNA) was added to all tubes with the exception of a control reaction containing primers only, to ensure products resulted from the amplification of template DNA. The DNA in the reaction mixture was denatured at 100°C for 7 minutes before 1 unit of enzyme was added and the tubes placed in the thermal cycler. Amplifications were carried out in a Pharmacia LKB Gene ATAQ Thermocycler under various programs as indicated for each experiment in the results sections. Products were visualised by acrylamide or agarose gel electrophoresis of 10% of the reaction mix before the remaining products were gel purified.

2.2.12. Synthesis of cDNA from poly(A) RNA.

Double stranded cDNA was synthesised from poly(A)$^+$ mRNA using a cDNA synthesis kit (Pharmacia) which utilised cloned MuLV reverse transcriptase. Both first and second strand syntheses were performed using 1-5μg of poly(A)$^+$ RNA (procedure A of the accompanying protocol). The products of the second strand synthesis were purified on Sephacryl S-300 spun columns using ligation buffer (BRL), and the DNA precipitated and resuspended in distilled H2O, prior to PCR amplification.

2.2.13. Agarose gel electrophoresis.

1% w/v agarose/TEA gels were used to separate DNA fragments of a size greater than 1kb for analysis. 20, 100 of 200ml gels were prepared as appropriate. DNA solutions were adjusted to 3% glycerol, 0.02% bromophenol blue, 0.02% xylene cyanol green, and electrophoresis carried out at about 5V/cm. After electrophoresis, the DNA fragments were stained with 3µg/ml ethidium bromide for 20 minutes then using a 312nm transilluminator.

For preparative electrophoresis of particular DNA fragments, low melting point agarose was used to separate fragments over 600bp, and for the separation of small fragments of DNA ranging from 200-600bp, 1% agarose/3% NuSieve GTG agarose (FMC) gels were used and the fragments purified by electroelution.

2.2.14. Purification of DNA from agarose gels.

(i) DNA of less than 2kb was simply purified from agarose by overnight incubation of the gel fragment in TE pH 8.0 then filtration/centrifugation of the DNA through a SpinX DNA filtration column (Costar)(12,000xg). The DNA was precipitated using 0.3M NaOAc and 2.5 volumes of ethanol.

(ii) DNA fragments greater than 2kb were separated on low melting point agarose gels, and the fragment of choice purified from the gel by first re-melting the fragment at 70°C in two volumes of 1xTEA buffer, and after cooling, repeated phenol extractions at room temperature. The DNA was precipitated using 0.3M NaOAc and 2.5 volumes of ethanol.

(iii) This most efficient method of purifying DNA from gels was used when only small amounts of DNA were available. The gel fragment was placed in a small piece of dialysis tubing, a small volume of the appropriate running buffer added, and the tube sealed. The tube was placed in an electrophoresis tank filled with buffer, the gel placed at the tube wall nearest to the cathode. A 50mA current was passed through the tank for 3 hours. The current was reversed once for 60 seconds then again for 30 seconds. The liquid was removed from the bag, and the transfer of DNA from the gel could be checked by looking for ethidium bromide fluorescence

of the DNA in the liquid and not the gel fragment. The DNA was precipitated, generally using 1µg of glycogen as carrier, dried and resuspended in an appropriate volume of distilled water.

2.2.15. Acrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis was the method of choice for the separation and accurate size estimation of DNA molecules of less than 1kb in length using acrylamide concentrations of 4-8%. The monomer:bis ratio was 19:1, and polymerisation was effected by the addition of 10% ammonium persulphate, w/v, and 0.08% TEMED, v/v. 13cm vertical gels of 1.5mm thickness were used (Atto) and run in TBE buffer, at 10V/cm. Products of sequencing reactions were electrophoresed on 6% acrylamide denaturing gels (containing 42% urea) of 2mm thickness and 48cm long (Flowgen) at about 30V/cm.

2.2.16. Purification of DNA from acrylamide gels.

This procedure was used when agarose gel electrophoresis gave insufficient resolution, particularly in the purification of PCR amplified DNA. DNA was eluted from the gel slice in 4 volumes of acrylamide elution buffer overnight at room temperature. The DNA was precipitated as normal, using 1µg glycogen as carrier and resuspended as required.

2.2.17. Enzymatic manipulation of nucleic acids.

Restriction endonucleases, T4 polynucleotide kinase and calf intestinal alkaline phosphatase were all used according to the manufacturers' recommendations in the buffers supplied.

(i) 100 units of enzyme were used to digest high molecular weigh DNA, the first 50 units incubated with the DNA overnight then the second 50 units added for a final 2 hours of digest. Generally, 1-5 units of enzyme were used per µg of plasmid DNA to be digested for 1 hour.

(ii) 1 unit of T4 polynucleotide kinase was incubated with the appropriate buffer, 1µl of 10mM ATP and approximately 0.5µg of DNA in a kinase reaction to add 5' terminal phosphates to synthetic DNA molecules. The reaction was carried out at 37°C for 1 hour.

(iii) 0.01 units of calf intestinal alkaline phosphatase (CIAP) in the appropriate buffer, to remove the 5' terminal phosphate groups from 1pM of vector DNA with 5' protruding ends at 37°C for 30 minutes. For 5' recessed ends or blunt ends, 1 unit per pM was used at 50°C for 1 hour. The CIAP was inactivated by heating at 75°C for 10 minutes.

(iv) Ligations were carried out according to the protocols supplied by BRL Ltd together with their T4 DNA ligase and 5x buffer. 50ng of vector and generally a 10 fold molar excess of insert DNA was used in a minimal volume (20µl) and ligations carried out at 12-14°C overnight. Blunt end ligations, which are much less efficient than sticky end ligations, were incubated at 4-6°C overnight.

2.2.18 Growth and manipulation of bacteria.

Stocks of bacteria were stored in growth medium adjusted to 10% glycerol at -20°C. E.Coli strain JM101 was maintained in a state that supported M13 infection by selection for the proAB gene on the F plasmid using minimal medium. JM101 was grown in 2xTY medium overnight prior to use for the propagation of recombinant M13 phage. DH5α competent cells (BRL) were stored at -70°C and grown in L-Broth.

(i) Preparation of competent cells.

JM101 strain E.Coli were grown to an OD_{660} of 0.4-0.6 and then pelleted by centrifugation at 1,430xg for 10 minutes. The cells were resuspended in half the growth volume of ice-cold 50mM $CaCl_2$.6H20 and incubated on ice for 15 minutes. After pelleting at 4°C, the cells were resuspended in one tenth of the original growth volume of ice-cold 50mM $CaCl_2$. Competent JM101 cells were used on the day of preparation. Competent E.Coli host cells for plasmid DNA transformation (DH5α) were purchased from BRL.

(ii) Transformation with phage or plasmid vector DNA.

To 300µl of competent JM101 cells was added 10µl of ligation mix containing 25ng of phage vector DNA, or 5ng of uncut vector as a control. The mixture was incubated in a polycarbonate tube for at least 40 minutes (the longer the incubation, the higher the efficiency) then the cells were heat shocked by incubation at 42°C for 3 minutes. The cells were then returned to the ice bath. 200µl of fresh exponentially growing JM101 cells was added to each tube of heat shocked cells followed by 40µl of 100mM IPTG and 40µl 2% X-gal in dimethylformamide. 3ml of molten (42°C) H-top agar was added and mixed by rolling before pouring immediately onto prewarmed (37°C) H plates. After the plates had set they were incubated overnight at 37°C. Recombinants were selected by virtue of their colourless plaque phenotype in the presence of X-gal.

For plasmid cloning, the ligations were diluted 5-fold, and one twentieth (5µl) added to 20µl of DH5α cells. Each sample was incubated on ice for 30 minutes, heat shocked for 45 seconds at 42°C then 180µl of L-broth containing 10mM MgCl, 10mM $MgSO_4$ added and each sample incubated with shaking for 1 hour at 37°C. 100-200µl of cells were spread on L-broth agar plates containing 100µg/ml ampicillin. If colour selection was required, X-gal (5-bromo-4-chloro-3-indoyl-βgalactoside) was included the plates as a top agar layer containing 300µg/ml X-gal using a 2% solution in dimethyl formamide. As before, the plates were incubated overnight at 37°C. Recombinants were selected by virtue of their colourless colony phenotype in the presence of X-gal.

2.2.19. Sequencing of nucleic acids.

Sequencing of both single and double stranded DNA was carried out according to the chain termination method of Sanger *et al.* (1977) using the reagents and protocol of the "Sequenase" version 2.0 reagent kit (United States Biochemical). Plasmid DNA was first denatured by the addition of 2µl 2M NaOH and 8µl TE to 10µl of DNA solution (about 5µg). After 5 minutes at room temperature the solution was neutralised with 8µl of 5M NH_4OAc and precipitated. The DNA was dried and

Fig.5a Oligonucleotide sequencing primers

Oligonucleotide primers used to sequence enFeLV *env*. Numbers refer to position relative to cfe-16 *env* (Fig. 18) with with the start of the signal peptide as zero.

2/3	-32	5' TCAGACGACCCCAGCTCAGA 3'	-13
70A	94	5' GGAATGGCCAATCCTAGTCC 3'	107
2/4	286	5' TACGGATGTGATCAGCCTAT 3'	306
2/1	531	5' CGATAAAGCTGTTCACTCCT 3'	551
2/2	540	5' TGTTCACTCCTCGACAACGG 3'	559
3/5	107	5' GGACTAGGATTGGCCATTCC 3'	94
70B	372	5' ACATTGCTTCCGGTTGGCAT 3'	352
3/1	551	5' AGGAGTGAACAGCTTTATCG 3'	531
3/2	559	5' CCGTTGTCGAGGAGTGAACA 3'	540
4/1	887	5' GCATATTATGACAGGGGGTC 3'	868
4/2	963	5' GGAGCTAAACGGTTGACTTA 3'	944
C	1084	5' CCAGAATGAGGGGAACAAAC 3'	1065

Additional oligonucleotide primers used to sequence FeLV-B/GM1 gp70^{env}. Numbers refer to the position relative to pOF-3 (Fig.24)

1	708	5' TATCCCGGCAAGTAATGACC 3'	727
9	845	5' AAACAGGGTCCAAAGTGGCG 3'	864
3	1102	5' CTTAGGTACCTACAGCAACC 3'	1121
4	1303	5' CTGTAACACTGGACTCACCC 3'	1322

resuspended in distilled water, and one third (1-2µg) used per sequencing reactions. 6% denaturing acrylamide gels were used to separate the labelled, terminated fragments and gels were run at 1500V/ 30-40mA. Aliquots of each reaction were run for 2, 4 and 6 hours, usually allowing the reading of 400bp of DNA sequence per primer. The sequencing primers are listed in Fig.5a

G or A tracking involved a miniaturisation of the procedure, using only half the DNA mix and only one chain terminating dideoxynucleotide in order to allow the screening of larger numbers of samples. Those samples exhibiting dGTP or dATP motifs as expected, were fully sequenced.

2.2.20. Production of bacterial fusion proteins.

(i) Small scale

To test for the production of the appropriate sized fusion protein from cloned vectors, individual colonies were picked, and grown overnight in 10ml of L-broth with 100µg/ml ampicillin. In 24 well plates (1cm diameter) 2x1ml of a 1:10 dilution of each culture was incubated for 2 hours at 37°C then production of fusion protein induced in one of each pair of cultures by the addition of 23µg/ml IPTG. The cultures were incubated for a further two hours before the cells were transferred to 1.5ml Eppendorf tubes, pelleted in a microcentrifuge, and resuspended in 100µl of 2x protein sample buffer, boiled for 5 minutes to disrupt the cells and 30µl loaded on each lane of a 13% SDS polyacrylamide gel with stacker. Protein standards (116-30kD, BRL) were also run on the gels. Electrophoresis was carried out at 12V/cm. The proteins were visualised by staining with protein gel fix-stain and destaining before drying the gel under vacuum at 80°C.

Gels to be immunostained were run with prestained protein standards (200-14.3kD, BRL) and transferred to nitrocellulose by Western blotting.

(ii) Large scale

Large quantities of fusion proteins were purified according to a modification of that used in the procedure of Smith & Johnson, (1988) for the production of purified GST fusion proteins. 10 litres of a 1:10 overnight culture of pGEX vector

clones in L-broth were incubated with shaking, for two hours at 37°C. IPTG (23mg/l) was added to induce fusion protein production and the incubation continued for a further two hours. The cells were pelleted by centrifugation at 11,300xg for 5 minutes and resuspended in as small a volume as possible (usually 10-20ml) of ice cold 1xPBS/10mM EDTA/20mM benzamidine. The cells were lysed by sonication, 2x30 seconds at maximum amplitude. PMSF to 0.1μM was added to inhibit protease activity, and the lysates centrifuged at 27,200xg for 30 minutes to remove the cellular debris. The soluble fraction was incubated with glutathione bound agarose beads, at room temperature for 30 minutes, with shaking. The bead and protein mixture was placed in a chromatography column and the unbound fraction washed through with 1x PBS. The pure GST fusion protein was eluted from the column by competition with 10mM glutathione. Aliquots of sample at each stage of the purification were collected and run on 13% SDS polyacrylamide gels as in 2.2.20(1). The amount of protein produced was calculated on the basis that a typical 0.5mg/ml solution should have an OD_{260} of 1 and the protein was concentrated by lyophilisation.

2.2.21. Western blot analysis of proteins.

Proteins that had been separated by SDS-PAGE were transferred to nitrocellulose membranes by Western blotting in a Biorad Trans-Blot Cell. The blot was set up by layering first a piece of "Scotchbrite" soaked in Towbin buffer, then two layers of Whatman 3MM paper, again presoaked. The gel was carefully placed on the paper and a presoaked and precut piece of nitrocellulose placed on top. The sandwich was completed by the addition of a double layer of 3MM paper and a second piece of "Scotchbrite" before the blotting frame was closed over and inserted into the tank filled with Towbin buffer, with the gel at the cathode end of the tank. Electrophoresis was carried out at 20V overnight at 4°C.

2.2.22. Immunostaining of immobilised proteins

Proteins immobilised onto nitrocellulose by Western blotting were immunostained using a secondary antibody conjugated with alkaline phosphatase. The blot was removed from the tank and washed 2x30 minutes in 3% gelatine/ 1xTBS/ 0.01% NaAzide w/v at 37°C. The membrane was then washed twice in 1xTBS/ 0.01% NaAzide w/v/ 0.05% Tween-20 (TTBS) to remove excess gelatine block. The primary antiserum, goat anti-F422 gp70 was diluted 1:1000 in TTBS with 1% gelatine and incubated with the membrane for 1 hour at 37°C. After two washes of TTBS the second antiserum (anti-goat alkaline phosphatase conjugate, Pierce Warrener) was diluted according to the manufacturers instructions in TTBS and 1% gelatine and incubated with the membrane for 1 hour at 37°C. The membrane was washed again in TTBS then in TBS, and the colour reaction was developed by the addition of 50 ml substrate buffer containing 0.44mM tetrazolium Nitro Blue/ 0.44mM 5-bromo-4chloro-3-indoyl phosphate. The reaction was neutralised by washing the membrane extensively with water.

2.2.23. Immunisation of rabbits.

Rabbits were immunised sub-cutaneously with 500µg of protein in Freund's complete adjuvant, then boosted 3 weeks later with the same dose of protein in Freund's incomplete adjuvant. Pre and post immune sera were collected by bleeding the main ear vein. The blood was allowed to clot and the clot to shrink overnight at 4°C, then the serum separated by centrifugation at 1,430xg for 10 minutes. Serum was aliquoted and stored at -20°C.

2.2.24. Enzyme linked immunesorbent assay (ELISA)

The protein to be tested was dissolved at 1µg/ml in coating buffer and 100ng bound to each well in a 96 well Dynatech "Immulon" plate except for a negative control well to test for antibody binding to the plastic. This plate was incubated at 4°C overnight. Each well was washed thoroughly with 1xPBS/ 0.05% Tween-20. Each well was blocked with 1% BSA in PBS for 1 hour at room temperature then

washed as before. The antiserum to be tested was placed in the first well, and successive dilutions in PBS made in the remaining wells of each row. The plate was then incubated for 1 hour at room temperature and washed again as before. The secondary antibody conjugated with alkaline phosphatase was diluted 1:1000 in PBS and 100µl added to each well and this was incubated for 1 hour at room temperature. The wells were washed as before and then the colour reaction performed. The colour reaction solution was Sigma 104 phosphatase substrate at 1mg/ml in 0.1M glycine buffer with 1mM $MgCl_2$, 1mM $ZnCl_2$, pH 10.4. 100µl was added to each well. After a maximum of 30 minutes the reaction was stopped by the addition of 30µl 1N NaOH. The intensity of each reaction was measured on the MR7000 plate reader (Dynatech) at 405nm.

2.2.25. Growth and manipulation of eukaryotic cells.

Eukaryotic cells were maintained on plastic, at 37°C in an atmosphere of 5% CO_2. Suspension cells were grown up to densities of $5x10^6$ cells/ml. Adherent cells were grown to sub-confluence then rinsed briefly in PBS before trypsinisation with 0.25% trypsin for 3 minutes, and after washing the cells, replating at the required density in fresh medium. For long term storage, fibroblast cells were resuspended at approximately $3\text{-}5x10^6$/ml in medium adjusted to 10% glycerol and cooled overnight to -70°C in plastic vials, before being transferred to liquid nitrogen storage. Lymphoid cells were stored in the same way but with the replacement of 10% glycerol for 10% DMSO in the medium.

2.2.26. Infection of cells with FeLV.

BHK cells producing FeLV-A were grown and the supernatant containing virus harvested and filtered through 0.4µm filters to remove cells. $5x10^6$ cells were pelleted and resuspended in 5mls of the virus-containing supernatant. The medium was replaced after 2 hours and the cells grown for 2-3 weeks to allow virus spread.

2.2.27. Preparation of virus from cell supernatants.

The medium from 2 litres of exponentially growing cells was collected by pelleting the cells at 4,420xg for 5 minutes. The supernatant was mixed with an equal volume of saturated ammonium sulphate to precipitate the virions. After 10 minutes incubation at 4°C, the ammonium sulphate precipitate was pelleted by centrifugation at 4,420xg for 20 minutes at 4°C. The pellet was resuspended in a small volume PBS and any remaining debris pelleted by centrifugation at 3,000xg for 10 minutes. The virus was purified through a sucrose gradient, banding at the interface between the 50% and 20% sucrose layers after centrifugation at 53,800xg for 2 hours at 4°C. The virus band was removed using a syringe and needle, and diluted 1:2 in PBS. The purified virus was pelleted at 272,400xg in 1 hour, and the viral pellet resuspended in 1ml RNasol to prepare RNA.

2.2.28. Metabolic labelling of cellular proteins.

Exponentially growing cells were rinsed in PBS and 10^6 cells incubated in 1ml of pre-gassed methionine free medium/ 4mM glutamine for 30 minutes at 37°C in sealed eppendorf tubes. This was done to deplete the cell's amino acid reserves, and to allow efficient incorporation of the labelled amino acids. 100µCi of [^{35}S] met/cys label (NEN) was added and the cells labelled for 30 minutes to examine the immediate products of protein synthesis.

2.2.29. Immune-precipitation of radio-labelled cell proteins.

Labeled cells were pelleted by microcentrifugation, rinsed twice in 1x TBS pH 7.4 and lysed by the addition of 500µl of 1x eukaryotic lysis solution. The samples were incubated on ice for 15 minutes before being spun in a microcentrifuge for 5 minutes to precipitate the insoluble debris. Each sample was split in two and either control serum or test serum added to each aliquot. The reaction mixture was allowed to stand on ice for 60 minutes. 100µl of protein A sepharose (Sigma 100mg/ml preswollen in 1x lysis buffer) was added and mixed by inversion for 1 hour at 4°C. The sepharose beads were pelleted in a microcentrifuge for 1 minute,

rinsed once in 500µl 0.5M LiCl and 3 times in 500µl lysis solution. The adsorbed proteins were released from the beads and denatured by incubation at 100°C for 3 minutes before analysis by SDS polyacrylamide gel electrophoresis.

For optimal resolution of the immuno-precipitated proteins, the samples were electrophoresed through an 8-15% gradient SDS-polyacrylamide gel. Both an 8% and 15% acrylamide (30:0.39 monomer:bis) were prepared, the 15% containing 5% glycerol to prevent the gel form cracking when it was handled and dried. The gradient gel was poured using a gradient former. [^{14}C] labeled protein molecular weight standards (Rainbow Markers, Amersham) were used as markers. Electrophoresis was carried out at 5V/cm using 1x tris glycine buffer. Following electrophoresis, gels were fixed in 5% methanol, 7.5% acetic acid v/v for 1 hour, then incubated with 5 volumes of autoradiographic enhancer solution ("Enlightning" NEN research products) for 30 minutes prior to drying under vacuum at 80°C and autoradiography.

CHAPTER 3 EXPRESSION OF enFeLV ELEMENTS.

3.1. INTRODUCTION.

The expression of endogenous viral elements related to FeLV was studied in feline cells and tissues from a variety of sources using short, highly specific DNA probes. Little was known of the factors controlling expression of these enFeLV elements at the start of this project, although the influence of flanking DNA sequences had been implicated. Expression of enFeLV transcripts had been clearly demonstrated only in placental and embryonic tissues of specific pathogen free cats and a small number of virus free lymphomas. These studies had found no evidence of endogenous *gag*-related proteins but did not address the expression of an envelope protein product. (Niman *et al.*1980, Niman *et al.*1977b)

Because of the endogenous origin of the FeLV-B *env* gene, the B/S FeLV-B *env* probe was suitable to detect enFeLV transcripts (Stewart *et al.*1986). However, in order to detect enFeLV transcripts against a background of exogenous virus expression, it was necessary to design a more specific probe. Casey *et al* (1981) showed that the U3 portion of the LTR of infectious (exogenous) FeLV is not endogenous to domestic cats. In an attempt to identify the reciprocal endogenous-specific sequence, an enU3 probe comprising a 600bp KpnI-HindIII fragment of an endogenous LTR had been tested in preliminary experiments however problems arose, presumably due to homology with exogenous FeLV LTR promoter sequences as well as the presence in the probe of host genome sequences 5' to the start of U3. (Fig. 6)

The publication of the sequences of three endogenous LTR elements by Berry *et al* (1988) allowed the design and synthesis of a more specific probe using polymerase chain reaction (PCR). Using these probes a number of cell lines were examined for enFeLV expression, as were a number of virus positive and negative tissues. The results indicated that enFeLV expression is more widespread than had previously been suspected.

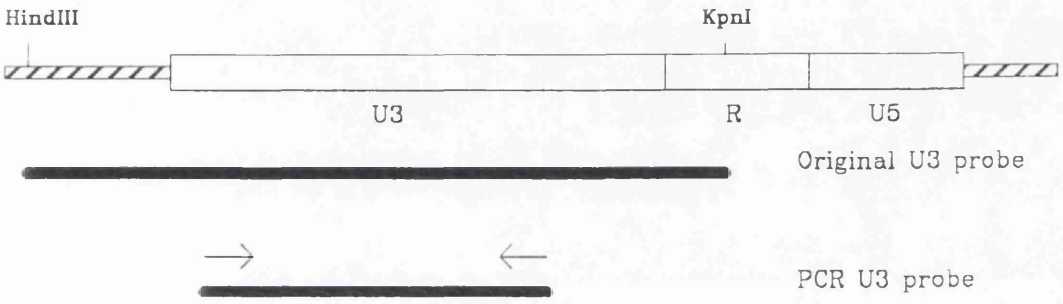


Fig.6. Production of a DNA probe specific for enU3 by PCR amplification.

The figure shows the restriction fragment enU3 DNA probe as shown in Fig.5, containing both U3, R and non-specific flanking DNA sequences, and the relative position of specific oligonucleotides (arrows) designed from the sequence of other endogenous LTRs (Berry *et al.*1988) to amplify a portion of the U3 region from a cloned endogenous element, pLCM1-KO.

3.2. RESULTS.

3.2.1. Design of an endogenous specific LTR probe.

To prepare a specific endogenous U3 probe the DNA sequences of three endogenous LTRs were compared and a region with minimal homology to exogenous U3 was selected. Two oligonucleotides were prepared with the following sequences:

oligo A 5' GACCCCCTGTCATAATATGC 3'

oligo C 5' CCAGAATGAGGGGAACAAAC 3'

Oligo A was situated 25bp downstream from the 5' end of U3. Oligo C was situated 36bp upstream of the CAAT box and the orientations of the oligonucleotides were chosen to amplify the intervening 236bp. (Fig. 6)

Following the standard PCR protocol (Chapter 2.2.11.), 10ng of plasmid pLCM1-KO was amplified. pLCM1-KO is derived from an enFeLV proviral clone pLCM-1 (supplied by J.Mullins, Stanford University) which was modified by digestion with KpnI and religation, removing the entire viral coding sequence and leaving a solo LTR derived in part from 5' and 3' elements.

The PCR reaction was carried out at 94°C for 1 min., 50°C for 1 min. and 72°C for 2 mins. The cycle was repeated thirty times. The size of the product was confirmed on a 6% acrylamide gel. The product was cut from the gel and eluted in acrylamide elution buffer. After Spin-X column purification, the eluate from the gel was phenol extracted, then precipitated with ethanol and dried under vacuum. The DNA was then resuspended in a small volume of ddH_2O. The PCR product was blunt end ligated into the pBluescript vector at the SmaI site after the vector had been phosphatased to prevent it religating with itself, and the insert incubated with T4 polymerase kinase to add a 5' terminal phosphate to the synthetic DNA. Ligation was carried out for 16 hours at 4-6°C using a vector to insert ratio of 1:10. A number of white colonies were picked, grown overnight in 10ml L-broth with ampicillin, and extracted to prepare sequencing grade plasmid DNA. These DNAs

```
                                                                    50
                    .         .         .         .         .
enU3      GACCCCCTGTCATAATATGCTTAGCAATAGTAACGAAATTTGCAAGACAG
CFE-6     ..................................CC.........G...
CFE-16    ..................................CC.........G...
CFE-14    ..................................................
exU3                            AGCAAG      GCCATTT CAAG

                                                                   100
                   .         .         .         .         .
enU3      CACCAAGAAGTTCAGGGGTCTTATCCTAAGTCCACCGTTTAGCTGCCAAA
CFE-6     .....G..C.............T.......................
CFE-16    .....G................T......A................
CFE-14    ..................................................
exU3                                              TTAG     AAA

                                                                   150
                   .         .         .         .         .
enU3      CAGGATATCTGTGGTCAGCCACCCGGCCCTAAGATAGCCACCTGGCCCTA
CFE-6     ......................T.... .C.....
CFE-16    ......................T............
CFE-14    ........................ ................. ..
exU3      CAGGATATCTGTGGT A  CACCTGG

                                                                   200
                   .         .         .         .         .
enU3      AGATGGGAATGGAAAGTACTGACTCCACCCGATAGACCCTAGAGATGAGC
CFE-6         ..G.........................A.................
CFE-16         ....G.....................T.................
CFE-14    ..................................................
exU3

                                                 236
                   .         .         .
enU3      CTAGTCAGCCACCCATGTTTGTTCCCCTCATTCTGG
CFE-6     .AC.............................
CFE-16    .AT........T.....................
CFE-14    ..................T.C............
exU3
```

Fig.7. The sequence of the enU3 probe generated by PCR amplification.

The nucleotide sequence of the enU3 probe generated by PCR amplification is compared to the relative sequences from three endogenous LTRs (Berry *et al.*1988). The dots represent sequence identity to the enU3 probe sequence and the spaces represent gaps inserted to maintain to homology. The specific oligonucleotides used to amplify the enU3 probe, and designed from the sequence of CFE-6 and 16, are underlined. The regions of homology found in exogenous U3 are shown in the bottom line, in italics.

were sequenced (G-tracked) using universal primer. Two clones were selected for complete sequencing. (Fig. 7) The sequences of both clones were identical and were very similar to a previously published sequence, CF-14, but had an additional residue in each copy of the direct repeat and two further point mutational differences. The figure also shows the limited residue homology to exU3. As all enFeLV U3 sequences were 86% homologous to each other it was expected that this probe would recognise all known enFeLV LTRs. The probes for experiments were prepared from CsCl purified plasmid by BamH1-Pst1 digestion and gel purification.

3.2.2. Expression of enFeLV in cell lines.

A number of FeLV positive and negative cell lines were tested for enFeLV expression by Northern blot analysis. (Fig. 8/8a) The origins of these cell lines are given in chapter 2.1.5. The virus status of the cells was confirmed by hybridisation of total RNA to an exogenous U3 probe (exU3) (and an FeLV-A *env* probe, results not shown). T3, F422 and FL74 cells are tumour cell lines infected with exogenous FeLV, both full length and defective viruses. The presence of RNA in all lanes was confirmed by re-hybridisation of the blot to the GAPDH probe. A duplicate blot was hybridised to enU3 (the B/S probe was unsuitable here due to the presence of exogenous virus.)

No enFeLV expression was detected in AH927 or FEA fibroblast cells (there is no RNA in the AH927 lane of Fig.8, as determined by GAPDH hybridisation, but Fig. 8a confirms AH927 cells to be negative for enFeLV expression). Expression was detected in all lymphoid cell lines tested, both virus positive (T3, F422 and FL74) and negative (3201B and MCC). The pattern of transcripts varied with a larger transcript from 4.5-3.5kb and a smaller more consistent form at 2kb. Three transcripts were detected in the FL74 cell line. (The smear seen below the major transcripts of RNA from 3201B cells was not a consistent feature, and appeared only in older preparations of RNA. It was therefore considered to be the result of partial degradation of the major transcripts) There was some evidence of differential expression of the transcripts, with MCC and FL74 cells having stronger

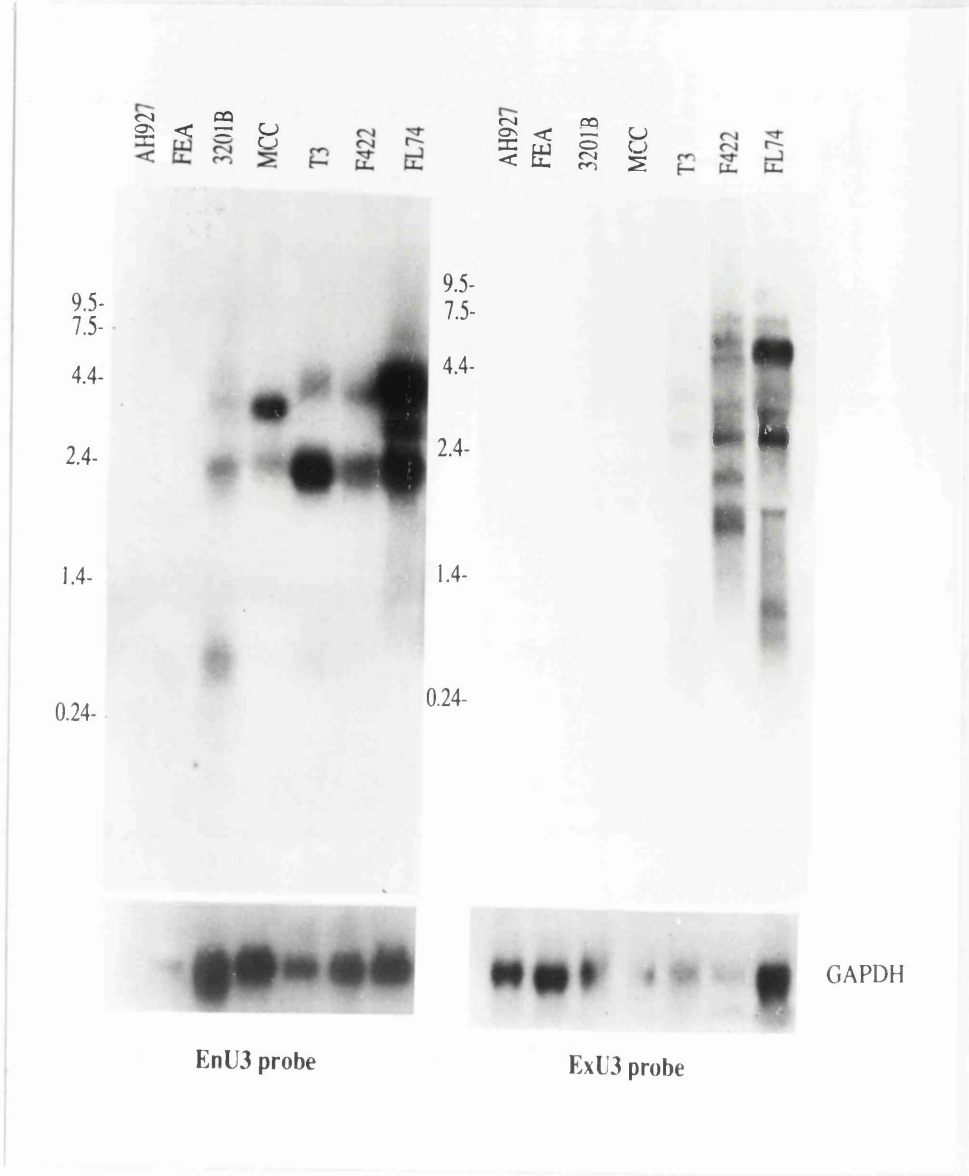


Fig.8. Expression of enFeLV in cell lines.

20µg of total cellular RNA from a number of feline cell lines were separated on two 1% agarose gels containing formaldehyde. Each gel was blotted onto a nitrocellulose membrane and hybridised with either [^{32}P] labelled enU3 or exU3 probes. Once the activity on the membranes had decayed, the blots were re-hybridised with [^{32}P] labelled GAPDH probe. The positions of the molecular weight markers are indicated on the left of each blot and the sizes are in kb.

The size of the GAPDH transcript is 1.8kb

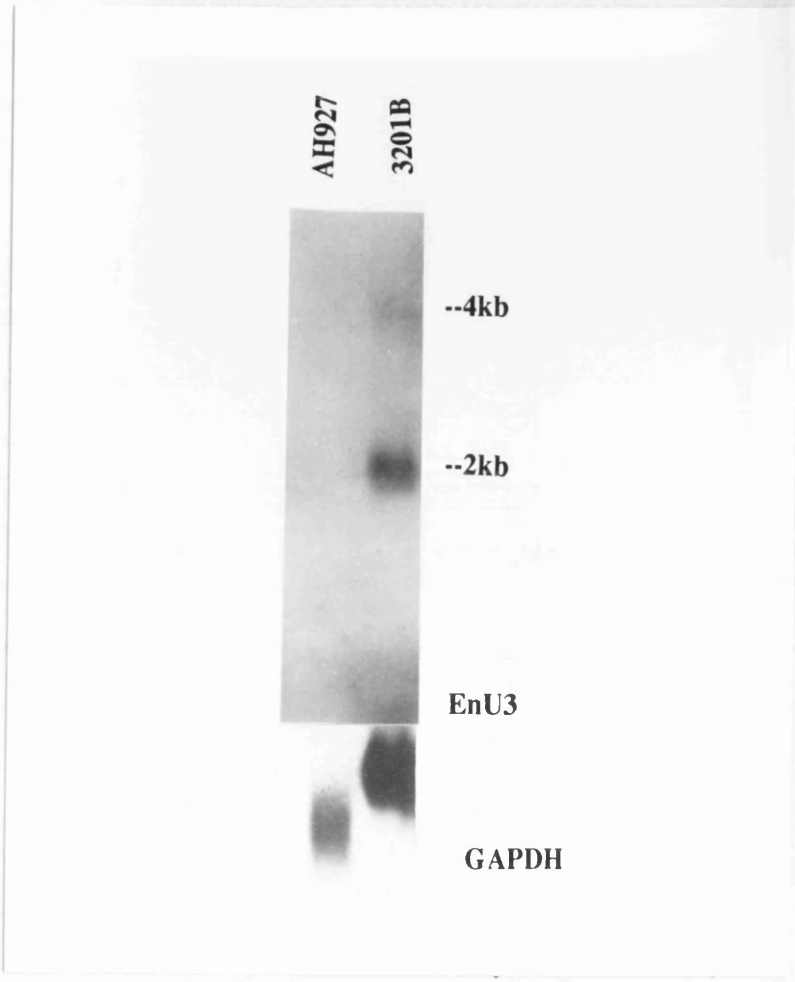


Fig.8a. EnFeLV expression in AH927 cells.

20μg of total cellular RNA from AH927 and 3201B cell lines were separated on a 1% agarose gel containing formaldehyde. The gel was blotted onto a hybridisation filter membrane and hybridised with [^{32}P] labelled enU3 probe. The enU3 probe was stripped from the blot by high temperature washes in 70% formamide before the blot was re-hybridised to a [^{32}P] labelled GAPDH probe. The two enFeLV transcripts found in 3201B cells are indicated.

signals for the larger transcripts and 3201B, T3 and F422 cells having more abundant smaller transcripts.

3.2.3. Expression of enFeLV in feline tissues.

(i) Expression of enFeLV in FeLV positive tumours.

Tumours were obtained from cats experimentally inoculated with FeLV. (T17 strain (Fulton *et al.*1987, Terry *et al.*1992)) The samples were collected between 24 and 28 weeks post inoculation, all animals having presented with thymic lymphosarcoma. Thymic tumour and kidney total RNA were tested for enFeLV expression by Northern blot analysis. (Fig. 9)

The exU3 probe detected exogenous viral transcripts in all tumours. The enU3 probe detected expression of endogenous transcripts in all tumour tissues. Samples J49/1 and J53/1 were too heavily degraded for specific band sizes to be seen but in the others, two bands were detected with sizes similar to those of the 3201B cell lines except for the larger transcript of the J53/2 tumour, which appeared to be smaller than the others.

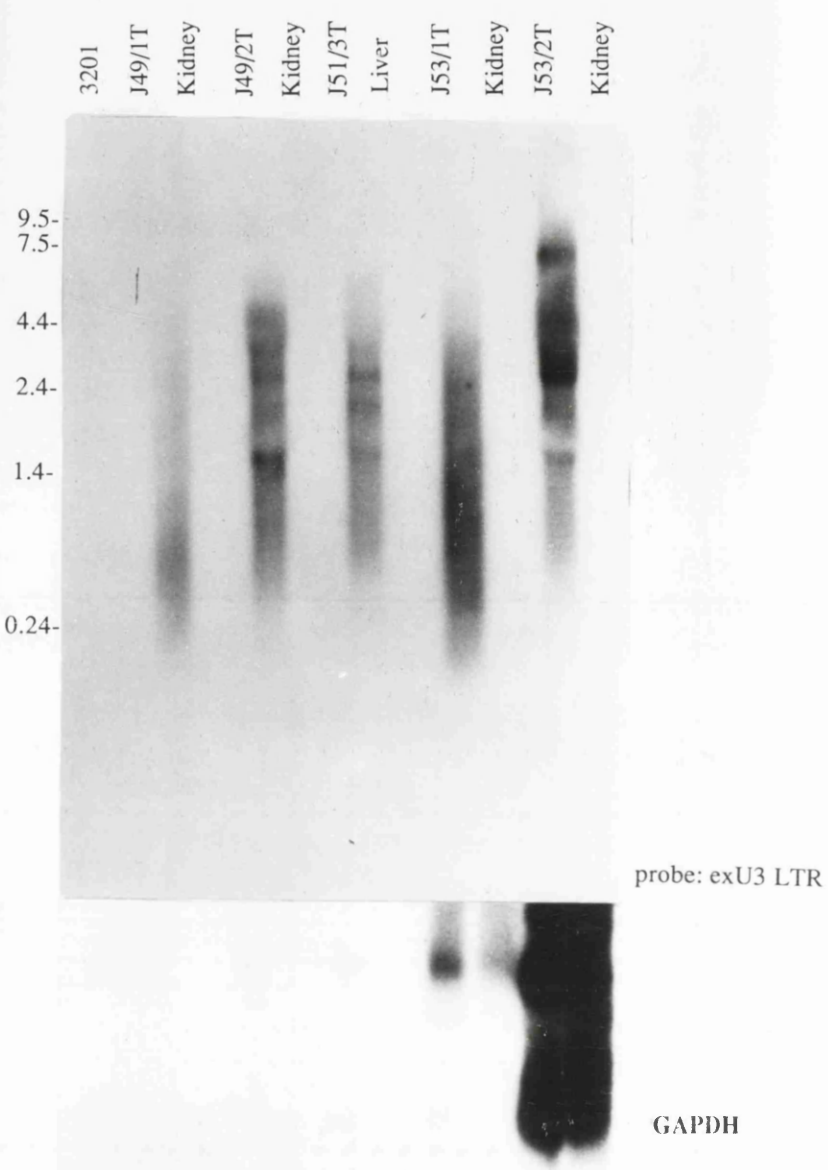
The presence of RNA was checked by reprobing the blots with the GAPDH probe. Unfortunately, a number of the kidney RNA samples appeared to be degraded. However in those samples with intact RNA, no expression could be detected in kidney tissue.

There was again evidence of differential expression of the two transcripts, in all cases here, as in 3201B cells, the smaller transcript having a stronger signal than the larger.

(ii) Expression of enFeLV in FeLV positive healthy tissue.

FeLV positive thymic and kidney tissues from a number of apparently healthy, but viraemic cats were obtained, and total RNA tested for enFeLV expression by Northern blot analysis.

The enU3 probe detected expression in the thymic tissues of the four healthy virus positive cats. (Fig.10) All transcripts were of a similar size. Cat P112


3201
J49/1T
Kidney
J49/2T
Kidney
J51/3T
Liver
J53/1T
Kidney
J53/2T
Kidney
9.5-
7.5-
4.4-
2.4-
1.4-
0.24-
probe: exU3 LTR
GAPDH

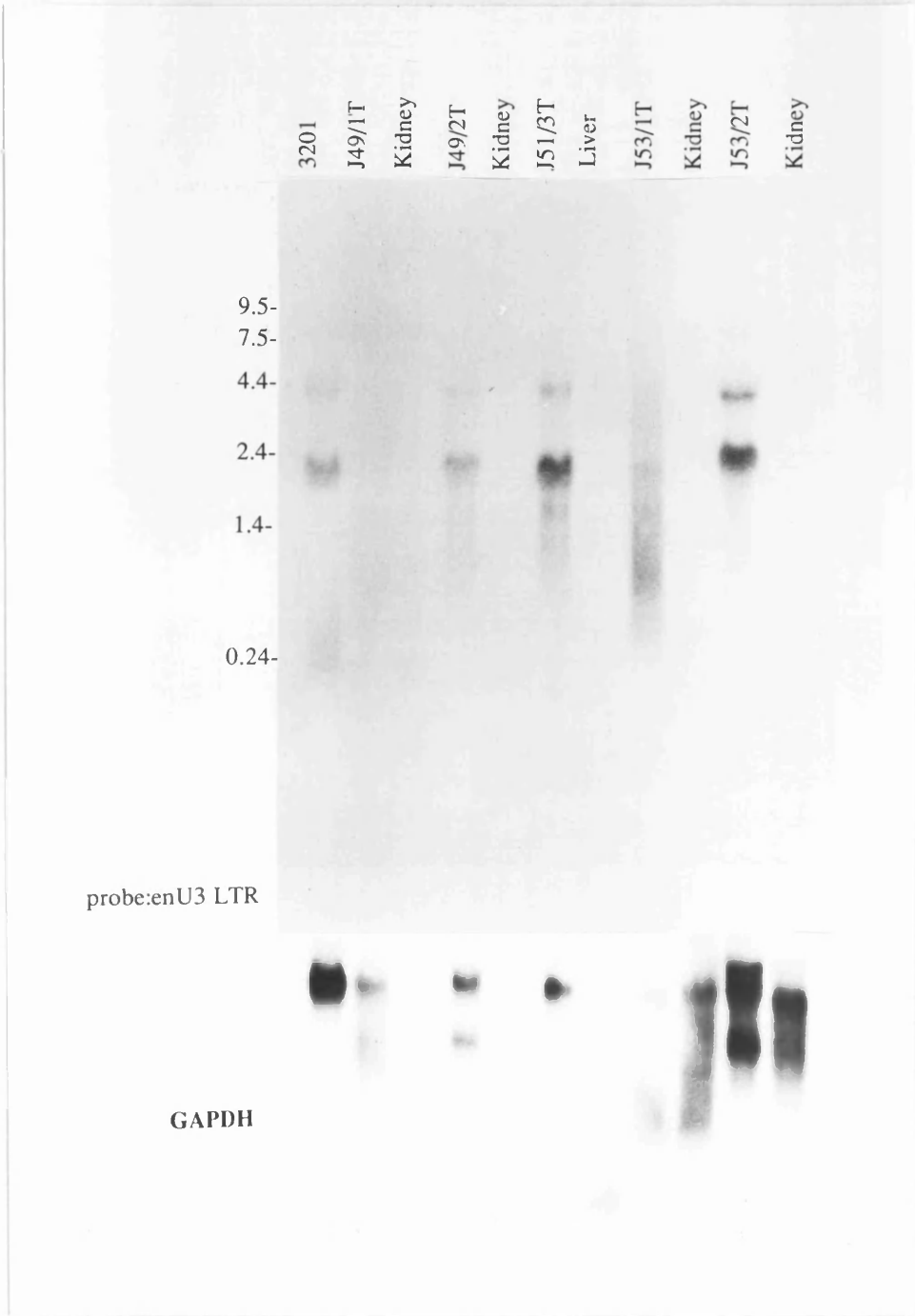


Fig.9. Expression of enFeLV in virus positive tumours.

20µg of total cellular RNA from thymic tumours and kidney or liver tissue from a number of virus infected animals were separated on two 1% agarose gels containing formaldehyde. Each gel was blotted onto a hybridisation filter membrane and hybridised with either [^{32}P] labelled enU3 or exU3 probes. Once the activity on the membranes had decayed, the blots were re-hybridised with [^{32}P] labelled GAPDH probe. The positions of the molecular weight markers are indicated on the left of each blot and the sizes are in kb.

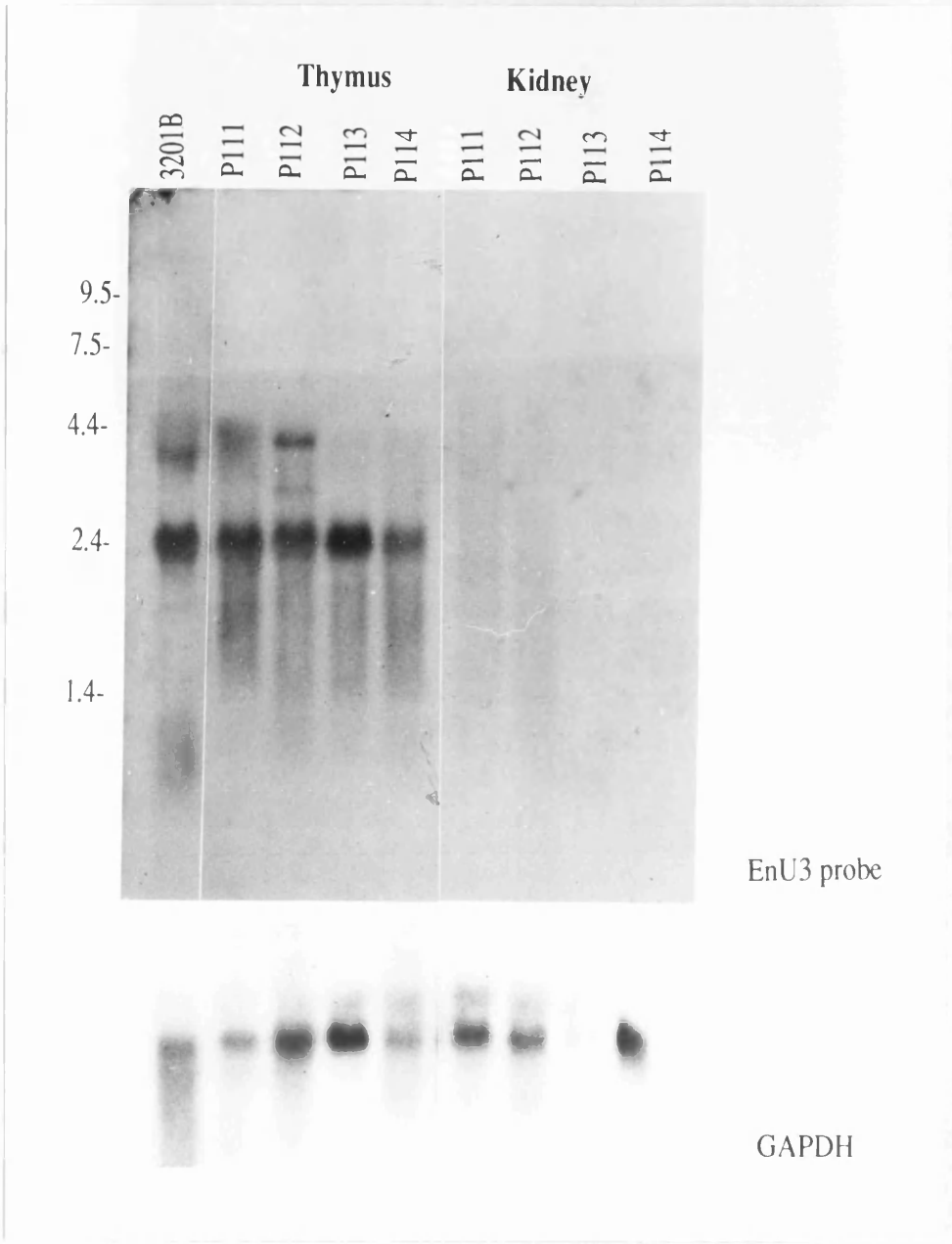


Fig.10. Expression of enFeLV in virus positive healthy tissue.

20μg of total cellular RNA from thymic and kidney tissues from a number of virus infected, but apparently healthy animals were separated on two 1% agarose gels containing formaldehyde. The gel was blotted onto a hybridisation filter membrane and hybridised with [^{32}P] labelled enU3 probe. Once the activity on the membrane had decayed, the blot was re-hybridised with [^{32}P] labelled GAPDH probe. The positions of the molecular weight markers are indicated on the left of the blot and the sizes are in kb.

displayed three transcripts. Again differential expression was apparent with the smaller transcript displaying a stronger signal. There also appeared to be low level expression in two of the kidney samples, P112 and P113.

3.2.4. Comparison of enFeLV genomic content of expressing and non-expressing tissues.

At this point the available evidence suggested that enFeLV expression might be induced by exogenous virus infection. This hypothesis was based on the observation that only virus positive tissues from infected animals expressed enFeLV and on the published studies of enFeLV expression which failed to show expression in virus free, healthy adult cats. It was considered that infection with exogenous FeLV might influence the expression of enFeLV loci by co-packaging endogenous transcripts which were expressed at low levels and thereby mediating the integration of the endogenous provirus into a new chromosomal position more favourable for expression. Such a process, if widespread, would result in unique integrations in individual cells and so be impossible to detect by Southern blotting analysis of provirus-host junction fragments. This problem might be overcome, however, if the same analysis was performed on clonal tumour cells expressing enFeLV, since these resulted from the expansion of one or a few virus infected cells.

To examine the chromosomal location of enFeLV elements a method of fingerprinting the feline genome was employed. (Fig.11) All the tumours tested were of clonal origin (as defined by rearrangement of the T-cell receptor β chain gene, not shown) and all expressed enFeLV to levels equivalent to 3201B cells. The genetic fingerprints of these enFeLV expressing tumours were compared to kidney tissues from the same animal which did not express enFeLV transcripts.

DNA was digested with BamHI and separated on a 0.8% agarose gel. The resulting Southern blots were probed with the B/S *env* probe. Most endogenous proviruses would be expected to be cut by this enzyme at a conserved site at the 5' end of the *env* gene, and in the 3' flanking DNA. The B/S probe would selectively detect these fragments. The difference in location of the BamHI site in the 3'

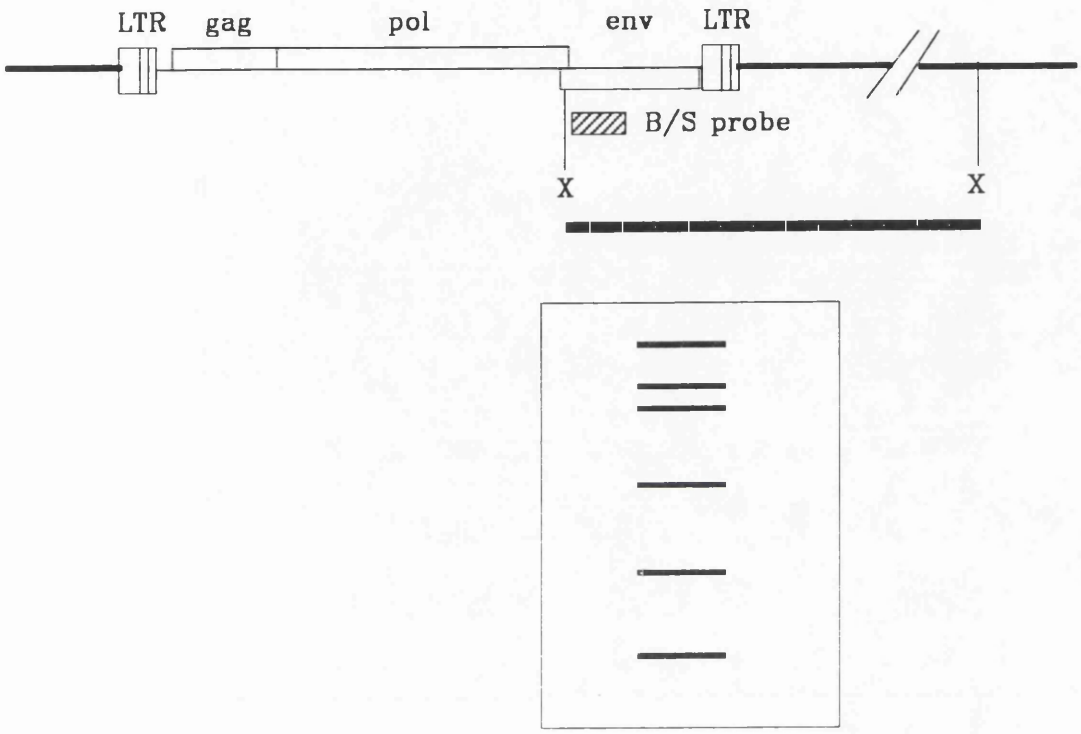


Fig.11. Identification of virus-cell junction restriction fragment polymorphisms.

This diagram shows the principle of the "fingerprinting" studies to identify individual enFeLV loci. The restriction enzyme BamHI cuts the enFeLV provirus at a conserved site within the *env* gene and at unique sites in the 3' flanking DNA. The size of the restriction fragment will depend upon the size of the *env* gene, and the specific region of flanking DNA. Each locus in a genome can therefore be separated on an agarose gel as unique sized fragments which can be identified by Southern blotting and hybridisation to the B/S *env* probe. The resulting pattern is a unique genetic fingerprint of the DNA sample tested.

flanking DNA would then result in a unique band for each provirus in a different chromosomal location. Between two tissues of the same cat then, any changes in bands would represent the acquisition of a new endogenous provirus or translocation to a new chromosomal location.

Kidney tissues generally had no detectable enFeLV transcripts while thymic tumour tissues were consistently shown to express enFeLV. These two tissues were therefore selected to look for evidence of a change in chromosome position of enFeLV loci which may be responsible for the tissue specific expression of enFeLV transcripts. Concomitantly, these studies allowed the characterisation of the genetic heterogeneity of enFeLV loci within the species. (Fig.12a,b) Figures 12a and b represent the endogenous virus fingerprint of a number of thymic tumours that had been found to express enFeLV (Fig.9 and other results not shown) and kidney tissues from the same animals. Fig.12a shows the fingerprints from a number of cases from a bank of feline tumour tissues. T15 and T19 were both virus positive samples, T16 and T20 were both free of detectable exogenous virus. Fig.12b shows the fingerprints of a number of FeLV positive cats that were related through the male parent. Each cat had a different proviral pattern, and there was no difference in the pattern between expressing and non-expressing tissues of the same cat. The genetic fingerprints in Fig.12b showed more similarities to each other and reflected the close genetic relationship of the animals. The J49 cats were from the same litter, and the J53 cats were from another litter. The patterns of these differed by only one or two restriction fragments.

3.2.5. Expression of enFeLV in specific pathogen free cats.

Wendy 3 and F6 were two specific pathogen free cats which had never been exposed to exogenous FeLV. A Northern blot using the exU3 probe confirmed that the Wendy3 samples were free of infectious virus. (data not shown)

From Wendy 3, four tissues were initially tested for enFeLV expression by hybridisation to the B/S *env* probe. (Fig.13a) EnFeLV transcripts were detected in thymus, mesenteric lymph node and bone marrow samples. Expression was not

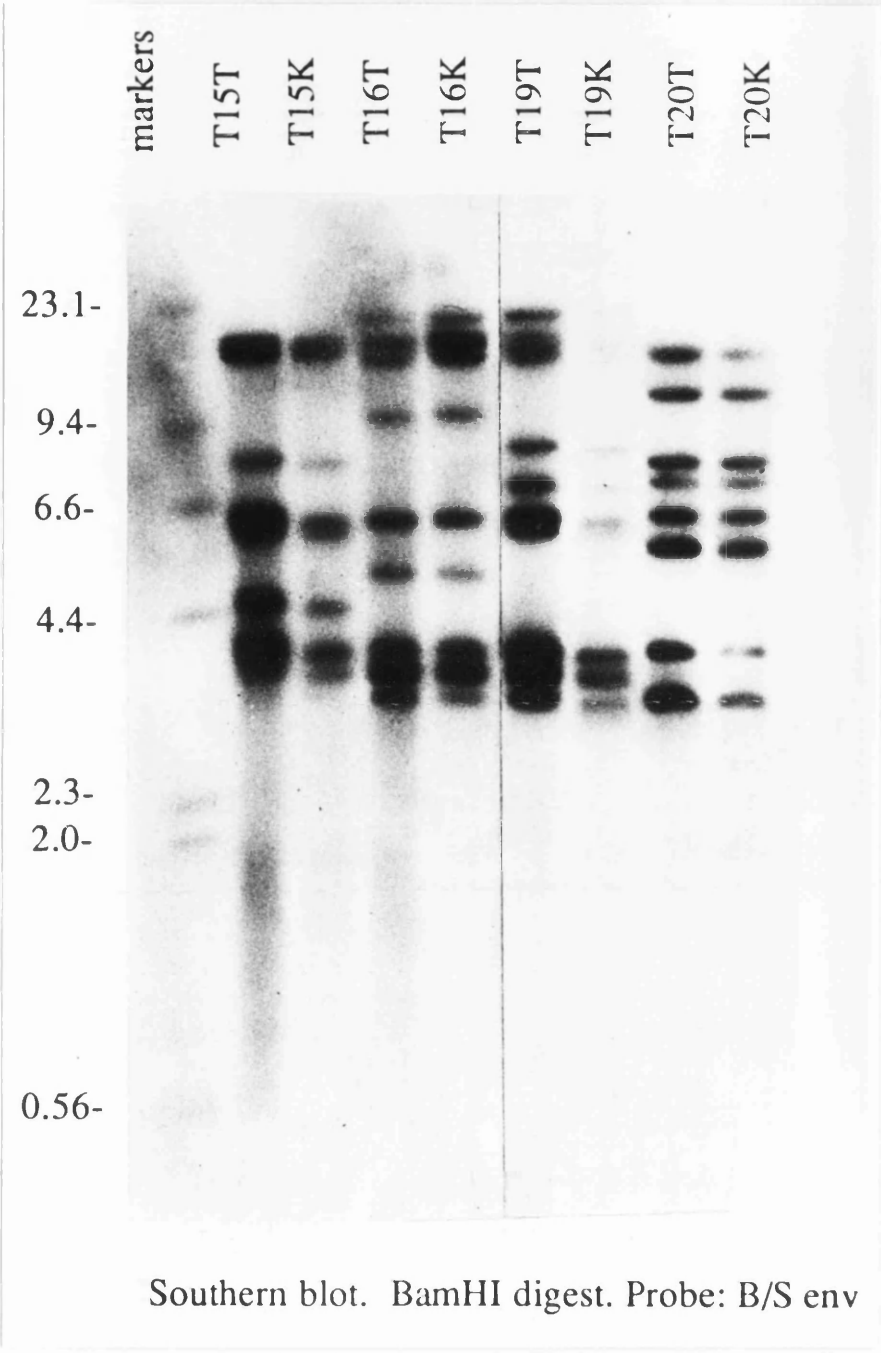


Fig.12a. Comparison of the enFeLV content and distribution in tissues with and without detectable enFeLV expression.

20µg of high molecular weight DNA from a number of thymic tumours that had been found to express enFeLV (T), and kidney tissues (K) from the same animals (T15, T16, T19, and T20) were digested with BamHI, and the resulting fragments separated on a 0.8% agarose/TAE gel. The DNA was transferred to a filter membrane by Southern blotting and hybridised with [^{32}P] labelled B/S *env* probe. The positions of the molecular weight markers are indicated on the left of the blot and the sizes are in kb.

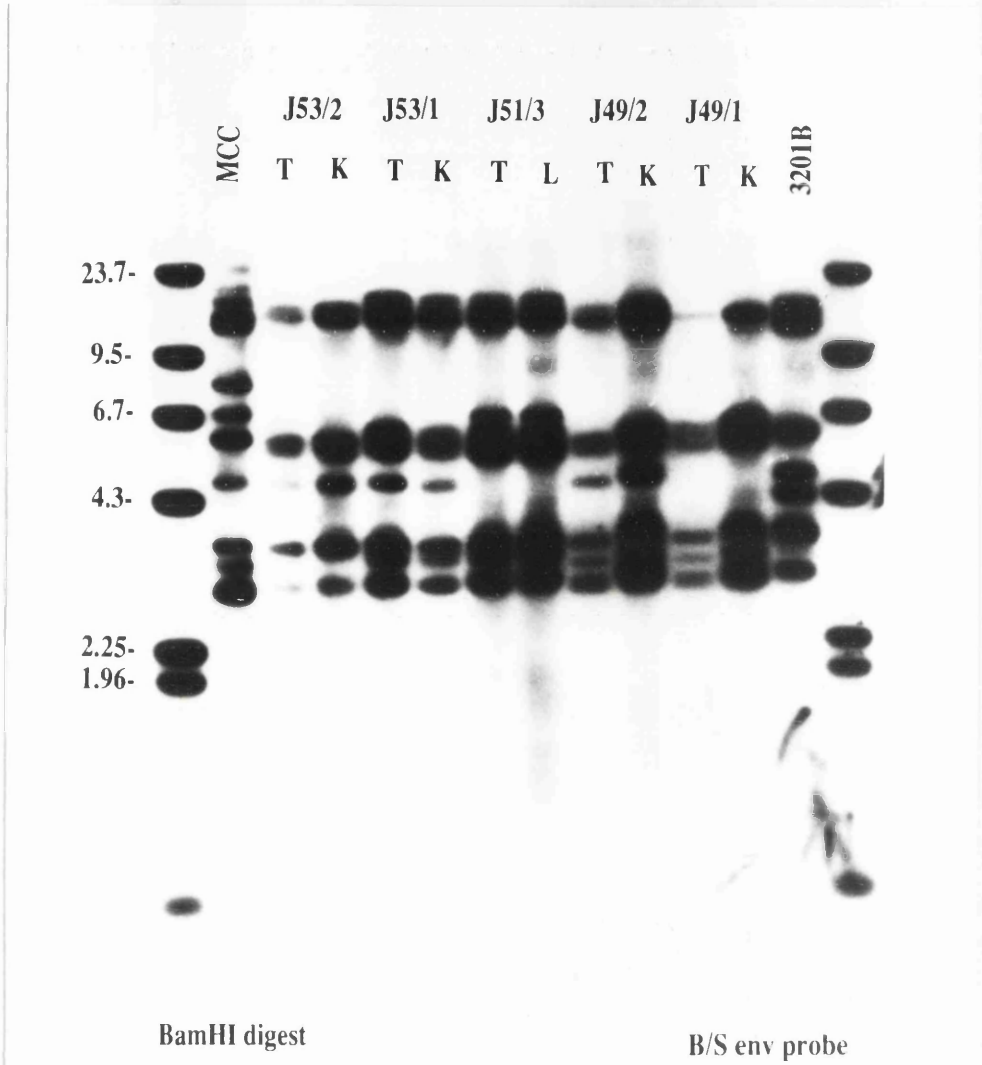


Fig.12b. Comparison of the enFeLV content and distribution in tissues with and without detectable enFeLV expression.

20μg of high molecular weight DNA from a number of thymic tumours that had been found to express enFeLV (T), and kidney or liver tissues (K or L) from the same animals (J49/1, J49/2, J51/3, J53/1, J53/2) were digested with BamHI, and the resulting fragments separated on a 0.8% agarose/TAE gel. High molecular weight DNA from 3201B and MCC cell lines were also tested. The DNA was transferred to a filter membrane by Southern blotting and hybridised with [^{32}P] labelled B/S *env* probe. The positions of the molecular weight markers are indicated on the left of the blot and the sizes are in kb.

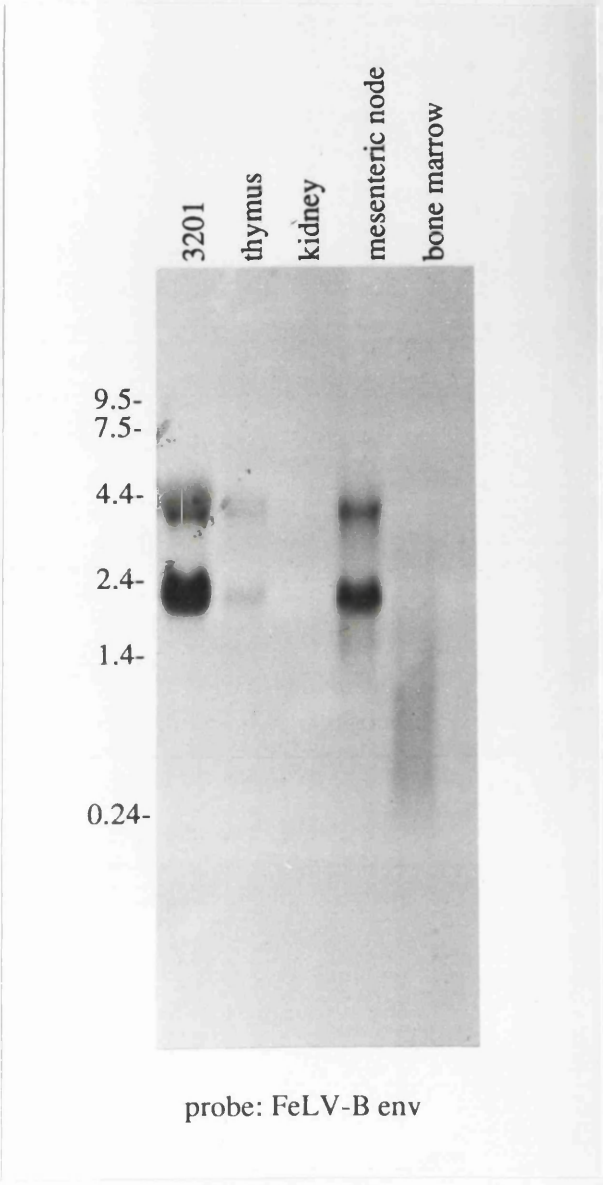


Fig.13a. Expression of enFeLV in specific pathogen free cats (1).

20μg of total cellular RNA from a number of tissues from a specific pathogen free cat were separated on a 1% agarose gel containing formaldehyde. The gel was blotted onto a hybridisation filter membrane and hybridised with [^{32}P] labelled B/S *env* probe. The positions of the molecular weight markers are indicated on the left of the blot and the sizes are in kb.

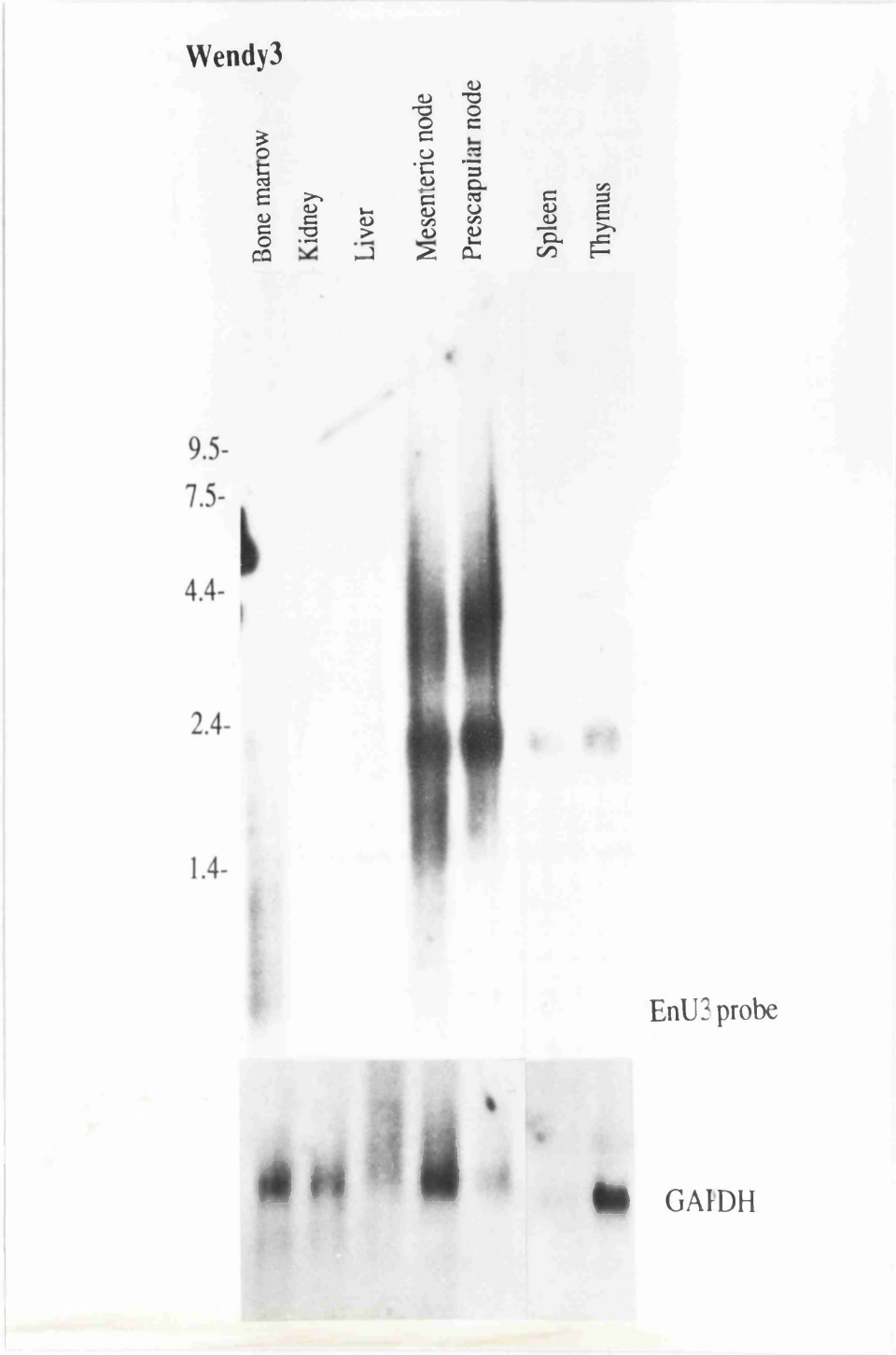


Fig.13b. Expression of enFeLV in specific pathogen free cats (1).

20µg of total cellular RNA from a number of tissues from a specific pathogen free cat were separated on a 1% agarose gel containing formaldehyde. The gel was blotted onto a hybridisation filter membrane and hybridised with [^{32}P] labelled enU3 probe. The enU3 probe was stripped from the blot by high temperature washes in 70% formamide before the blot was re-hybridised to a [^{32}P] labelled GAPDH probe. The positions of the molecular weight markers are indicated on the left of the blot and the sizes are in kb.

detected in the kidney RNA. In a subsequent analysis, this time using the enU3 probe, (Fig.13b) enFeLV expression was confirmed in bone marrow, mesenteric node and thymus and also detected in prescapular lymph node and spleen. Expression was not detected in liver and kidney. In those samples with undegraded RNA, two transcripts were detected. The smaller of these appeared to be relatively more abundant. The GAPDH probe analysis confirmed the presence of RNA in all lanes.

For the F6 samples, a wider range of tissues were tested using the enU3 probe. (Fig.14a) High levels of enFeLV RNA were detected in peripheral blood lymphocytes, bone marrow, and mesenteric node. Lower levels were seen in the thymus and spleen. Because of the poor quality of the results, (due to the inadvertent use of an excess of probe) the procedure was repeated using those RNA samples still available. (Fig.14b) EnFeLV expression was detected in the small intestine, liver and mesenteric node. It was possible to enhance the signals using a computer densitometer and image analyser (SUN Microsystems). (Fig.14c) The enhanced image revealed enFeLV transcripts in spleen, thymus, liver and lung tissue. In both experiments, no expression was detected in salivary gland, brain, and cardiac or striated muscle. GAPDH probing confirmed the presence of RNA in all lanes.

A number of tissues displayed more complex transcript patterns, and the size of transcripts appeared to vary from tissue to tissue. For example, the peripheral blood lymphocytes had three different transcripts. Also, the size of the relatively less mobile transcript in intestine and mesenteric node tissues was larger than in spleen, thymus and lung tissues, and in liver tissue it was smaller still.

3.3. DISCUSSION.

The results of this study indicate that enFeLV expression is more widespread than previously reported. This new finding was made possible, in part, by the development of a more specific and sensitive probe. The high sequence homology between the published sequences of three enFeLV LTR elements and the enU3 probe generated here suggests that the new probe will be broadly specific for enFeLV loci.

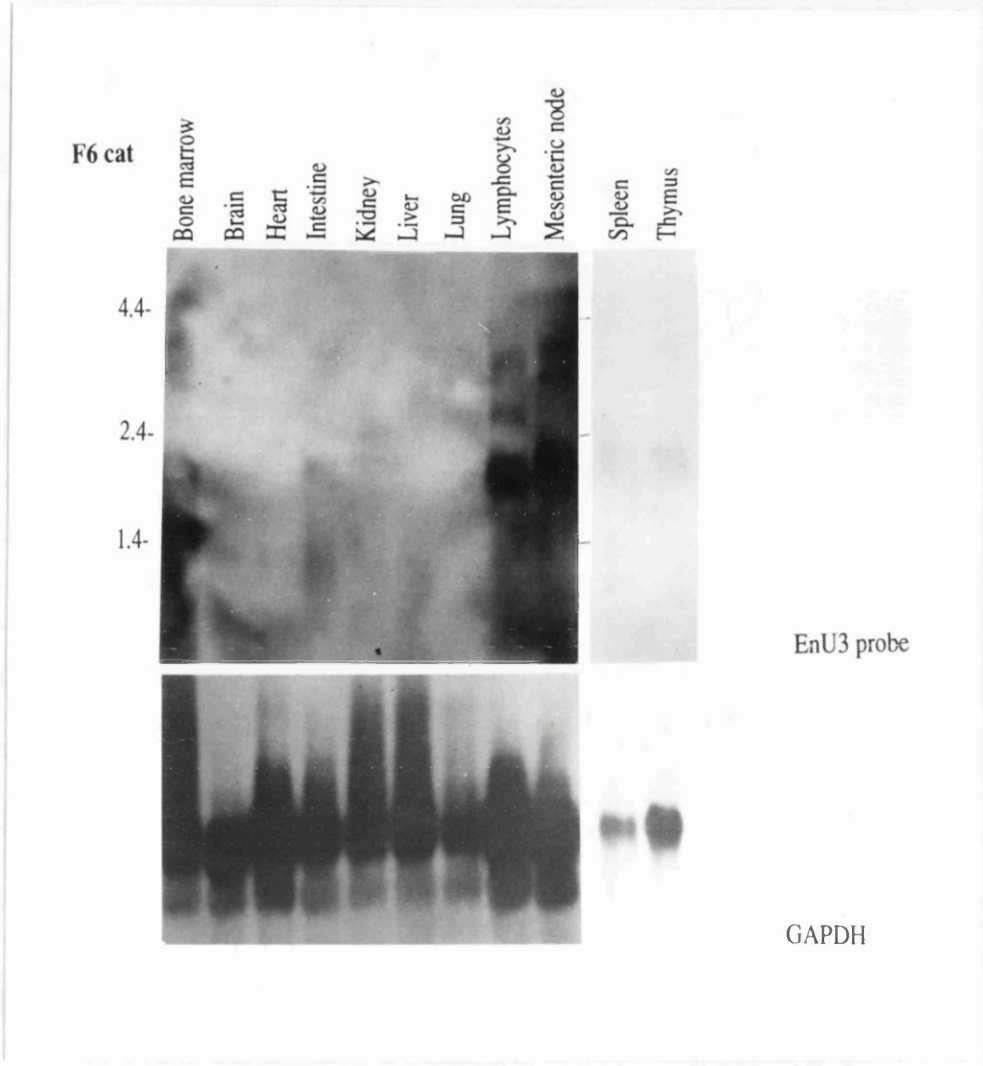


Fig.14a. Expression of enFeLV in specific pathogen free cats (2).

20µg of total cellular RNA from a number of tissues from a specific pathogen free cat were separated on a 1% agarose gel containing formaldehyde. The gel was blotted onto a hybridisation filter membrane and hybridised with [^{32}P] labelled enU3 probe. The enU3 probe was stripped from the blot by high temperature washes in 70% formamide before the blot was re-hybridised to a [^{32}P] labelled GAPDH probe. The positions of the molecular weight markers are indicated on the left of the blot and the sizes are in kb.

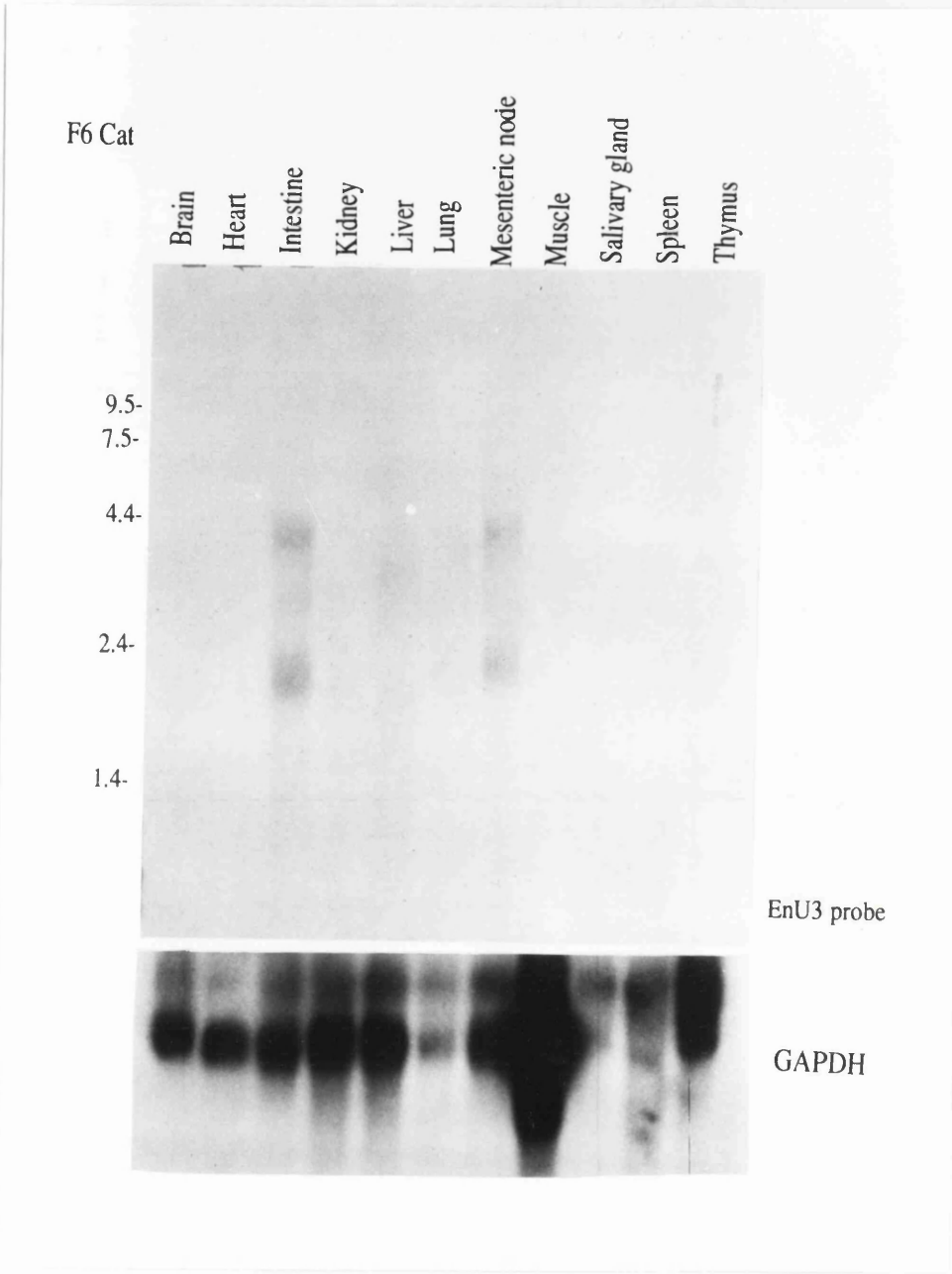


Fig.14b. Expression of enFeLV in specific pathogen free cats (2).

20µg of total cellular RNA from a number of tissues from a specific pathogen free cat were separated on a 1% agarose gel containing formaldehyde in a repeat of the experiment shown in Fig.14a. The gel was blotted onto a hybridisation filter membrane and hybridised with [^{32}P] labelled enU3 probe. The enU3 probe was stripped from the blot by high temperature washes in 70% formamide before the blot was re-hybridised to a [^{32}P] labelled GAPDH probe. The positions of the molecular weight markers are indicated on the left of the blot and the sizes are in kb.

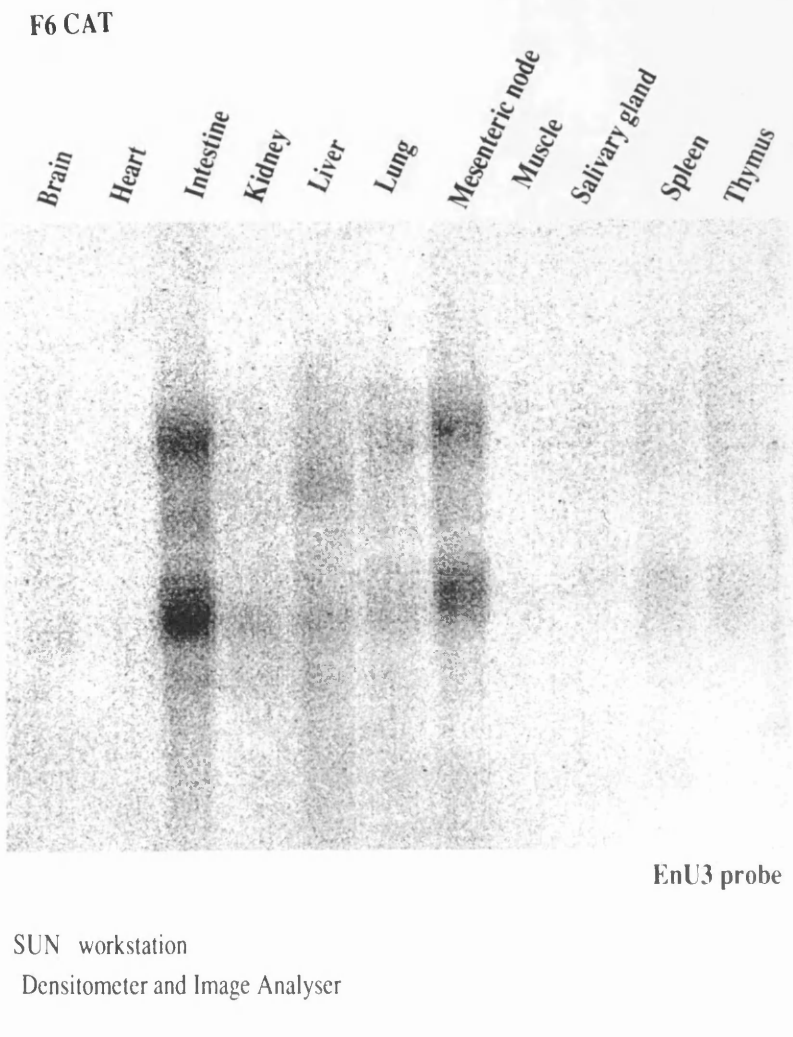


Fig.14c. Expression of enFeLV in specific pathogen free cats (2). Enhancement of enFeLV signals.

This figure shows the result of the enhancement of the enFeLV signals present on the blot shown in Fig.14b by a computer densitometer and image analyser (SUN Microsystems). The enhancement was performed by Lynn McGarry (Beatson Institute for Cancer Research)

There were a number of technical problems encountered regarding the isolation of RNA. Although flash-freezing of tissues immediately upon excision did, in general, give good quality RNA, this was not the case with bone marrow RNA which was consistently degraded. This may reflect the heterogeneous nature of the tissue, and the extraction procedure (scraping the marrow out of a crushed femur) which may have resulted in cell lysis and release of RNases. There was also an unexpected difficulty in obtaining intact RNA from kidney tissue which was apparent in some control hybridisation blots. This may have been the result of tissue degradation, in the J series cats, as these tissues were not immediately flash-frozen after excision (a few of the corresponding thymus RNA samples were also degraded). However the problem of kidney RNA isolation may not be tissue specific, but reflect the simple fact that it was commonly collected after the neoplastic tissues during the post-mortem procedure.

Initial analyses suggested that enFeLV expression might have been influenced by exogenous virus infection. However, a study of enFeLV expressing and non-expressing tissues revealed no difference in enFeLV provirus numbers or chromosomal location. Hence, novel insertions of enFeLV sequences did not appear to be responsible for expression. The possibility remained that the mobilisation of enFeLV sequences occurred in a small percentage of cells which were not detected. However the amount of endogenous specific RNA produced in these tissues is comparable to 3201B cells which suggests either the majority of cells express endogenous specific RNA or a smaller proportion of cells in these tissues express enFeLV at higher levels than 3201B cells. Therefore a further level of control was considered to be involved. The possibility that exogenous viral sequences could indirectly influence the transcription of enFeLV loci (e.g. via *trans* acting factors), was not considered further after the observation of enFeLV expression in specific pathogen free cats proved that enFeLV expression can occur independently of exogenous FeLV.

Overall, the data from both FeLV positive and negative cats showed that enFeLV expression was a consistent feature of lymphoid tissues, whether exposed to

exogenous FeLV or not. EnFeLV expression appeared to be controlled in a tissue specific manner. Thus, the fibroblast cell lines AH927 and FEA displayed little detectable expression as did kidney, muscle or brain tissues. It is conceivable that expression in non-lymphoid tissues may have been due to the presence of lymphoid cells in these tissues, and might explain the detection of low levels of expression in liver and lung tissue. Similarly, higher levels of enFeLV expression detected in the smooth intestine tissue was perhaps the result of enFeLV expression in gut-associated lymphoid tissue, including Peyer's patches which are located in the intestinal wall. It might also be considered that the tissues not displaying high enFeLV expression were less mitotically active than lymphoid cells, and so may be less transcriptionally active, but the observation that mitotically active fibroblast cell lines also failed to express enFeLV suggested that the tissue specific restriction is a more salient feature in the control of enFeLV expression.

In a number of samples, including FL74 cells, the thymus of P112 cat and the lymphocytes of F6 cat, more than two enFeLV transcripts were detected. These presumably arose either from the expression of a greater number of loci or from a more complex splicing mechanism. The study of a panel of tissues of F6 cat also indicated the expression of different sizes of transcripts in different tissues. This may reflect some tissue specific control of the activity of different enFeLV loci. The differences in the sizes of enFeLV transcripts expressed in different cell lines and tissues presumably indicated the presence of different active loci. However, in all RNA samples tested, from both a cellular and tissue origin, the size of the smaller transcript was considerably more conserved than the larger transcript(s). The possible significance of this is explored further in Chapter 8.

CHAPTER 4. COMPOSITION OF enFeLV TRANSCRIPTS.

4.1. INTRODUCTION.

The following studies were performed to characterise the composition of enFeLV transcripts and to identify possible translation products.

4.2. RESULTS.

4.2.1. Expression of sub-genomic transcripts in the 3201B cell line.

This experiment was performed by Robert McFarlane. (Beatson Institute for Cancer Research)

Northern blot strips of total RNA from 3201B cells were hybridised with DNA probes specific for the individual viral genes of FeLV to characterise the genetic content of the enFeLV transcripts. (Fig.15) 3201B cells were selected for this analysis because they were free of exogenous virus which would react with the subgenomic FeLV probes.

Both size classes were detected by enU3 and B/S *env*. Only the larger transcript was detected by the *gag* probe, and neither transcripts was detected by the *pol* or the exU3 probe. In subsequent Northern analyses, 3201B RNA was generally used as a positive control for enFeLV expression.

4.2.2. Identification of candidate enFeLV proteins using polyclonal antisera.

The initial work to identify the protein products of the endogenous envelope open reading frame was performed by Anne Terry. (Beatson Institute for Cancer Research)

FeLV positive and negative lymphoma lines, which had been shown to express endogenous related proviruses, were labelled with [^{35}S] met/cys, and immune-precipitated with an anti-p15^{gag} or anti-gp70^{env} polyclonal antisera. (Fig.16a/b) No *gag* related proteins were detected in the virus negative cell lines (3201B, MCC). Therefore, although the larger RNA transcript in the 3201B cells contains *gag* related sequences these do not appear to be translated into a stable protein. As expected, *gag* related proteins were readily detected in the FeLV positive T-cell lines (T3, FL74).

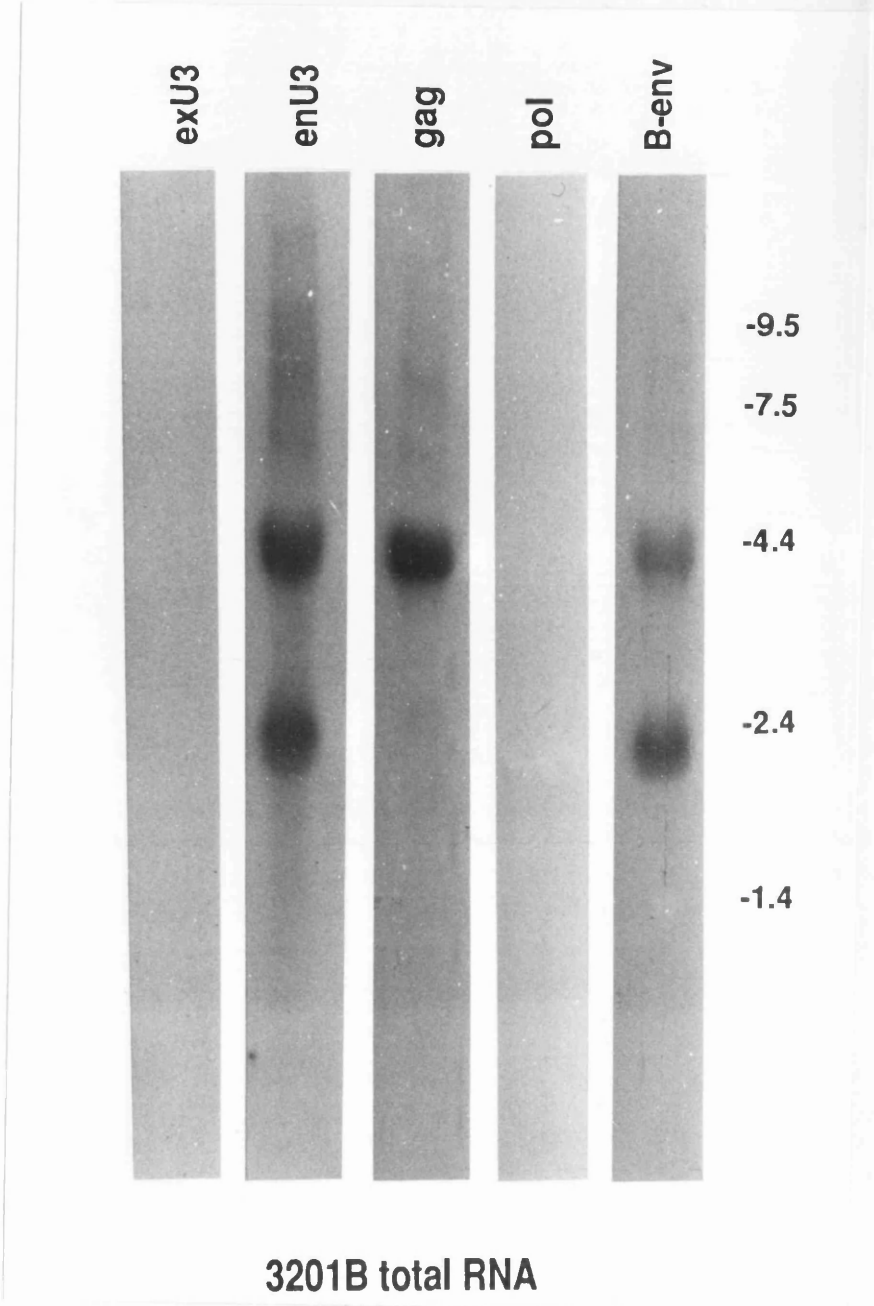


Fig.15. Expression of subgenomic transcripts in 3201B cell line.

5 aliquots of 20µg of total cellular RNA from 3201B cells were separated on a 1% agarose gel containing formaldehyde. The gel was blotted onto a hybridisation filter membrane and strips hybridised with [^{32}P] labelled probes specific for *gag*, *pol*, *env*, and ex or enU3.(see Fig.5) The positions of the molecular weight markers are indicated on the right and the sizes are in kb.

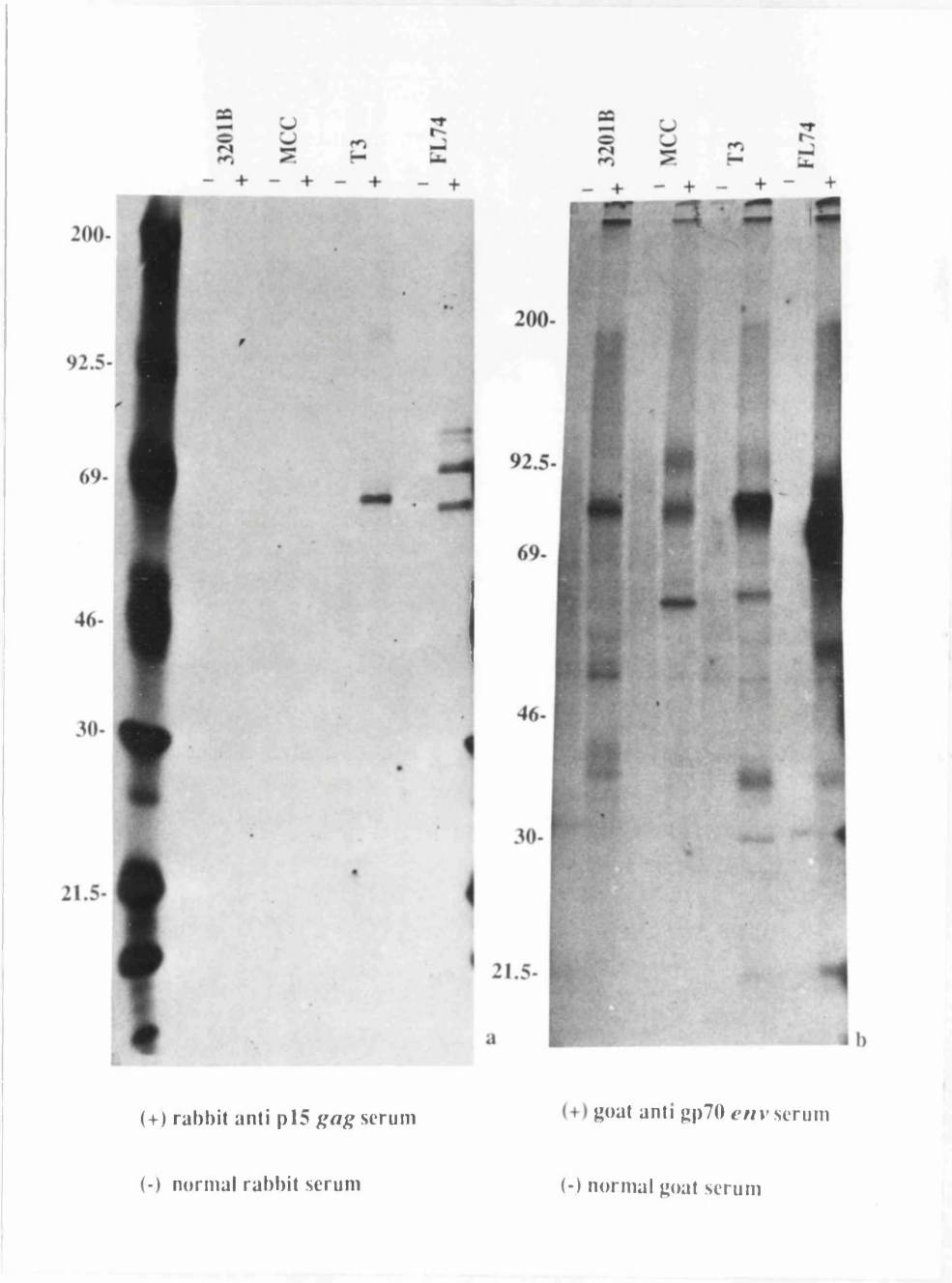


Fig.16 Identification of candidate endogenous proteins using polyclonal antisera.

10^6 cells from enFeLV expressing lines were metabolically labelled with [^{35}S] met/cys and the cellular proteins precipitated with polyclonal anti-p15^{gag} (a) or anti-gp70^{env} (b) sera. The precipitated proteins were separated by denaturing electrophoresis through a 8-15% gradient SDS-polyacrylamide gel and visualised by autoradiography. The positions of the molecular weight markers are indicated on the left of each blot and the sizes are in kD.

The polyclonal *env* antiserum precipitated a number of proteins in these cell lines. The exogenous envelope gene precursor polyprotein, $Pr80^{env}$ was detected in virus positive cells. The sizes of the proteins precipitated from both the FeLV negative and positive cell lines were of 70-80kD, 60kD, 50kD and 35kD.

4.2.3. Amplification of endogenous envelope gene sequences from feline cDNA

The technique of polymerase chain reaction was used to amplify cDNA prepared from enFeLV expressing cells using oligonucleotide primers designed from endogenous *env* sequences published. (Kumar *et al.*1989) By amplifying cDNA, only those genes which were expressed would be isolated. Oligo 2/3 corresponded to DNA sequences 33bp 5' to the signal peptide ATG of the *env* gene.

Oligo 2/3 5'TCAGACAGACCCCAGCTCAGA3'

Oligo C was previously described for the amplification of enU3.

The use of a 3' oligo primer located within enU3 was to ensure the amplification of the entire *env* coding sequences.

Poly-$(A)^+$ RNA was isolated from total cellular RNA using poly-d(T) bound magnetic beads. Double stranded cDNA was synthesized from this mRNA using a commercially available cDNA synthesis kit (Pharmacia). This was then used as the template for PCR amplification.

Amplification was carried out under the following conditions:

10ng of control plasmid or 1µl of cDNA were amplified using 1µg of each primer per reaction. The reaction cycle consisted of 94°C for two mins, 60°C for 2 mins and 72°C for 10 mins. The longer extension times were chosen to allow the amplification of full length envelope genes. One tenth of the reaction products were run on a 6% acrylamide gel and visualised by ethidium bromide staining and UV fluorescence. (Fig.17)

Lanes 1 and 2 show the products of amplifying two full length endogenous clones pLCM-1 and pBCM-3 respectively. These gave products of the size expected

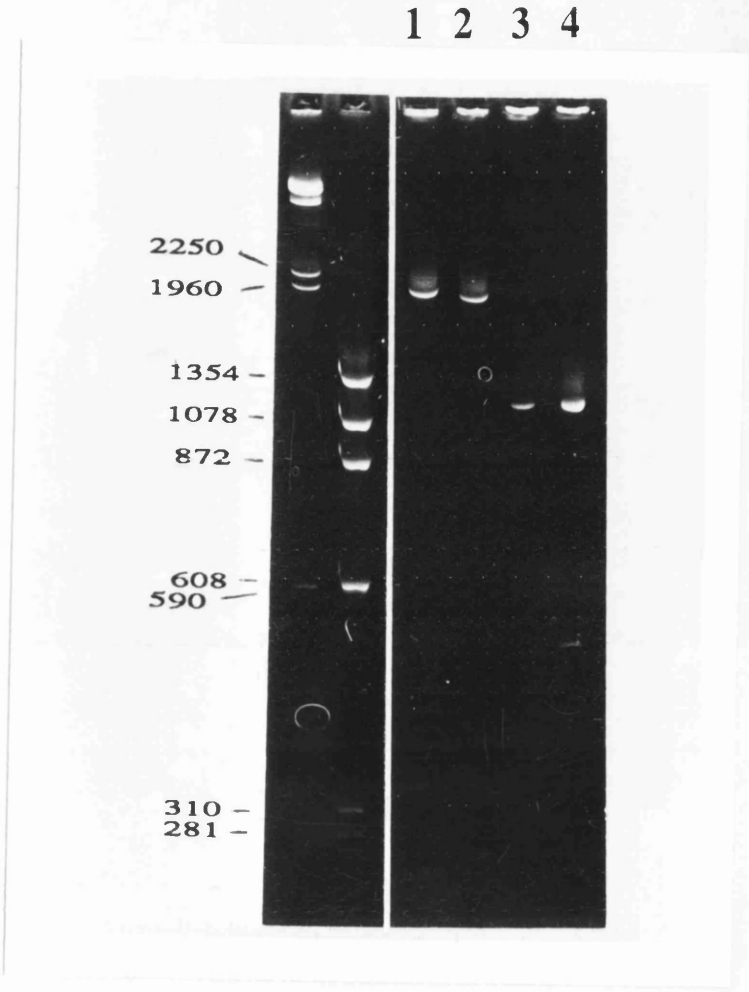


Fig.17. Amplification of endogenous envelope gene sequences from feline cDNA.

Oligonucleotides were designed to specifically amplify the entire *env* gene using PCR. The endogenous *env* gene sequences from two full length endogenous clones (pLCM1, lane 1, and pBCM-3, lane 2) or from the prepared cDNA from 3201B (lane 3) and MCC cells (lane 4) were amplified by PCR using these probes. One tenth of the products are shown after electrophoresis on a 6% acrylamide gel, and visualising by ethidium bromide fluorescence. The positions of the molecular weight markers are indicated on the left and the sizes are in bp.

for full length *env* genes. Lanes 3 and 4 show the products of amplifying the cDNAs of 3201B and MCC. these products were much smaller, at only 1.1kb.

4.2.4. Cloning of endogenous envelope gene DNA and sequence analysis.

The 1.1kb DNA fragments from the 3201B and MCC cDNA amplifications were independently gel purified and cloned into an M13 mp10 vectors. Before ligation, the synthetic DNA was incubated with T4 polynucleotide kinase to add 5' terminal phosphates. It was found that contaminants of gel-purified DNA inhibited the kinase enzyme and it was therefore necessary to kinase the PCR products prior to gel purification. The DNA was then eluted from the gel and precipitated with sodium acetate and ethanol, dried and resuspended in ddH2O. The inserts were then blunt end ligated into SmaI digested, phosphatased M13 mp10 vector. (Amersham) Plaques were picked and single stranded DNA phage prepared. These were then sequenced, firstly by A-tracking then by full sequencing. In total, 1 envelope clone from 3201B cells and 2 from MCC cells were fully sequenced. (Fig.18) These clones contained an open reading frame (orf) coding for a 273 amino acid polypeptide, which would produce a truncated envelope protein consisting of the 5' half of the FeLV SU, $gp70^{env}$ with a predicted size of 33.5kD, excluding the first 32 residues comprising the hydrophobic signal peptide. (Fig.19)

4.3. DISCUSSION.

The detailed study of the enFeLV transcripts in 3201B cells with sub-genomic probes indicated that the larger transcript contained LTR, *gag* and *env* sequences, while the smaller had LTR and *env* sequences only. This suggests that the enFeLV locus (or loci) involved is a truncated provirus, substantially deleted in *pol*, that is transcribed into genomic length RNA. The smaller RNA could be a spliced envelope mRNA derived from the larger form. Inspection of the published enFeLV 5' sequences indicated that in all cases studies, the splice donor site 5' of the glycosylated *gag* gene sequence was intact. (Berry *et al.*1988) Sequence information on the *env* splice acceptor is not available at present. The possibility that the two

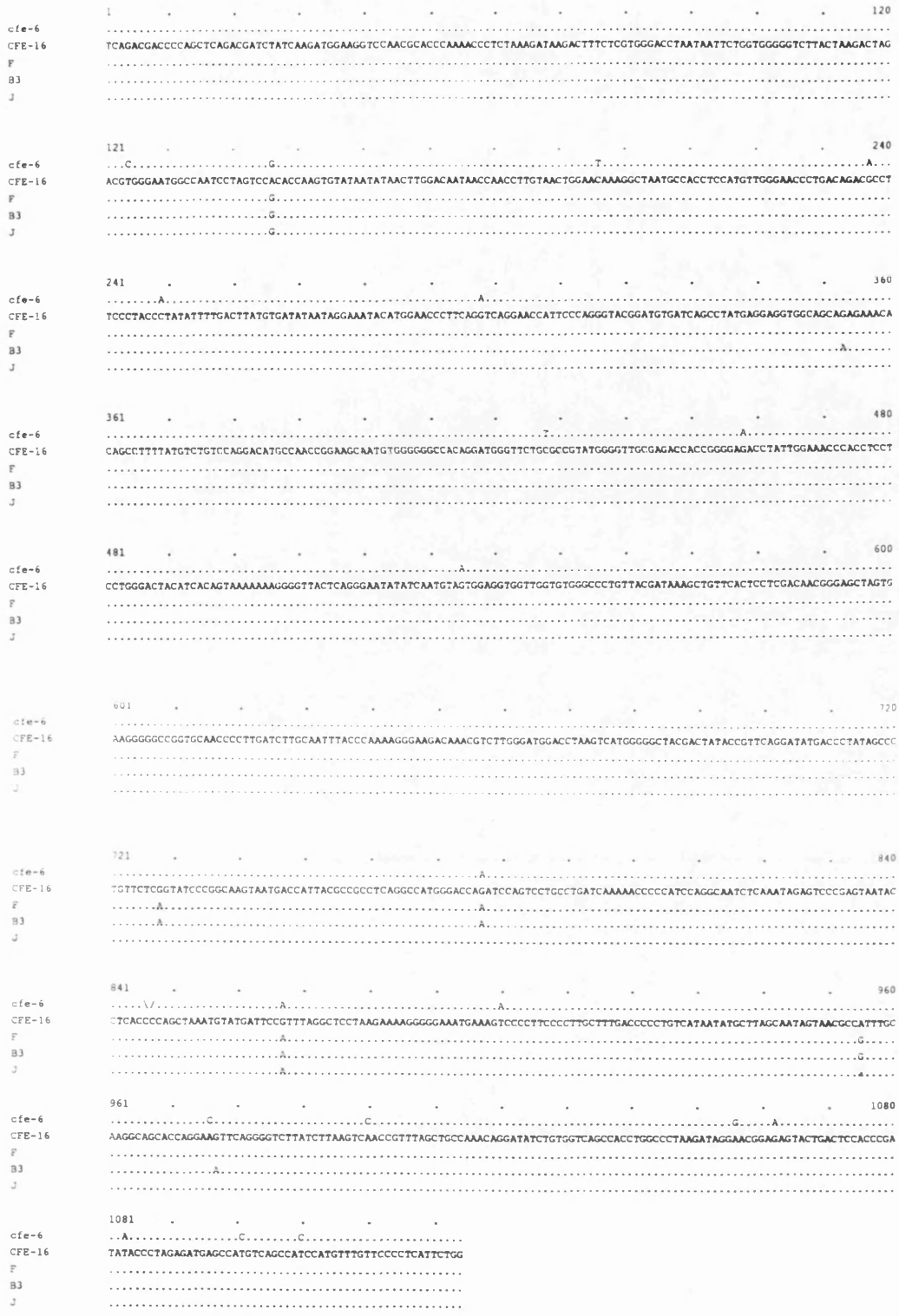


Fig.18. Sequence analysis of the endogenous *env* genes isolated by amplification of feline cDNA.

The nucleotide sequence of the three individual enFeLV *env* clones, isolated by the specific amplification from cDNA, was determined by chain termination sequencing and is shown here. F and B3 are the sequences of the *env* genes isolated from MCC cDNA and J is the sequence of the *env* gene isolated from 3201B cDNA. The sequences are compared to that of CFE-16 and CFE-6 (Kumar *et al.*1989). The dots represent identity to CFE-16. The arrow in the sequence of CFE-6 at position 845 represents the site of the deletion of *env* gene sequences in both CFE-16 and the enFeLV *env* genes isolated in this study compared to CFE-6 and to other full length FeLV *env* genes.

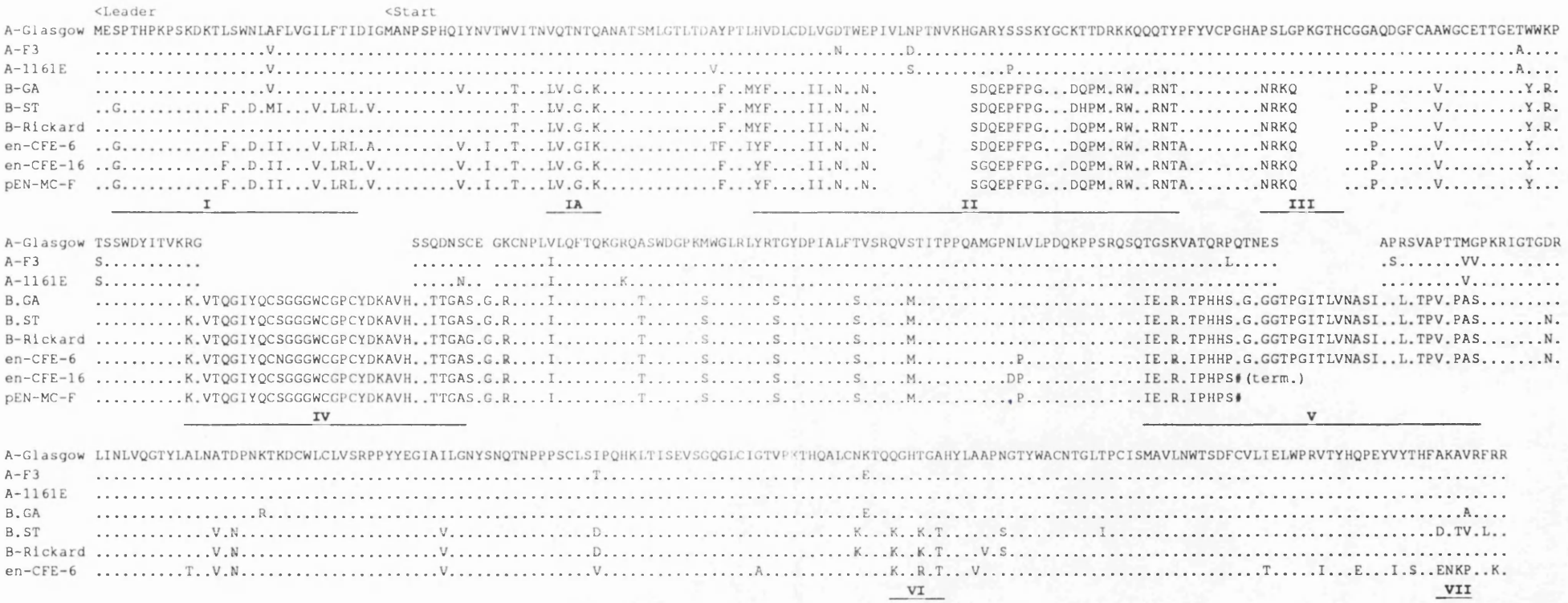


Fig.19. Comparison of the amino acid homologies of the SU coding sequences of FeLV-A, FeLV-B and the expressed enFeLV *env* genes.

The figure compares the amino acid homologies between different isolates of FeLV compared to the sequence of FeLV-A/Glasgow1 and in particular, shows the amino acid identity of the coding potential of the *env* gene isolated from MCC cells (p-EN-MC-F) and CFE-16. Dots represent identities, spaces represent gaps inserted to maintain the homology. The regions of variation between endogenous sequences and FeLV-A are underlined and designated Vr.I to VII. The termination of the open reading frame of *env* in enFeLV CFE-16 and p-EN-MC-F is marked.(#) The sources of the FeLV SU sequence data are as for Fig.3.

transcripts arose from separate proviral loci cannot be formally excluded. However, the proposal that a single locus is transcribed is consistent with published findings that only one of a panel of enFeLV LTRs displayed significant promoter activity. This clone, CFE-16, was a truncated provirus of a size consistent with the major transcript observed here. (Berry *et al.*1988)

A number of proteins were precipitated in tumour cell lines by polyclonal antisera to gp70^{env} and p15^{gag}. *Gag* related proteins were detected, as expected, in the virus positive cell lines T3 and FL74. No *gag* related proteins were detected in the 3201B and MCC cells suggesting that although *gag* related RNA is present, at least in 3201B cells, it is not translated into a stable product. A number of proteins were precipitated in tumour cell lines by the polyclonal antiserum for gp70^{env}, including proteins of approximately 70-80kD, 60kD, 50kD and 35kD. The proteins of 70-80kD precipitated in the virus positive cell lines were of the expected size for the glycoproteins of exogenous FeLV. The 3201B and MCC cells were free of exogenous virus (as confirmed by precipitation with the polyclonal antiserum, anti-p15^{gag}) and so this could not explain the 70-80kD bands precipitated in these cells. Each cell line produces a number of prominent envelope related bands and different sized proteins were found in different cell samples. These proteins were not seen in the AH927 fibroblast cell line. It was suspected that some of the bands may have been due to the presence of contaminating antibodies to other endogenous feline retroviruses, in particular RD114. The gp70^{env} protein used to raise the antiserum in goat was prepared by lentil lectin fractionation of the viral proteins produced by another T-cell lymphoma line, F422. The expression of another endogenous retrovirus, RD114, has been shown to be significantly increased in cats with lymphomas (Niman *et al.*1977a,b) perhaps due to some aspect of the neoplastic condition induced proviral expression, or simply the expansion of an expressing cell. This virus although genetically unrelated to FeLV (Livingston & Todaro, 1973) does have an envelope glycoprotein that is immunologically unrelated, but of similar size to FeLV gp70 and so it is possible that RD114 is released by F422 cells and RD114 gp70^{env} contaminated the FeLV gp70 used for immunisation. It is therefore not

clear whether the unexpected reactivities were due to the expression of RD114 related proteins in these lymphoma cells, or were non-viral contaminants. Molecular characterisation of the endogenous RD114 proviruses in the domestic cat showed many of the *env* genes to be substantially deleted or substituted and generally more divergent than RD114 *gag* and *pol* genes (Spodick *et al.*1984, Reeves & O'Brien, 1984) or the restriction maps of *env* genes of enFeLV clones. (Soe *et al.*1983, 1985) This may therefore explain the heterologous proteins precipitated from different cell lines. Although it was apparent that no *gag* related proteins were translated from enFeLV transcripts, it was possible that endogenous *env* proteins were translated, but I cannot be certain that all the observed species were encoded by enFeLV. It was clear that the identification of the coding potential of the expressed *env* genes would help to distinguish between the protein products precipitated by the polyclonal antiserum, anti-gp70^{env}. Also, it was known that enFeLV *env* genes recombine with infectious FeLV-A to produce a new virus subgroup (FeLV-B) with an expanded host range. (Stewart *et al.*1986) However, it was not known which enFeLV loci contribute to the recombination. It was considered that the highly expressed enFeLV loci may be more likely to become involved in retroviral recombination. For these reasons, it was decided to characterise those *env* genes being expressed in feline cells. The choice of cDNA cloning allowed the specific isolation and analysis of those envelope sequences that were expressed. The 3201B and MCC cell lines were chosen because both are from virus negative lymphomas, and although the PCR amplimers were designed not to amplify exogenous viral sequences (by choosing a 3' amplimer homologous only to endogenous U3 sequences), the use of FeLV negative cells was a further safeguard against isolating exogenous sequences. These cell lines were also selected because they appeared to express different proviruses, the larger transcript from MCC cells being approximately 0.5kb shorter than the most typical transcript in 3201B cells. The smaller transcripts were of apparently identical size. The cloning and sequencing analysis would therefore address two questions: the first, whether the different large transcripts represented the expression of *env* genes of different sizes or different loci with the same size of *env* gene (the sub-genomic

analysis done on 3201B cells had not been performed on MCC cells) and the second, how conserved the *env* genes expressed in these two different cell lines were.

The envelope genes cloned from the two feline cell lines were found to be almost identical. The sequences isolated from the 3201B cells compared to the MCC cells showed only a few single nucleotide differences. (Fig.18) All three cloned envelope gene sequences almost exactly match the sequence of the truncated *env* gene from CFE-16, a truncated endogenous provirus isolated from a placental DNA library. (Soe *et al* 1983, Kumar *et al.*1989) The predicted amino acid sequence of these genes were calculated and compared to those of exogenous FeLV-A and B. (Fig.19) The 3201B clone encoded a protein of precisely the same primary sequence as CFE-16, while the MCC clones has just one amino acid change. This change from aspartic acid to asparagine just before the C-terminus is also found in the amino acid sequence of CFE-6, a full length envelope gene from a complete provirus isolated from the same DNA library as CFE-16. (Kumar *et al.*1989) All display an identical, large deletion involving the 3' half of gp70 (SU) and the entire coding sequence for the FeLV TM protein, p15E, with the exception of the final 15bp of p15E. There are several point mutations relative to CFE-16 which appear in CFE-6. In the two MCC cell derived clones there are two points of difference, the first a conservative change in the *env*-orf and the second, in the non-coding sequence of the LTR. Whether these represent the expression of two closely similar loci in the MCC cells or the artefacts of PCR amplification and cloning cannot be determined without the isolation of more clones or the direct sequencing of the products of further PCR amplifications.

The truncated endogenous *env*-orfs encode a protein of approximately 33.5kD assuming the removal of the signal peptide, but discounting glycosylation. All potential N-linked glycosylation sites are conserved. The 35kD protein immuno-precipitated from the lymphoid tumour cell lines (Fig.16) was therefore considered a candidate product of the truncated *env*-orf.

The use of PCR to isolate the endogenous envelope genes had the major advantage of speed. The construction of a full cDNA phage library and the

subsequent screening for *env* containing elements would have resulted in perhaps a larger number of clones being isolated and may have determined whether a number of different proviruses were expressed in the same cell line. However, both should have resulted in the isolation of the predominant species. Any enFeLV proviruses lacking primer complementary sequences would not be isolated by the specific PCR technique although the primers were selected from relatively conserved regions of enFeLV proviruses. It was uncertain whether any PCR products amplified from low abundance transcripts would be seen given the sensitivity of ethidium bromide fluorescence, but the immediate aim of the experiment was to isolate *env* genes from those enFeLV proviruses that were highly expressed and so the process was considered satisfactory. The use of PCR may also have generated point mutations or other artefacts in the sequences of the envelope genes due to the misincorporation of bases by Taq polymerase. Taq polymerase has been reported to have an error rate for single base paired substitutions of 1 per 9000 bases, but the rate is higher if the level of nucleotide substrate is limiting. (Tindal & Kunkel, 1988) This would be a greater problem in PCR experiments involving a large number of cycles without adding more nucleotide to the sample, but in the protocol involved here, may not be so important, as the number of cycles was comparatively small and the nucleotides always in excess. The consistency of the derived sequences suggest that there has been very little, if any misincorporation. Sequencing of the PCR products of known cloned genes would show the percentage misreading due to the procedure as would the sequence analysis of additional independent amplifications of target DNA. Direct sequencing of a larger number of enFeLV *env* genes may identify other variants of these transcripts. Because the full length proviruses were successfully amplified by the PCR procedure, it did not appear to bias against the amplification of longer *env* sequences. However, the possibility that the synthesis of cDNA from full length *env* genes may have been less efficient could not be discounted. The highly expressed enFeLV sequences seen by Northern blot analysis would be expected to predominate in cDNA libraries, but it is also possible that other enFeLV transcripts expressed at low levels were amplified in the PCR reaction but not detected by

ethidium bromide staining of the DNA on a polyacrylamide gel. However, the DNA amplified appeared to represent the major expressed species in both cell lines.

CHAPTER 5 EXPRESSION OF enFeLV: POSSIBLE ROLE IN RECOMBINATION.

5.1. INTRODUCTION.

The involvement of expressed endogenous transcripts in recombination of murine and avian viruses has already been discussed. (Chapter 1/4.2.) Recombination of endogenous viral sequences requires the expression of ERV elements in virally infected cells, their co-packaging into exogenous virions, and the transfer of reverse transcriptase from one template to another during the synthesis of the DNA provirus in newly infected cells. In the following studies, the ability of the expressed endogenous transcripts to be co-packaged into virion particles, as the putative first step in recombination, was tested. The sequence analysis of a novel FeLV-B isolate suggested that loci resembling the expressed transcripts are involved in some recombinations.

5.2. RESULTS.

5.2.1. Comparison of the coding potential of enFeLV *env* genes.

The characterisation of enFeLV *env* genes expressed in feline lymphoma cell lines allowed the comparison of these sequences with the published gp70 sequences of other enFeLV *env* gene sequences and exogenous FeLV of subgroups A and B. Comparison of the gp70 (SU) coding regions of enFeLV *env* genes to those of FeLV-A identifies a number of sequence features unique to endogenous viruses. (Fig.20) Some of these are also found in FeLV-B viruses as a result of their recombinational origin, and are hence implicated in the binding of FeLV-B gp70 to its cell receptor. The expressed enFeLV *env* genes encoded only endogenous specific regions, Vr I, Ia, II, III and IV in full. However, all FeLV-B viruses for which sequence information was available, contain the entire endogenous derived Vr V region and some, a Vr VI region. This analysis suggested that the expressed enFeLV *env* genes contain insufficient information to account for all enFeLV derived sequences found in the FeLV-B viruses and therefore could not produce these viruses by a simple recombination. Of the enFeLV proviruses so far sequenced, only the full length CFE-6 clone had all the appropriate sequences present in the FeLV-B viruses.

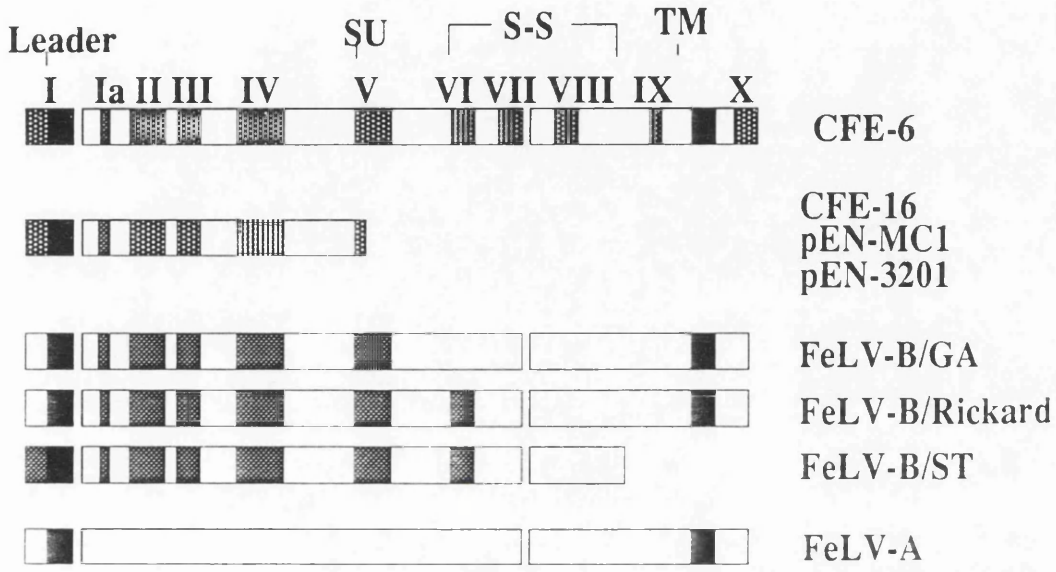


Fig.20. The variable domain structure of FeLV-B *env* genes.

The figure shows the structure of a number of FeLV-B and enFeLV *env* genes that have been described in Figs.3 and 19. The endogenous derived variable domains are shown as shaded boxes and are numbered I to X. The black boxes represent hydrophobic sequences (signal peptide and transmembrane domains). The *env* gene of FeLV-A is shown below for comparison.

5.2.2. Packaging of enFeLV transcripts into FeLV virions.

In order to test the packaging of the endogenous proviral transcripts contained in 3201B cells, FeLV-A/Glasgow-1 virus from a productively transfected BHK line (BHK21-pFGA-5) was used to infect 3201B cells at high multiplicity. The infected cells were cultured and expanded for 3 weeks before viral particles were harvested from 2 litres of exponentially growing cells. RNA was isolated and tested for FeLV-B *env* related sequences by Northern blot analysis. (Fig.21) Using an exogenous FeLV-A envelope probe, A/HH, the vast majority of the RNA was shown to be of exogenous FeLV origin. The B/S probe picked up a faint signal corresponding to the 4kb transcript of 3201B cells. The faint genomic length band detected by this probe may have resulted from cross hybridisation of the FeLV-B related envelope sequences with the FeLV-A envelope sequences, which were in vast excess. Unfortunately that meant that any evidence of co-packaging of full length transcripts or novel FeLV-B recombinants would be hidden by this cross hybridisation. This problem might be solved by using the enU3 probe instead of the B/S *env* probe to detect enFeLV specific sequences.

5.2.3. Sequence analysis of FeLV-B/GM1.

The GM-1 strain of FeLV was isolated from a naturally occurring case of myeloid leukemia and induces severe haematopoetic abnormalities, including myeloblastic leukemia, on inoculation into cats. (Tzavaras *et al.*1990) The strain contains a mixture of at least two viruses, including a replication competent FeLV of subgroup A, resembling other low or minimally pathogenic FeLV-A isolates and a replication defective virus of subgroup B. Restriction enzyme analysis of this FeLV-B virus showed extensive deletions in *gag* or *pol* but the envelope gene appeared to be intact. (Fig.22) Because this virus had extensive deletions in *gag* and *pol* similar to those seen in the truncated endogenous viruses it is conceivable that it resulted from recombination between exogenous virus and a truncated enFeLV element similar to the ones that have been shown to be expressed in lymphoid cells. Therefore studies were initiated to characterise the GM-1 provirus with particular

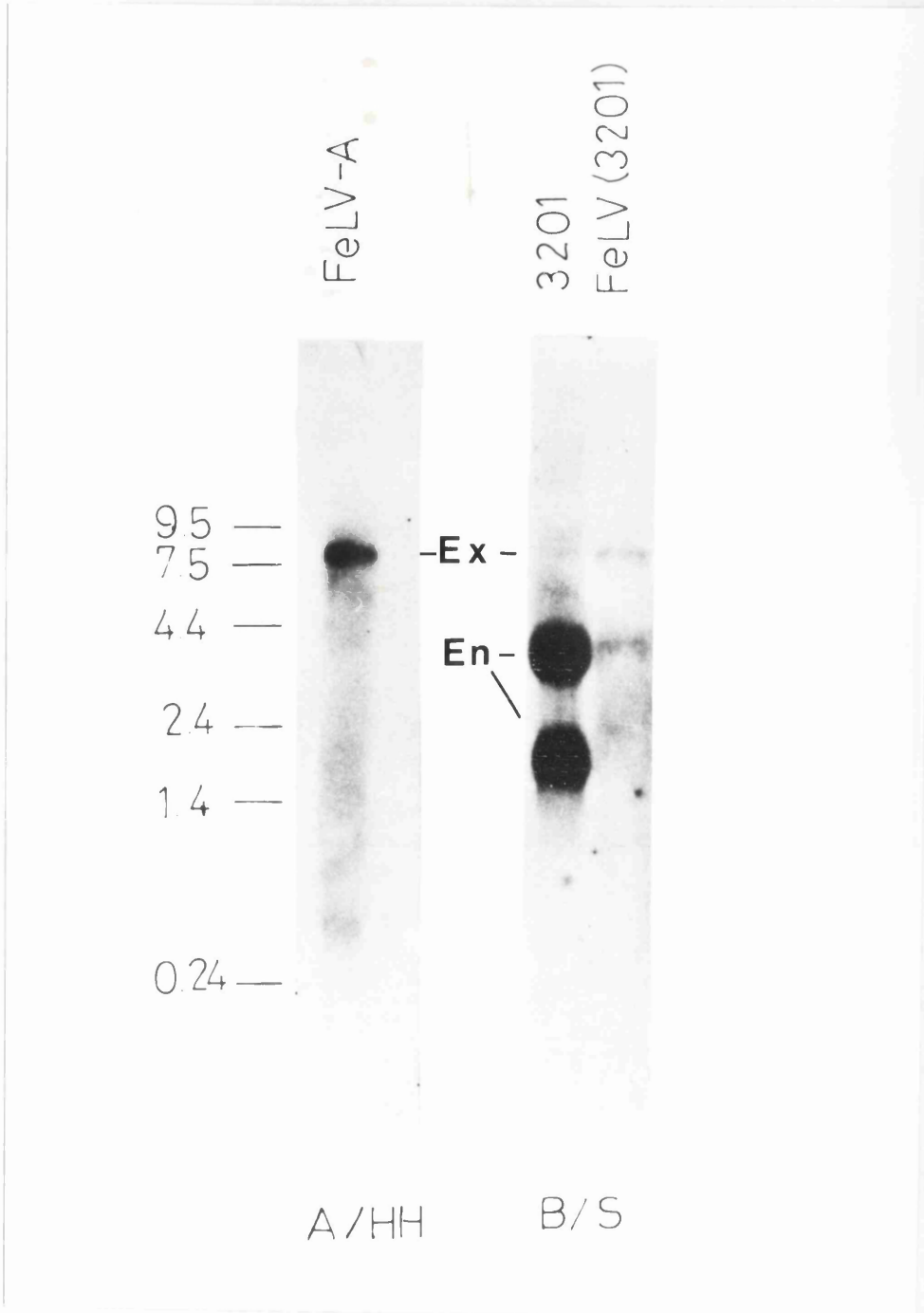


Fig.21. Identification of enFeLV transcripts in exogenous virions.

3201B cells were infected with FeLV-A/Glasgow1 and the virion particles harvested from 2 litres of infected cells after 3 weeks of culture and RNA isolated. The RNA was split into two aliquots and separated on a 1% agarose gel containing formaldehyde, as was 20µg of 3201B total RNA for comparison. The gel was blotted onto a hybridisation filter membrane and strips hybridised with [^{32}P] labelled probes specific for FeLV-A *env* (A/HH) or FeLV-B *env* (B/S). The positions of the molecular weight markers are indicated on the left and the sizes are in kb.

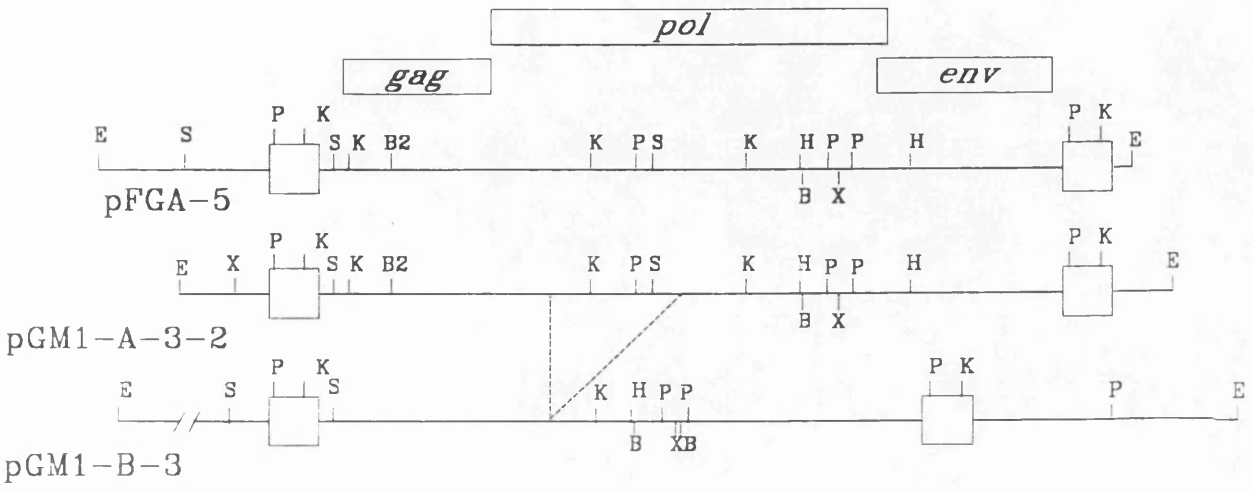


Fig.22. Molecular structure of viral clones isolated from FeLV-GM1.

The structures of the replication-competent viral clone, pGM1-A-3-2, and a replication defective clone, pGM1-B-3 are presented. For comparison, the restriction map of a clone of FeLV-A/Glasgow1 (pFGA-5) is shown at the top of the figure, below a gene map of the provirus. Host cell sequences flanking integrated proviruses are not to scale. From sequence analysis, the precise location of a 1.6kb deletion in *pol* of pGM1-B-3 has been determined, and is indicated by the dashed lines between the pGM1-A-3-2 and pGM1-B-3 structures. Restriction enzyme site abbreviations are: B, BamHI; B2, BglII; E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SstI; X, XhoI.

emphasis on those areas of the genome likely to contain the 5' and 3' sites of recombination and the intervening enFeLV-derived sequences.

To determine the 5' point of recombination and the extent of the deletion a 2.6kb SstI-KpnI *gag-pol* fragment was sub-cloned into M13 vectors in both orientations and partially sequenced from either end of the fragment using a series of oligonucleotide primers designed as the sequence was established. Sequencing from the KpnI site in an upstream direction identified a 1.6kb deletion in the coding sequence of the *pol* gene. (See fig.22, sequence not shown.) Comparison with the DNA sequence of 1161E, a weakly leukaemogenic FeLV-A isolate (Donahue *et al.*1988) showed the deletion to involve the reverse transcriptase coding region. The sequence of the LTR was analysed previously and shown to be typical of the exogenous LTR of FeLV-A/Glasgow 1 and 1161E. (Tzavaras *et al.*1990) In the 610bp 3' to the SstI site, the sequence diverged from exogenous FeLV but showed a closer match to enFeLV in the coding sequence of the glycosylated *gag* leader and more markedly in the $Pr65^{gag}$ precursor. (Fig.23) FeLV-B/GM1 and CFE-6 shared a 9bp insert and multiple point mutations relative to exogenous FeLV-A sequences, but also had numerous individual base pair substitutions. The FeLV-B/GM1 glycosylated *gag* leader was in frame with the main body of the *gag* gene but terminated prematurely within the $p15^{gag}$ coding sequence. These termination codons would also cause the premature termination of the $Pr65^{gag}$ protein, 316 bases downstream of its initiation site. The sequence information suggested that the 5' point of recombination of FeLV-A with endogenous sequences was in the region between the primer binding site and the 9bp insert located in the glycosylated *gag* precursor coding region. The exact point of recombination could not be determined since enFeLV and exogenous FeLV are closely matched here.

The entire $gp70^{env}$ sequence was determined by double stranded plasmid sequencing of a subclone of FeLV-B/GM1, pEPP-3 which contained the envelope gene and a portion of the 3' LTR coding sequence. This proved to be more easily sequenced than the plasmid containing the entire genomic sequence, possibly due to the smaller size making denaturation of double stranded DNA more efficient, or the

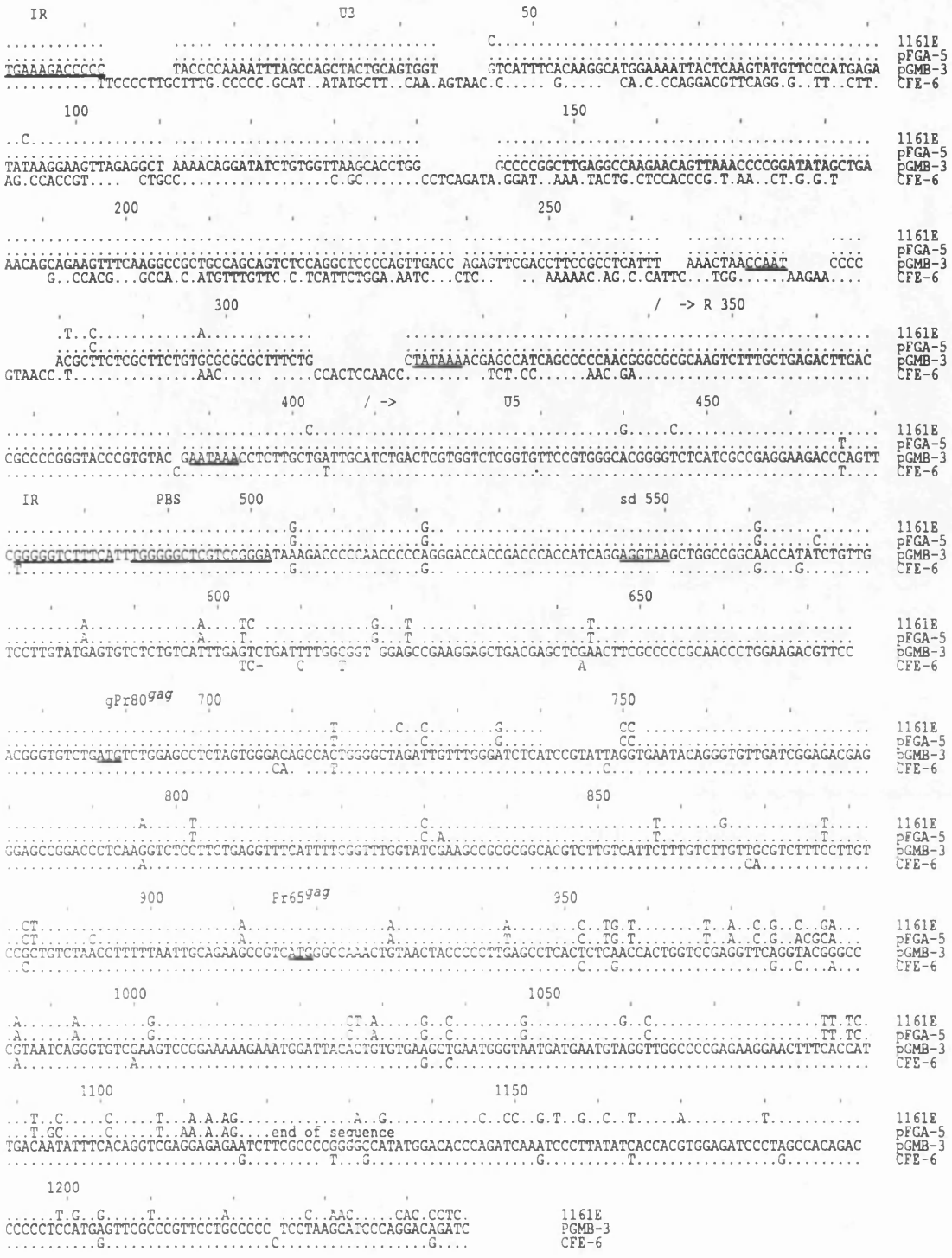


Fig.23. Nucleotide sequence analysis of the 5' end of FeLV-B/GM1.

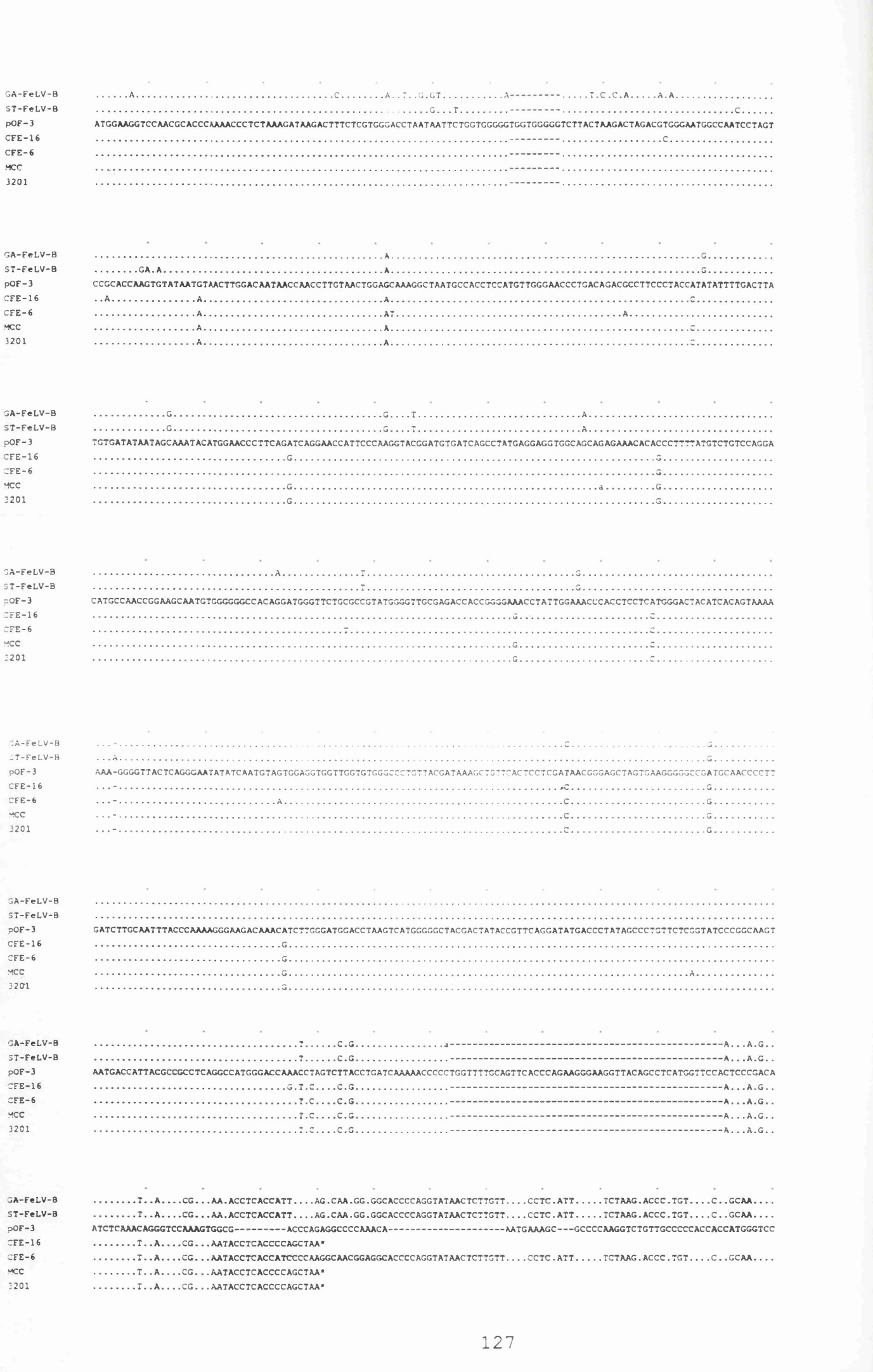
Sequence of 1247bp of pGM1-B-3, extending from the 5' LTR. The GM1 sequence is shown in full. Matches in the other sequences are indicated by dots, whereas differences are shown in letter code or as gaps in the sequence. The sequences compared to pGM1-B-3 are from two exogenous FeLVs, FeLV-A/Glasgow1 (pFGA-5), (Tzavaras *et al.*1990) and FeLV-A/1161E (Donahue *et al.*1988), and one enFeLV sequence, CFE-6 (Kumar *et al.*1989). The boundaries of U3, R and U5 sequences are indicated, as are the inverted repeats (IR) marking the termini of the LTR, the tRNA primer-binding site (PBS), the consensus splice donor (sd) site for *env* mRNA and the initiation codons for glycosylated and non-glycosylated forms of the *gag* precursors (gPr80 and Pr65^{gag}).

reduced genetic information present, reducing the chance of the oligonucleotides mis-priming. Sequencing primers were constructed where required. The nucleotide and predicted amino acid sequences when aligned with those of other FeLV gp70^{env} genes and their endogenous counterparts showed the region of recombination where the sequence ceased to be like endogenous *env* and to have sequences present only in exogenous *env* lay just downstream of the Vr 5 region. (Figs.24 and 25) The exact point of recombination where the sequences cease to resemble enFeLV *env* appeared to be at an ACCCC nucleotide motif. After this point, the sequence appeared to be derived from FeLV-A *env*.

5.3. DISCUSSION.

Analysis of the sequences of the enFeLV *env* genes expressed in 3201B and MCC cells and comparison with FeLV-B virus isolates suggested that the expressed *env* genes did not contain sufficient enFeLV sequences to generate any of the FeLV-B viruses so far characterised. All the FeLV-B viruses contain enFeLV sequences that must have come from more complete enFeLV *env* genes such as that of CFE-6. However, no expression of these sequences has been detected in any tissue examined so far.

The result of the co-packaging experiment in section 5.2.2. shows that the 4kb transcript in 3201B cells does possess sequences sufficient for packaging although the packaging efficiency appears to be low. Given the homology between exogenous FeLV and enFeLV, recombination might be expected to be favoured by co-packaging. It can be concluded that the apparent restriction of expressed enFeLV proviruses in recombination is not due to an inability to be co-packaged into virions. A recent examination of the *pol* gene sequence of the enFeLV clone CFE-6 by Roy-Burman's group, (Pandey *et al.*1991) revealed a complete open reading frame, with high homology to exogenous FeLV *pol*. The 5' point of recombination involved in the production of FeLV-B viruses may therefore be within the *pol* gene. It may be that the highly expressed enFeLV loci are less prone to recombine due to the extensive deletion of *pol* sequences as in the 3201B cell transcripts, and the CFE-16


GA-FeLV-B
ST-FeLV-B
pOF-3
CFE-16
CFE-6
MCC
3201

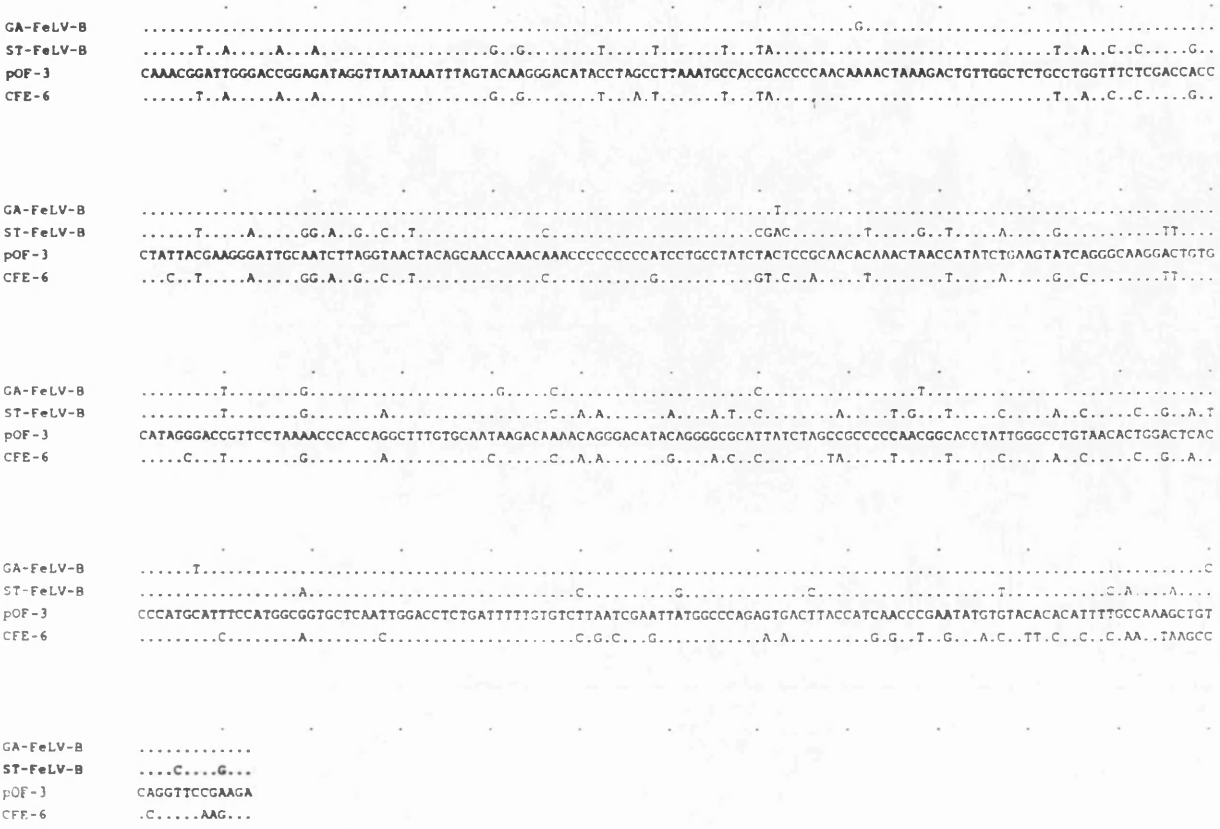


Fig.24. Nucleotide sequence analysis of FeLV-B/GM1 gp70.

Sequence of gp70 of pGM1-B-3 (pOF-3) is shown in code. Matches in the other sequences are indicated by dots, whereas differences are shown in letter code or as dashes to indicate gaps in the sequence. The sequences compared to pGM1-B-3 are from two exogenous FeLVs, FeLV-B/Gardner Arnstein (GA-FeLV-B)(Elder & Mullins, 1983) and FeLV-B/Snyder Theilen (ST-FeLV-B)(Nunberg *et al.*1984), two enFeLV sequences, CFE-6 and CFE-16 (Kumar *et al.*1989) and the sequence of the expressed truncated *env* genes isolated from 3201B and MCC cells (Chapter 4).

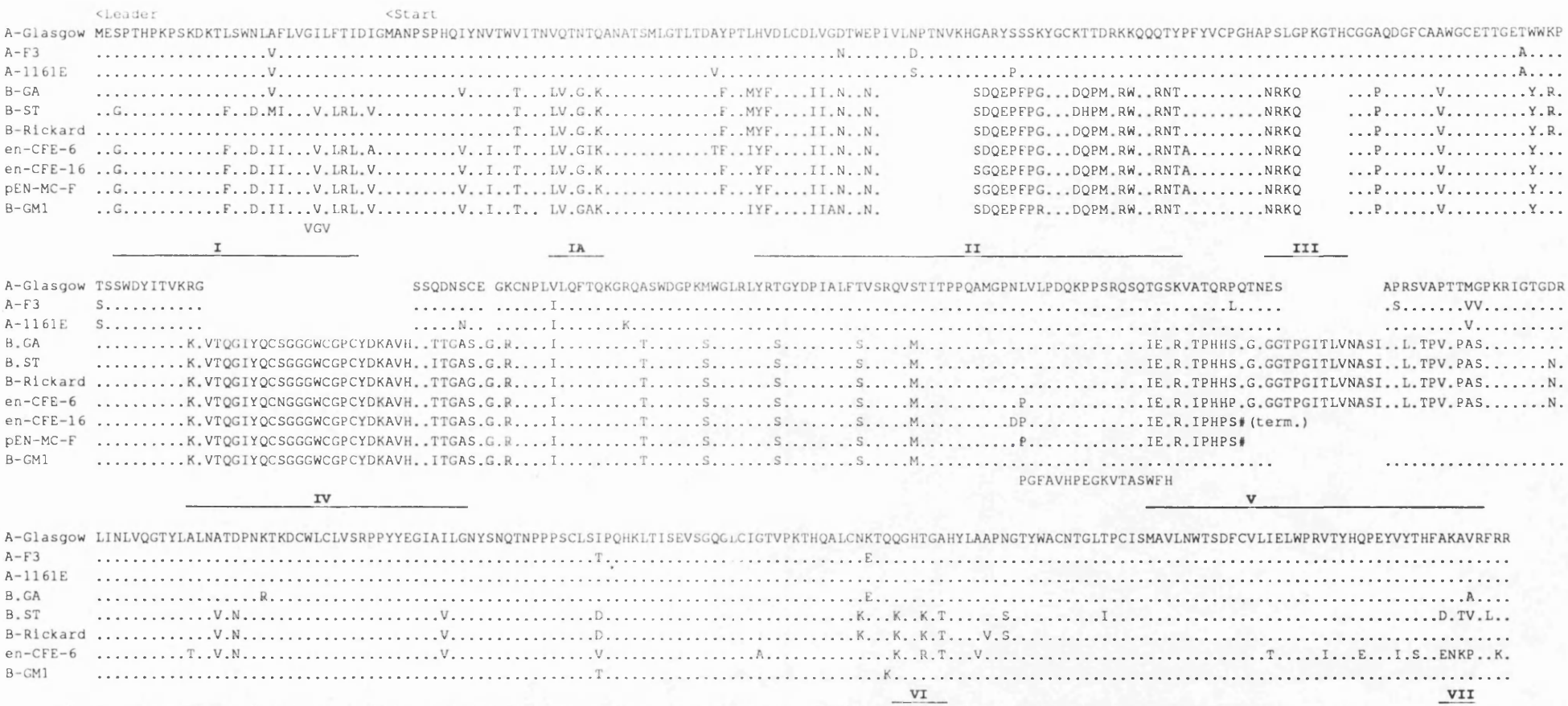


Fig.25. Comparison of the amino acid homologies of the SU coding sequences FeLV-B/GM1 to FeLV-A, FeLV-B and the expressed enFeLV *env* genes.

The figure compares the amino acid homologies between the SU coding region of the *env* gene from FeLV-B/GM1 to those FeLV SU sequences compared in Figs.3 and 19. Dots represent identities, spaces represent gaps inserted to maintain the homology. The sequence of FeLV-B/GM1 contains two inserts relative to the other FeLV SU sequences and are indicated below the main sequence. The regions of variation between endogenous sequences and FeLV-A are underlined and designated Vr.I to VII. The termination of the open reading frame of *env* in enFeLV CFE-16 and p-EN-MC-F is marked.(#) The sources of the FeLV SU sequence data are as for Fig.3.

clone. Nevertheless, it is possible that the recombination of truncated, expressed loci occurs, but that the resultant viruses are defective, due to the extensive deletions found in the endogenous parent sequence. Such a virus may not be able to replicate and therefore expand to become a significant virus population. The observed preference for full length, but apparently non-expressed sequences may simply represent the competence of these recombinants to replicate. The key to this restriction may be that envelope chimeras of endogenous and exogenous sequences are functional only when the recombinant contains a particular portion of the enFeLV *env* gene missing from expressed enFeLV. The GM1-B virus may be the exception to this rule by virtue of the unique sequences inserted at the recombination site. The construction of envelope chimeras would be necessary to define further the exogenous sequence requirements for virus function.

The GM1-B virus is an example of a defective recombinant that propagates efficiently along with its non-defective FeLV-A helper virus. The sequence of the GM1-B *gag* gene is homologous to the *gag* sequence of an endogenous provirus, CFE-6. (This was the only published *gag* sequence from the enFeLV clones isolated by Roy-Burman's group.) The GM1-B virus contained multiple defects including the premature termination of the *gag* open reading frame and the deletion of 1.6kb of DNA from the reverse transcriptase coding region of *pol*. The fact that the FeLV-B/GM1 *gag* gene sequences are highly homologous to enFeLV *gag* suggests that the 5' end of the genome was a site of recombination. This recombinant therefore contains a greater extent of the enFeLV derived sequences than has been previously been reported for FeLV-B viruses.

The 3' point of recombination occurs after ACCCC, a motif commonly seen at recombination sites between FeLV and cellular oncogenes (Neil *et al.*1987), and is complicated by the presence of a 50bp insert at the 3' recombination site. 30bp of this insert were a reiteration of FeLV-A specific sequences. This sequence also occurs after ACCCC found 184bp upstream in FeLV-A. This sequence in FeLV-B/GM-1 is in an alternative reading frame resulting in a completely novel stretch of amino acids. The remaining nucleotides from this insert were of unknown origin.

Examples of this sort of illegitimate insertion have been recorded, (Pulsinelli & Temin, 1991) and a number of studies of retroviral recombination have identified many examples of deletions, insertions and other mutations resulting from the process of reverse transcription. (Pathak & Temin, 1990 a,b)

The GM1-B *env* gene contains the endogenous specific variable regions Ia, II, III and IV, which are found in both full length and truncated enFeLV *env* genes. (Fig.26) Therefore the recombination leading to the generation of FeLV-B/GM1 could have involved a truncated enFeLV, with a large deletion in *pol*, and an incomplete *env* gene. In fact, the predicted genetic structure of the enFeLV locus responsible for the GM1-B virus is similar to those of the transcripts expressed in 3201B cells and isolated from placental DNA (CFE-16). There are a number of point mutations in the gp70 coding region present in the GM1-B virus compared to CFE-16 and pEN-MC-F to suggesting that these are not the actual progenitor loci. However, polymorphisms of enFeLV in different animals and viral sequence evolution after capture may have obscured the true origin of this *env* sequence. It should be recalled that the mutational frequency of viral sequences is much higher than that of cellular genes, because of the lower fidelity of reverse transcriptase due to its lack of proof reading systems and the subsequent rounds of replication. (Gojobori & Yokoyama, 1985) However, it cannot be ruled out that FeLV-B viruses are the result of multiple recombinations with the expressed enFeLV playing a role in the first steps. The analysis of GM1-B shows that Vr Ia, II, III and IV are, in this case, sufficient to confer B-subgroup specificity. However, it is conceivable that the novel insert at the *env* recombination site containing FeLV-A *env* sequences compensated for the lack of enFeLV derived *env* sequences. It is unlikely that this sequence is required for FeLV-A envelope function in the same way as it may be for FeLV-B/GM1 function, because although the nucleotide sequence is reiterated, it is in an alternative reading frame in FeLV-B/GM1 than in FeLV-A, and so would produce a novel peptide sequence. The GM1-B virus therefore proves to be an exception to the general observation that FeLV-B viruses contain more enFeLV derived sequences that are present in the expressed, truncated transcripts. In this

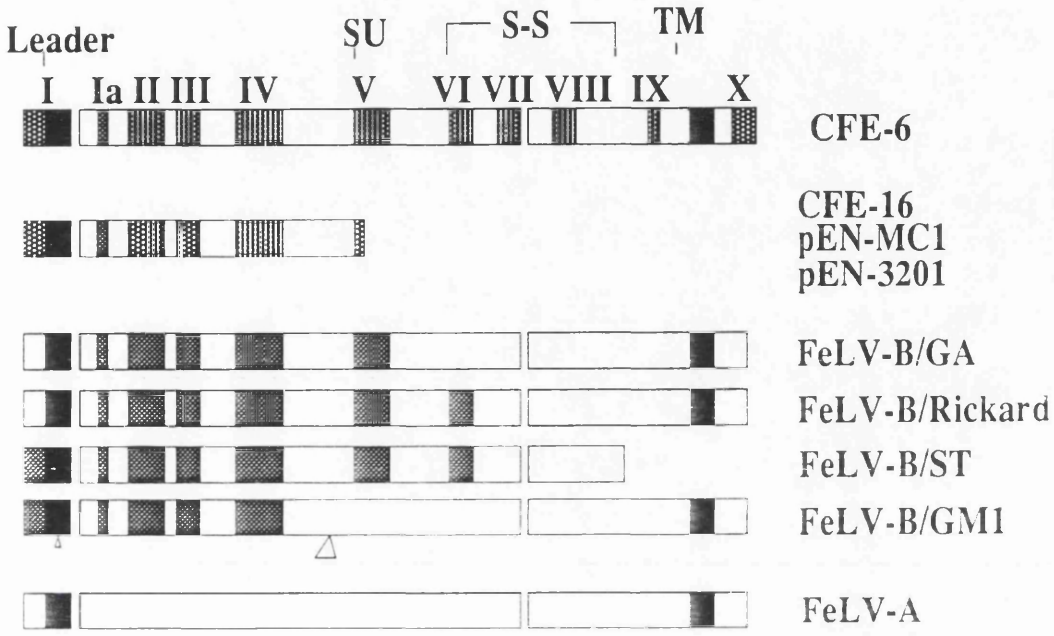


Fig.26. The variable domain structure of FeLV-B *env* genes.

The figure shows a modification of Fig.20. to include the *env* gene sequence isolated from FeLV-B/GM1 The endogenous derived variable domains are shown as shaded boxes and are numbered I to X. The black boxes represent hydrophobic sequences (signal peptide and transmembrane domains). The *env* gene of FeLV-A is shown below for comparison. The positions of the inserts present in FeLV-B/GM1 are shown as triangles.

case, the defective virus expanded to become a major virus population perhaps due to some unusual advantage of this particular recombinant. FeLV is frequently associated with T-cell abnormalities. The GM1 complex, however induces a number of severe haematopoetic abnormalities including myeloblastic leukemia. Experimental inoculation of the FeLV-A/GM1 component alone or with the FeLV-B component of the GM1 isolate demonstrated that the FeLV-A virus was minimally pathogenic, and caused persistent viremia in only one sixth of the cats involved whereas the AB complex induced persistent viraemia in all cats inoculated. It is possible that the FeLV-B specific envelope gene with its novel sequence insert may have altered the tropism of the FeLV-B/GM1 virus but this remains to be proved. What is clear is that the GM1-B virus, although defective, has a significant effect on viral pathogenicity.

CHAPTER 6 CHARACTERISATION OF A PROTEIN PRODUCT OF THE ENDOGENOUS ENV-ORF

6.1. INTRODUCTION.

Immune-precipitation analysis using a polyclonal anti-F422 $gp70^{env}$ showed the presence of a candidate product of the *env*-orf but was inconclusive due to the apparently extraneous reactivity of the antiserum. Two monoclonal antibodies were tested for reactivity to *env* related proteins in 3201B and MCC cells (results not shown). The failure of these to recognise *env* related proteins in MCC and 3201B cells was unremarkable in view of the loss of epitopes the truncated *env* gene. Knowing the DNA sequence encoding the truncated envelope protein, it was possible to construct synthetic peptides and to raise a specific antiserum.

6.2. RESULTS.

6.2.1. Development of endogenous specific antisera.

(i) Design of pGEX constructs.

The pGEX vector is a bacterial expression vector that directs the synthesis of foreign polypeptides in *E.coli* as C-terminal fusions of Sj26, a 26kD glutathione-S-transferase (GST) encoded by the parasitic helminth *Schistosoma japonicum* (Smith & Johnson, 1988). At the 3' end of the GST-orf in pGEX is a cloning site followed by translational termination signals. Different versions of the vector have the cloning sites in three different reading frames. Protein synthesis is under the control of the IPTG inducible tac promoter. In the absence of inducer, the plasmid-encoded $lacI^q$ gene efficiently represses transcription from the tac promoter regardless of the lacI status of the host cell strain.

In deciding which fragments of *env*-orf DNA to clone into the pGEX vectors, a number of factors had to be considered.

1. Although the pGEX system has been reported to accommodate proteins up to 84kD, problems with solubility of larger proteins have been encountered by others using the vector. (M.Rigby, N.Spibey, University of Glasgow. pers.commun.) Better success rates were achieved with small fragments encoding around 100 amino acids.

2. It was decided to exclude regions of high hydrophobicity to avoid problems with solubility and hence difficulties in recovering the products.

3. Finally it was necessary that the reading frame of the protein should be preserved.

The *env*-orf is deficient in restriction enzyme sites useful for cloning into the vector sites, BamHI, EcoRI and SmaI. The sites of a number of 4 base pair cutting enzymes were analysed and five fragments selected which, if successfully cloned, would encompass the complete gene apart from the leader peptide, which was avoided due to its hydrophobic nature. Because the enzymes selected were frequent cutters, it was necessary to remove the *env*-orf from its vector before digesting to isolate the fragments of choice. (Fig.27)

(ii) Construction of recombinant pGEX vectors.

The truncated *env*-orfs from 3201B and MCC cell lines were transferred from phage to plasmid vector by digesting M13 RF DNA with BamHI and EcoRI, removing the *env*-orf, and ligating it into BamHI and EcoRI digested pIC20H. The ligated DNAs were transfected into competent E.coli DH5α and the white positive colonies grown o/n in 10mls L-broth with ampicillin. Test plasmids were prepared using the STET method, BamHI and EcoRI digested and the DNA separated on a 1% agarose/TAE gel to test for the presence of the insert. Plasmids containing an appropriately sized insert were prepared in bulk by CsCl gradient purification. 100mg of plasmid pEN-MC-F (containing the *env*-orf from MCC cells) was digested with BamHI and EcoRI and the insert separated from the vector by electrophoresis in a 1% agarose/TAE gel. The insert band was excised from the gel and the DNA extracted from the gel by electroelution. The DNA was precipitated with ammonium acetate and ethanol, dried under vacuum and resuspended in ddH_2O. The purified insert DNA was subsequently digested to isolate fragments for cloning into the pGEX vector.

One clone, H5A, representing the 3' fragment encompassing both *env* and LTR, encoded the terminal 30 amino acids of the *env*-orf. Because of the small size, it was thought that this might be likely to produce a stable product. The clones were tested for fusion protein production by the induction of bacterial miniprep cultures

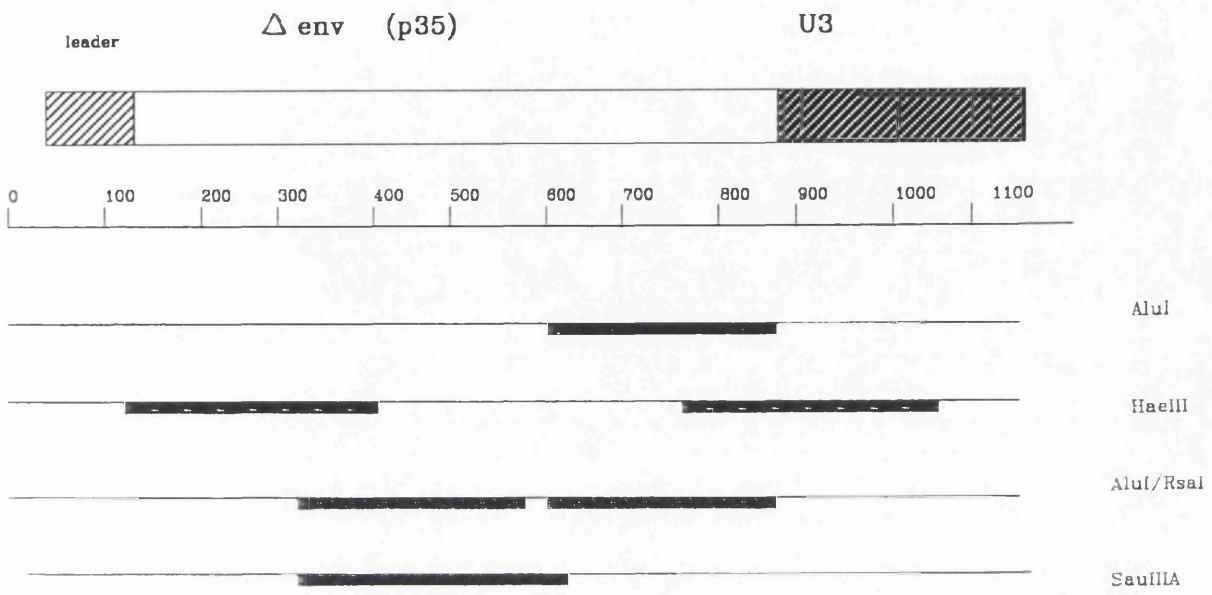


Fig.27. EnFeLV peptides selected for pGEX cloning.

The enFeLV *env* containing clone isolated from feline lymphoid cells is shown as a line marked at 100bp intervals, with the regions encoding the hydrophobic leader sequence, the envelope open reading frame (Δ*env*) and the U3 region indicated above. The bold lines below represent a number of fragments produced by the appropriate restriction enzymes (AluI,AluI and RsaI, HaeIII, SauIIIA) that were considered appropriate for enFeLV-fusion protein synthesis. The carboxy-terminal fragment resulting from HaeIII digestion successfully produced a GST-fusion protein.

with IPTG followed by lysis and protein separation on a 13% denaturing PAGE gel. Proteins were visualised by staining with Coomassie brilliant blue. (Fig.28) Bulk protein preparations were produced from the clone H5A. The fusion protein was analysed by Western blotting and immune staining using the polyclonal antiserum for gp70^{env} followed by an alkaline phosphatase conjugated secondary antibody. Fig.29 shows a protein gel and the corresponding Western blot to test whether the GST protein encoded by pGEX-2T alone was antigenic for the polyclonal antiserum for gp70^{env}. Both H5A and GST (pGEX-2T) were run in the same lane, and were stained by Coomassie blue, however only the *env*-orf fusion protein was stained in the Western blot by the antiserum conjugate.

Fig.30. shows samples taken at various stages of protein purification described in Methods, section 2.2.20, run on duplicate 13% SDS-PAGE gels, one stained with Coomassie blue, the other Western blotted and immuno-stained with the polyclonal antiserum for gp70^{env}. The fusion protein reacted with the antiserum, and so contained some epitopes cross-reactive with the viral envelope protein. There appeared to be some loss of fusion protein in the purification steps as shown by the presence of reactive protein in the unbound fraction and subsequent washes. This may have been due to a lack of bead excess in the binding step which was not optimised, or to the failure of a proportion of the fusion protein to bind to the glutathione beads. Nevertheless, 10 litres of induced bacterial culture provided sufficient protein for immunisation and further characterisation (approximately 5mg).

Of the other fragments chosen, two were not used, and two failed to produce stable proteins or envelope specific sera.

(iii) Immunisation of rabbits with GST fusion proteins.

Two rabbits were immunised subcutaneously with 500µg each of H5A protein, in an equal volume of Freund's Complete adjuvant. Serum was collected from each rabbit prior to immunisation for use as a control pre-immune serum. The immune response was boosted 3 weeks later by a second duplicate injection this time

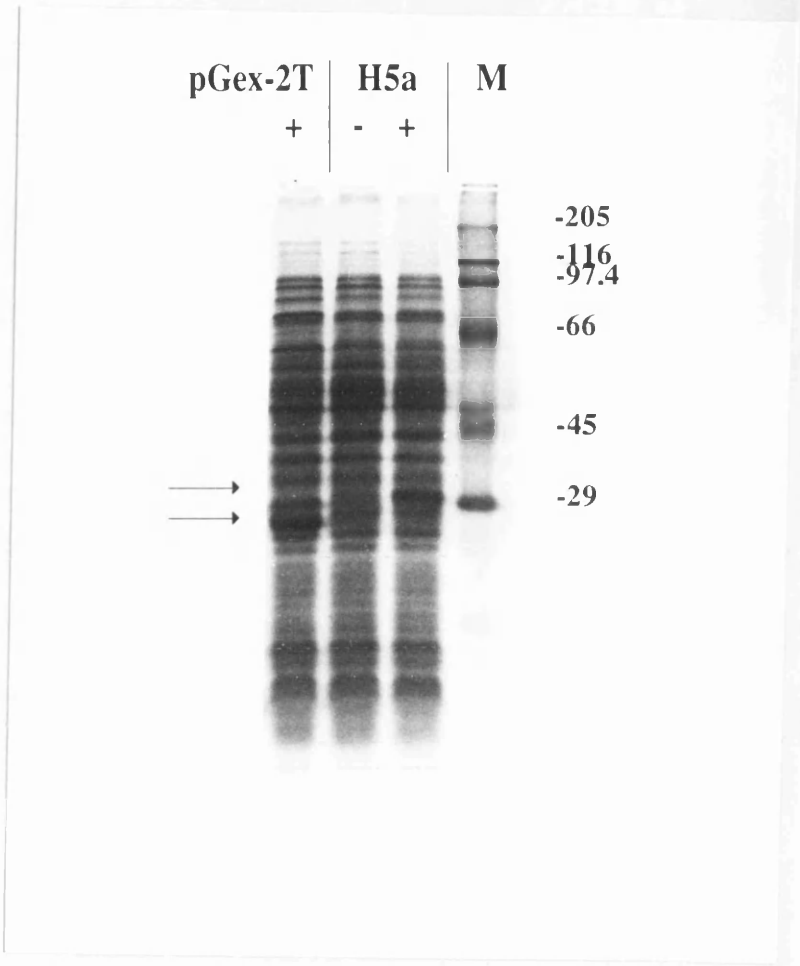


Fig.28. Identification of a GST-enFeLV *env* fusion protein.

1ml of exponentially growing E.coli containing either pGEX-2T or pGEX-enFeLV HaeIII digested *env* fragment (H5A) were incubated with (+) or without (-) 23µg/ml IPTG before the cells were pelleted, lysed and separated on a 13% SDS-polyacrylamide gel. The cell proteins were stained with Coomassie brilliant blue. The lower arrow identifies the 26kD GST protein, while the upper arrow identifies the 30kD enFeLV *env* fusion protein. The positions of the molecular weight markers are indicated on the right and the sizes are in kD.

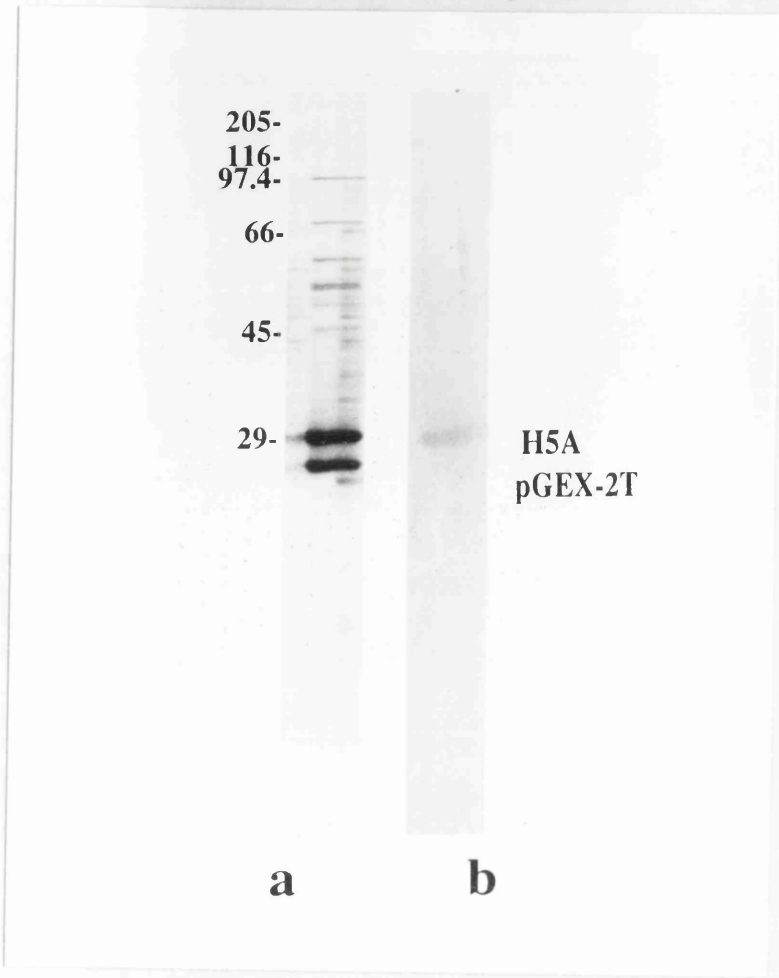
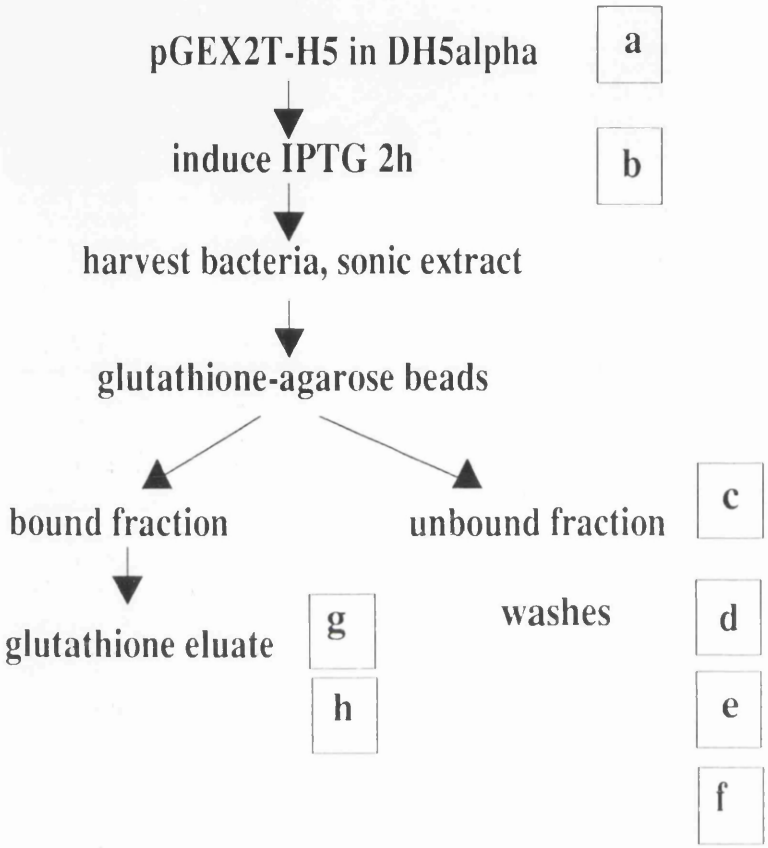


Fig.29. Reaction of GST and H5A enFeLV fusion protein to a polyclonal antiserum for gp70.

2 aliquots of 3µg of a equimolar mixture of purified GST (pGEX-2T) and H5A enFeLV fusion protein were separated on a 13% SDS-polyacrylamide gel. One half of the gel was stained with Coomassie brilliant blue. (a) The proteins on the half of the gel were transferred onto nitrocellulose by Western blotting and immunostained with anti gp70 polyclonal antiserum and a secondary alkaline phosphatase antibody conjugate. (b) The positions of the molecular weight markers are indicated on the left and the sizes are in kD.


pGEX2T-H5 in DH5alpha
a
induce IPTG 2h
b
harvest bacteria, sonic extract
glutathione-agarose beads
bound fraction
unbound fraction
c
glutathione eluate
g
h
washes
d
e
f

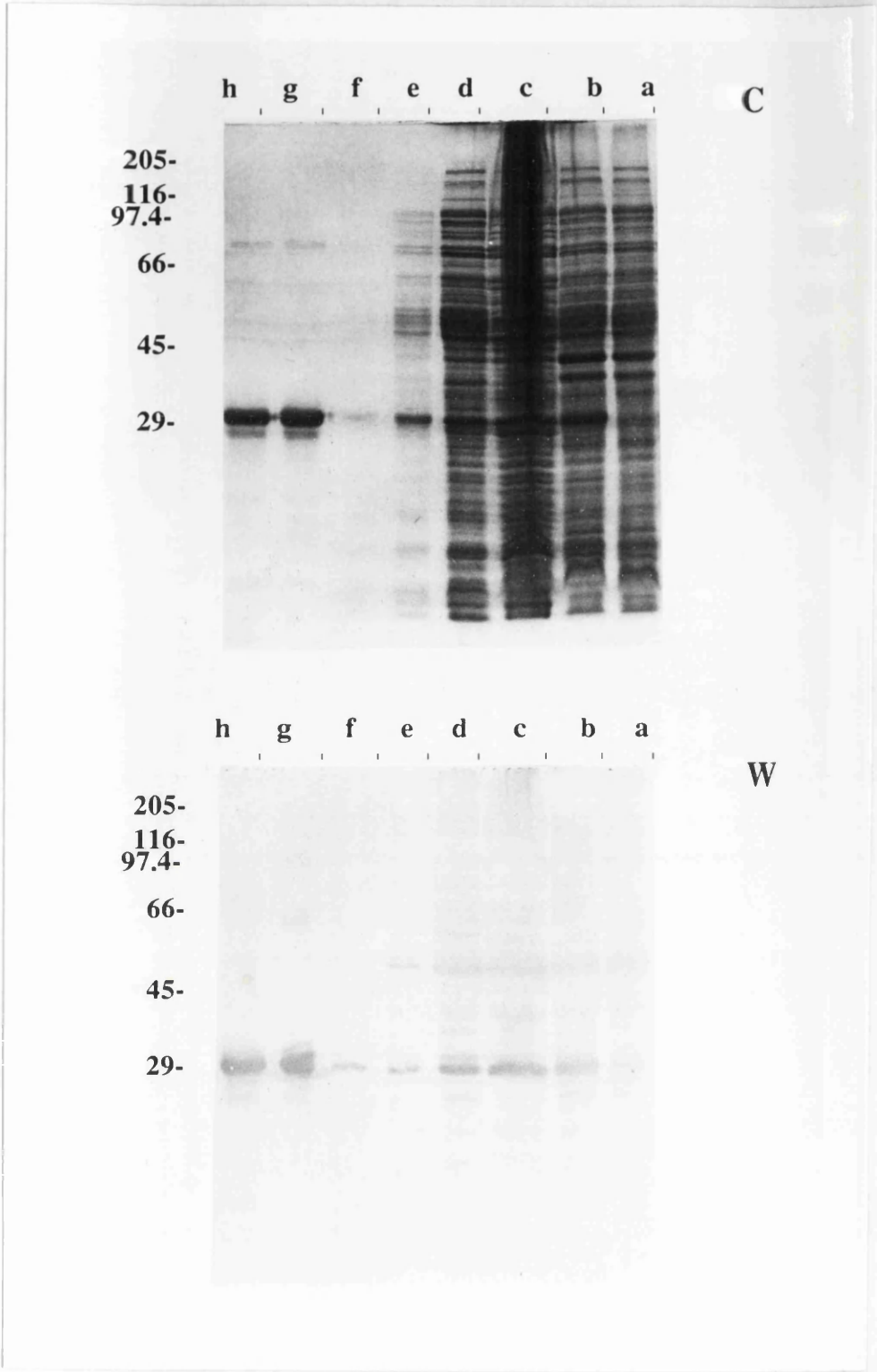


Fig.30. Purification of GST-H5A enFeLV *env* fusion protein.

The purification of GST fusion proteins is described as a flow diagram (left) and the purification of the enFeLV *env* fusion protein, H5a, is shown above. 50µl aliquots from each stage numbered in the flow diagram were collected and separated on duplicate 13% SDS-polyacrylamide gels. The proteins were stained by Coomassie Brilliant Blue (C) or after transfer of the proteins to a nitrocellulose filter by Western blotting, stained with polyclonal $gp70^{env}$ antiserum and an alkaline phosphatase conjugated secondary antibody (W). The positions of the molecular weight markers are indicated on the left and the sizes are in kD.

using Freund's incomplete adjuvant. Serum samples were collected 3 weeks later for use in the following studies.

(iv) Detection of reactivities to GST fusion proteins.

In order to establish the success of immunisation, an ELISA was performed in which serial dilutions of test sera were reacted with the fusion protein used for immunisation, to a 96 well plate. The results, summarised in Fig.31, indicated that both rabbit sera contained reactivity to the target pGEX proteins significantly greater than pre-immune sera. This confirmed that the rabbits had made an immune response to the GST-fusion protein.

6.2.2. Characterisation of a truncated endogenous envelope protein in feline lymphoma cells.

Metabolic labelling followed by immune-precipitation and SDS-PAGE were used to test the rabbit sera for activity to the reactive FeLV envelope protein, and to identify an endogenous viral envelope proteins in feline T-cell lines. (Fig.32)

Fibroblast cells uninfected or infected with FeLV-B/GA and two FeLV negative T-cell lymphoma lines were metabolically labelled with 100μCi/ml [^{35}S] met/cys by the method described. (Chapter 2.2.28.) Cells were lysed and the precipitation of envelope proteins carried out using polyclonal serum against gp70 or the test serum from rabbit 573 and compared to pre-immune controls. No envelope-related proteins were specifically precipitated by either anti-gp70^{env} or serum 573 in uninfected AH927 cells. Serum 573 precipitated the FeLV-B envelope protein from infected AH927 cells but with a reduced efficiency compared to anti-gp70^{env}. This demonstrated that the serum reacted with full length FeLV gp70^{env}. The 35kD protein, one of several originally detected by the anti-gp70^{env} serum, was also precipitated from both 3201B and MCC cells by the new antiserum. The other proteins precipitated by the anti-gp70^{env} serum were not precipitated by the new

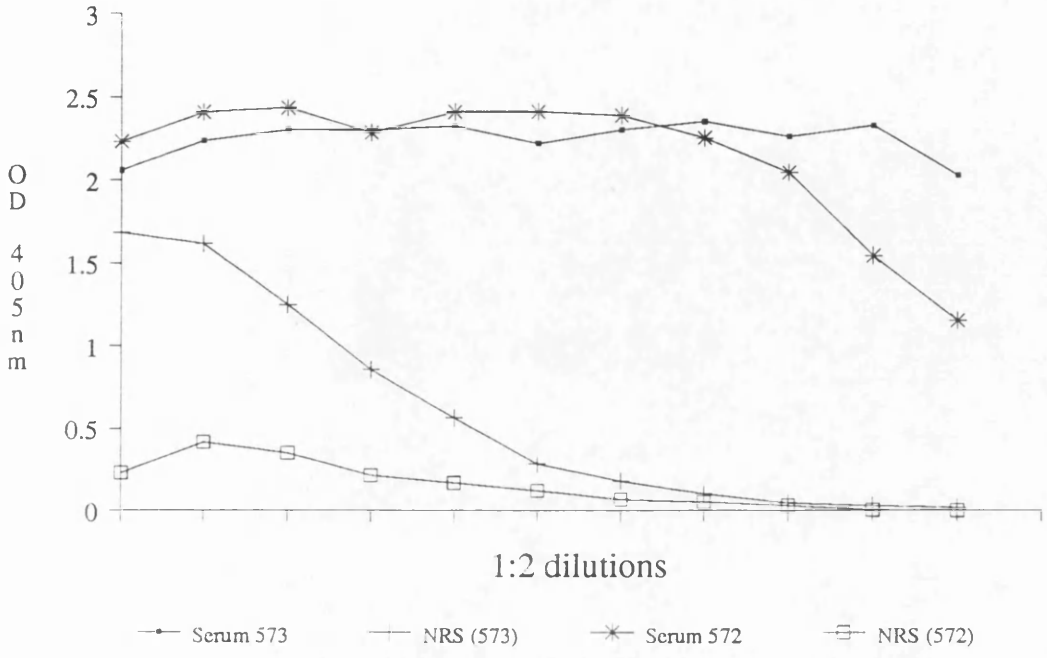


Fig.31 Detection of reactivity to enFeLV fusion protein by ELISA

The H5A-pGEX fusion protein used for immunisation, was bound to a 96 well plate at a concentration of 1µg/ml. After blocking each well with 1% BSA, undiluted antiserum from rabbits 572 or 573 were placed in a well and serial dilutions (1:2) performed. This was repeated with the pre-immune serum from the rabbits immunised (NRS 572 and NRS 573). After binding, a secondary antibody-alkaline phosphatase conjugate was bound and the products of the colour reaction read as optical density at 405nm. The graph represents the OD_{405} plotted against the number of dilutions for both the pre-immune sera and the test sera 572 or 573 (see legend). The results show both rabbits have produced a significant immune response to the GST-enFeLV fusion protein compared to the responses of the pre-immune sera.

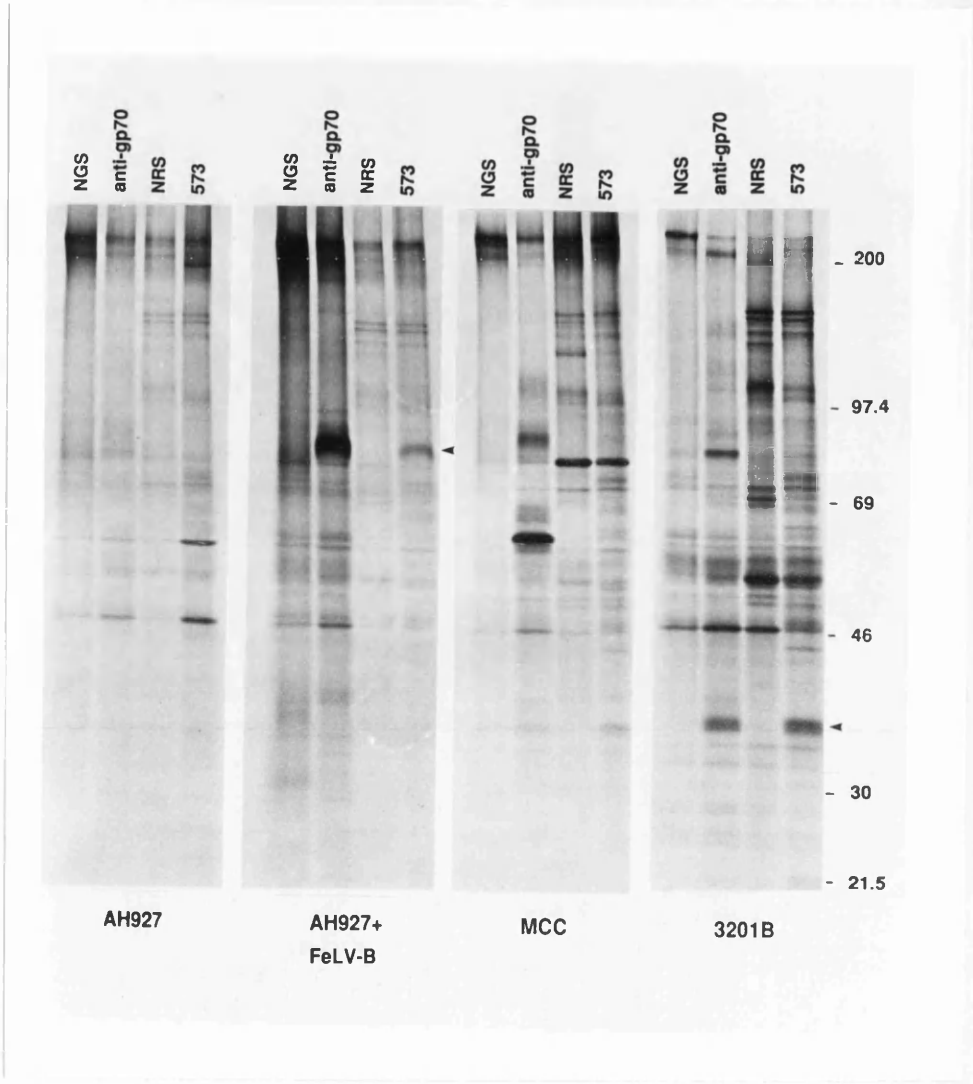


Fig.32. Identification of a truncated *env* protein using an enFeLV specific antiserum.

10^6 cells from virus free AH927, FeLV-B/GA infected AH927, 3201B and MCC cell lines were metabolically labelled with [^{35}S] met/cys and the cellular proteins precipitated with polyclonal anti-gp70*env* serum or the enFeLV specific antiserum, (573) together with the control serum for the gp70^{env} serum and the pre-immune serum from rabbit 573. The precipitated proteins were separated by denaturing electrophoresis through a 15-8% gradient SDS-polyacrylamide gel and visualised by autoradiography. The positions of the molecular weight markers are indicated on the right and the sizes are in kD.

serum. The 35kD protein corresponded with the size predicted from the truncated enFeLV *env*-orf and was therefore identified as its probable protein product. The serum from rabbit 572 failed to precipitate FeLV-related proteins. (Results not shown)

6.3. DISCUSSION.

A series of proteins were precipitated in tumour cell lines by the polyclonal antiserum, anti-F422 gp70^{env}, including proteins of approximately 70-80kD, 60kD, 50kD and 35kD. Only the 35kD protein corresponded with the size of the predicted protein from the *env*-orf. From these data however, the 35kD protein could not be conclusively identified as the product of the *env*-orf. In order to raise an antiserum to recognise the protein product of the expressed *env*-orf, a bacterial protein expression system was used. Although five peptides were examined, only one was ultimately successful in raising a specific antiserum. A number of factors contributed to this, including time limitation, and the instability of two of the fusion products. This is not an uncommon problem with fusion proteins in bacteria.

The development of a new FeLV envelope antiserum led to the demonstration that feline lymphoma cells produce a truncated enFeLV *env* protein, p35^{env}. The other proteins seen with the polyclonal antiserum, anti-F422 gp70^{env}, were not precipitated by the new serum and were considered to be the result of contaminant reactivities in the serum. There is no conclusive proof that any of the proteins precipitated by either antisera were full length enFeLV *env* genes expressed at low levels, although this remains a possibility. The enFeLV p35^{env} protein was not detected in fibroblast cells, but the specific enFeLV serum does have a weak reactivity with exogenous FeLV-B gp70^{env}. The lower reactivity of serum 573 to FeLV-B gp70^{env} compared to anti F422 gp70^{env} is presumably due to the fact that the immunising peptide is internal in the full length gp70^{env} molecule, but a free C-terminal peptide on both the truncated p35^{env} protein and the pGEX fusion protein. This explanation would also account for the efficient recognition of p35^{env} by serum 573.

In precipitations with both anti-F422 gp70^{env} and serum 573, the amount of p35^{env} protein detected in MCC cells was low compared to 3201B cells. Because the *env* coding sequences expressed in these cell lines have been shown to be almost identical, it is unlikely that the reduced level of precipitated protein in MCC cells is due to any physical difference in the protein. On the other hand, there is evidence of a correlation between the amount of protein produced and the level of the 2kb transcript. This presumptive *env* mRNA was significantly more abundant in 3201B than in MCC cells. The larger transcript expressed in MCC cells was significantly smaller than those found in most other cell lines and tissues, and consequently may be a variant enFeLV locus, having lost sequences required for efficient splicing. Alternatively host-cell specific mechanisms may reduce the levels of enFeLV splicing in MCC cells.

CHAPTER 7 AN ANALYSIS OF enFeLV GENETIC POLYMORPHISM.

7.1. INTRODUCTION.

This study showed that the putative *env* mRNA transcript of enFeLV is conserved throughout a variety of cell lines and tissues, and that the *env* genes expressed in two feline cell lines are highly conserved. The final experiments discussed here were performed to identify genetic loci within feline DNA that might be responsible for the expressed truncated *env*-orf and its corresponding protein, to examine the conservation of these loci in the feline population, and to compare this with the degree of conservation of loci containing larger amounts of *env* coding DNA.

7.2. RESULTS Distribution of truncated and full length *env* genes in feline genomes.

In order to discriminate between the endogenous proviral elements with full length and truncated envelope genes, a new probe was designed and constructed. (Fig.33) Oligonucleotides were designed to amplify the 3' half of the envelope gene which is missing from the truncated endogenous envelope genes responsible for the coding of $p35^{env}$. Oligo en1 was homologous to DNA directly downstream from the gp70 deletion site, and en2 directly upstream from the p15E (TM) deletion site.

Oligo en1 5'AATGCCTCCATTGCCCCTCTAAGC3'

Oligo en2 5'ACTGCACCAACCGGTTAAGGATGC3'

PCR amplification of the full length endogenous clones pLCM-1 and pBCM-3 using these two primers was carried out at 94°C for 1 min., 60°C for 1 min. and 72°C for 2 mins. and resulted in the amplification of a single DNA fragment of the predicted size of approximately 1kb.

In conjunction with the 5' *env* B/S probe, this Δ*env* probe should distinguish proviruses deleted in this region, and those which possess both 5' and 3' regions of gp70 and hence presumably represent full length envelope genes. Since this Δ*env* probe covers the entire deleted region, it should hybridise to endogenous loci

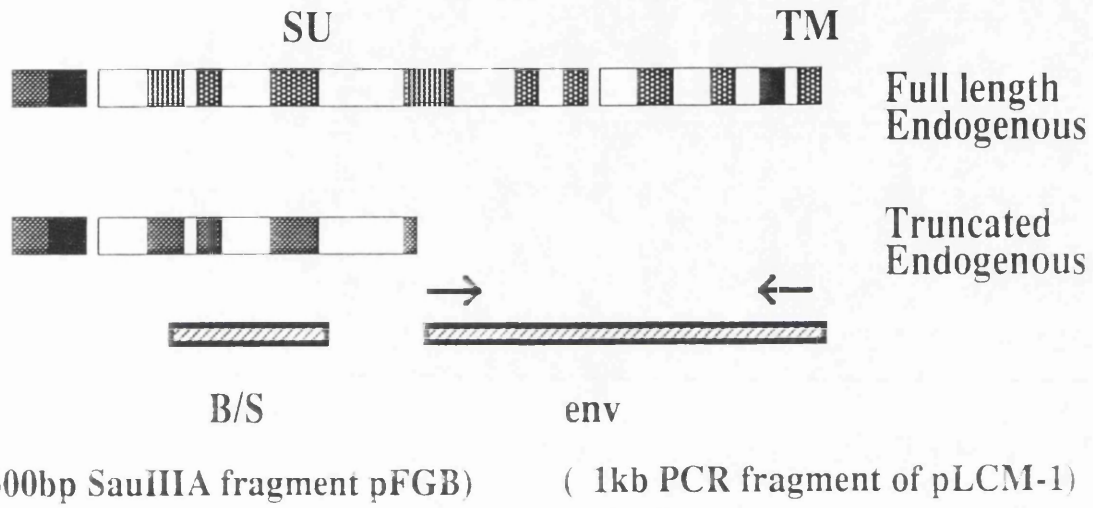


Fig.33. Production of a DNA probe specific for full length enFeLV *env* genes by PCR amplification.

The figure shows the structure of enFeLV *env* genes, both full length and truncated, that have been characterised. The restriction fragment B/S *env* DNA probe as shown in Fig.5. is marked. The arrows mark the relative position of specific oligonucleotides designed to amplify that portion of the full length *env* gene of the endogenous clone pLCM-1 that is not found in the expressed *env* genes.

containing smaller deletions in this region but not to those loci with the deletions found in the expressed transcripts. The amplified Δ*env* fragment was gel purified, precipitated and labelled with [^{32}P] by nick-translation. Identical Southern blots were prepared and hybridised with this probe or the B/S probe and the proviral patterns compared. (Fig.34) The loci were thereby distinguished as containing 5' *env* sequences, or 3' *env* sequences (underlined). Those loci which reacted with both 5' and 3' *env* probes, were classed as full length *env* genes, minor deletions notwithstanding. The most conserved loci appeared to be those containing 5' *env* sequences only.

7.3. DISCUSSION

High molecular weight DNA from a number of cell lines and tissues were digested with BamHI and probed with the B/S probe to examine the heterogeneity of proviruses and their chromosomal location in cats. Every cat revealed a unique pattern and this can be of practical use in determining the origin of a particular feline cell line or tissue. As can be seen in Fig.12b and the final 3 lanes of Fig.34, cats with one or both shared parents possessed quite similar patterns, with only a few differences. These analyses illustrate the Mendelian pattern of inheritance of enFeLV loci from each parent. In the J series, all the cats were sired by the same cat and the numbers 49, 51 and 53 refer to the female parent. Those cats with the same parents correspondingly had closely related fingerprints. However even DNA fingerprints from unrelated animals revealed a number of conserved bands.

Endogenous loci identified by both the B/S probe or Δ*env* probe contain both the 5' gp70^{env} and 3' gp70/p15E^{env} sequences and were in the majority. Of these, a proportion are conserved, but many are not. Two sets of bands hybridised to the 5' *env* B/S probe alone, and are thus identified as possible sources of enFeLV transcripts. The size and/or chromosomal location of these appear to be very highly conserved, with every sample tested having a band of about 13kb and 6kb. Therefore there appear to be only two endogenous proviruses capable of producing the p35^{env} protein, and these are highly conserved within the feline population.

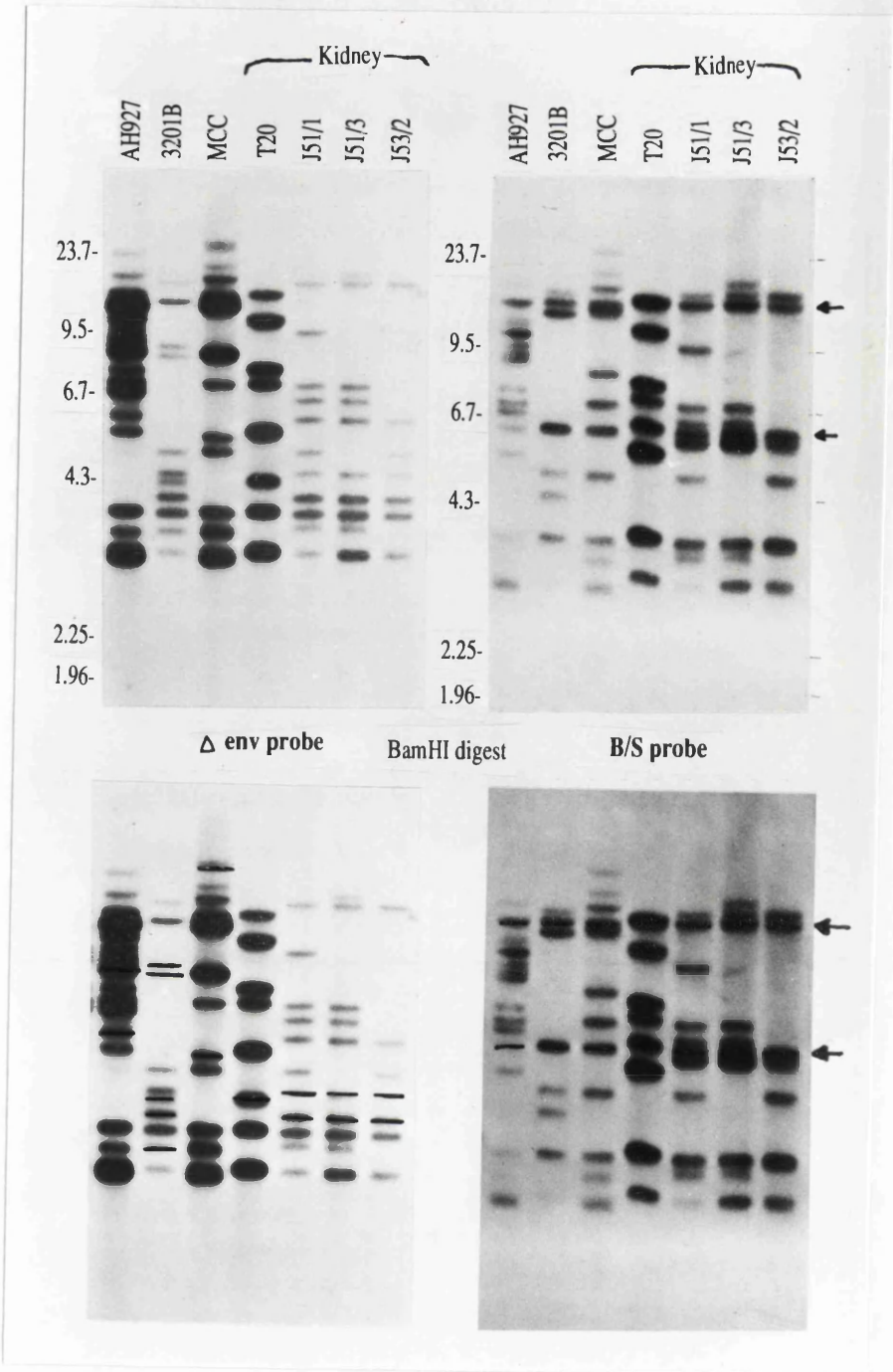


Fig.34. The relative distribution and conservation of truncated and full length *env* genes.

20µg of high molecular weight DNA from a number of feline cell lines (AH927, 3201B and MCC) and the kidney tissue from a number of virus negative (T20) and virus positive (J51/1, J51/3 and J53/2) were digested with BamHI, and the resulting fragments separated on duplicate 0.8% agarose/TAE gels. The DNA was transferred to a filter membrane by Southern blotting and hybridised with [^{32}P] labelled B/S *env* or Δ*env* probes. The positions of the molecular weight markers are indicated on the left of each blot and the sizes are in kb. Below each of the upper blots is a duplicate picture where the bands containing 3' *env* (Δ*env*) or 5' *env* (B/S) sequences only are highlighted.

AH927 cells (negative for enFeLV expression) possess both these bands further suggesting that the control of enFeLV involves tissue specific factors working in *trans* to either permit or inhibit expression.

Also of interest were a number of proviruses which appeared to react solely with the 3'Δ*env* probe. It was considered that these might represent exogenous FeLV because of the high homology between FeLV-A and B *env* sequences in the 3' half of the gp70 coding sequence and, although hybridisation was performed at high stringency, there was a possibility of cross hybridisation. This was discounted because although all the samples had one or more of these bands, only the DNA in lanes J51/1, J51/3 and J53/1 came from virus-infected animals, and these tissues appeared to be free from exogenous virus. (Chapter 3.2.3.(i)) Therefore it was concluded that these bands represented a new set of endogenous proviruses defective in the 5' half of gp70 but possessing the 3' half. A further implication of this finding is that enFeLV proviral number would be underestimated using the B/S *env* probe alone. There appeared to be less conservation of these endogenous proviruses, with similar sized restriction fragments seen only in closely related cats. If these represent intact proviruses with long terminal repeats, they are probably not expressed at detectable levels as they would have been seen in Northern analyses with the enU3 probe. If, however, the provirus had different LTR sequences than those tested for by the enU3 probe, or lacked LTR elements, transcription being driven by cellular sequences, the enU3 probe would not detect the transcript either. To answer this question further experiments should be performed using the 3' Δ*env* probe to test for expression.

CHAPTER 8 DISCUSSION

8.1. EnFeLV expression is a widespread feature in cats.

The analyses presented in this thesis showed that enFeLV expression is much more widespread than reported previously, being found in every animal tested. This expression was shown to be independent of exogenous virus infection since it was detected in two specific pathogen free cats. In total, my studies on enFeLV expression have included tissues from more than 16 animals, 11 of which feature in this thesis. Although enFeLV expression was detected in all cases, individual differences in the level and possibly the tissue specific sites of enFeLV expression cannot be excluded. While the sample size is not large enough to justify extrapolation to the entire feline population it can be concluded that the majority of cats express enFeLV elements.

8.2. Genetic structure of expressed enFeLV transcripts.

The analysis of the RNA from the 3201B cell line with subgenomic probes suggested that these cells express genomic length RNA from a locus substantially deleted in *pol* and a smaller RNA which is most likely its spliced *env* mRNA. This inference was further supported by the observation (Chapter 6) that the larger enFeLV transcript of 3201B cells (and not the smaller) could be packaged into virion particles. In exogenous FeLV, signals necessary for packaging lie downstream of the splice donor site in the provirus (M. Stewart, Beatson Institute for Cancer Research. Unpublished results.) and so the spliced mRNA does not have a functional packaging signal thus preventing its incorporation into the virion. The correlation between the level of the smaller transcript observed in the RNA of 3201B and MCC cells, and the amount of *env*-related protein precipitated from these cells also suggests that the smaller transcript only represents *env* mRNA.

8.3 Restrictions on enFeLV replication.

(i) Only a proportion of the enFeLV loci are detectably expressed.

Despite the simple pattern of enFeLV-related transcripts, the RNA data alone did not reveal whether these transcripts originated from one locus or multiple loci of similar structure. Nine different ERVs were cloned by Soe *et al* (1983, 1985) from the DNA of a single animal. Those studies indicated similarities between the restriction enzyme maps of the flanking DNA of a number of the truncated loci, suggesting a common origin, perhaps by gene duplication. However, the enFeLV proviruses cloned so far are heterogeneous in length and structure suggesting that the number of candidate loci cannot be large. It was also noted that the apparently full length enFeLV proviruses, the majority species, had unique flanking DNA. In this thesis, the fingerprinting experiments of Chapter 7 confirmed these findings, showing that those enFeLV loci containing both 5' and 3' *env* sequences (and assumed to be full length *env* genes) were in the majority.

Two enFeLV loci which could be considered possible sources of the truncated *env* gene products, and were detected in every feline DNA sample tested. The differences in the size of the flanking DNA, as judged by BamHI digestion, shows either these are unique proviral insertions or that the duplicated unit suggested by Soe and colleagues does not extend as far as the BamHI restriction sites. This issue could be clarified by more exclusive restriction enzyme analysis of the 3' virus cell-junctions. It appears likely that the expression of the other enFeLV elements present in the feline genome is repressed by the flanking DNA sequences. (Berry *et al.*1988)

Although most cell lines and tissues revealed a very similar pattern of enFeLV transcripts, at 4kb and 2kb, some heterogeneity was observed in the larger enFeLV transcripts in several cell lines and tissue samples. This may represent the expression of different proviral loci in these different cells, capable of expression perhaps because of their integration at favourable sites in the feline genome. Alternatively, expression of these loci may be linked to genetic differences of other controlling genes active in different animals in the feline population. However, the smaller

transcripts appeared to be much more conserved, at least in size, suggesting that the *env* genes of these different proviruses may be similar. Thus the transcripts of 3201B cells and MCC cells appeared to come from proviruses of different sizes but have very similar *env* gene sequences. Both cell lines had the same pattern of enFeLV proviruses which were B/S positive and Δ*env* negative and may represent the same original integrations. The presence of 3 transcripts in a number of samples, most obviously in FL74 cells, could represent the co-expression of two different loci. It would be of interest to investigate the genetic content of these transcripts and their inter-relationships by further analysis with subgenomic probes, cloning and sequencing. The expressed loci are likely to be different in genetic structure, and it would be of interest to see whether the *env* genes expressed were of similar structure, or if the different transcripts from FL74 cells were responsible for the expression of other enFeLV *env* genes. The conservation of enFeLV *env* genes observed in this thesis would suggest that the former is the more likely situation.

(ii). Only truncated enFeLV proviruses are detectably expressed.

In both cell lines and primary tissues, I found no evidence of expression of complete enFeLV loci: the sizes of the larger transcripts varied from 4.5-3.5kb and the smaller was generally about 2kb. A full length viral genome would be expected to produce a genomic transcript of approximately 8kb and a spliced *env* message of 3kb. No infectious virus has been isolated from enFeLV loci, and it may be that loci capable of producing infectious virus have been lost or silenced in the outbred feline population, due to their detrimental effects on the host.

The enFeLV transcripts from 3201B cells have not been shown unequivocally to have a U5 sequence. It is therefore not clear, at present, whether transcription is driven by a complete 5' LTR element, or like the murine *Fv-4* locus, by upstream cellular sequences. The isolation of the entire genomic transcript sequence from the cDNA of the 3201B cells would enable both the characterisation of the entire genome and test for the presence of U5 sequences. Alternatively, the 5' end of the mRNA from 3201B cells could be isolated by primer extension from a specific

oligonucleotide. The product of primer extension could be analysed for LTR derived sequences either by S1 nuclease protection analysis using an endogenous LTR probe, or by direct sequencing.

(iii) EnFeLV expression is controlled by tissue specific factors.

It was established that tissues expressing enFeLV transcripts had no obvious rearrangement or gain of ERV genetic information compared to non-expressing tissues of the same animal. This further level of control of the expression of enFeLV loci appeared to be tissue specific. It is possible that, like the *Rmcf* locus, enFeLV expression is limited to a subset of lymphoid cells that vary in abundance in high and low expressing tissues. (Buller *et al.*1989) Quantitative studies on the relative levels of RNA produced by each tissue could be performed to yield a more accurate measure of expression than the Northern analyses presented here. *In situ* analysis of enFeLV expression may be possible using the newly synthesised endogenous antiserum to stain enFeLV proteins in tissues. Lymphocytes represent a mobile cell population that are found in peripheral blood in a variety of tissues, and in high concentrations in specific lymphoid organs. It would be of interest to separate peripheral blood lymphocytes into a number of different subsets on the basis of the expression of cell surface markers, and then quantify enFeLV expression in each of these differentiated cell types.

8.4. The expressed *env* gene is highly conserved.

Despite some variations in larger transcript size, cell lines and tissues all appeared to have a conserved 2kb transcript, the putative *env* mRNA. This conservation was further shown to extend to the sequence of *env* genes expressed in two cell lines established from different cats. The *env* genes expressed in both 3201B and MCC cells were almost identical to each other and to the clone CFE-16, isolated by Soe *et al* (1983). The conservation of the expressed enFeLV *env* gene sequences is remarkable given the genetic heterogeneity of enFeLV loci in the domestic cat (as revealed by the DNA polymorphisms). The sources of the cells from which these

three *env* genes were isolated are all separated by both geography and time of isolation. The 3201B thymic lymphoma cells were isolated in the laboratory of William Hardy in New York, 1978 (Snyder *et al.*1978); the MCC cells were isolated from a cat with a large granular lymphoma in the laboratory of Jennifer Rojko in Ohio, 1988 (Cheney *et al.*1990); the CFE-16 clone was isolated from the placental DNA of a specific pathogen free cat by Pradip Roy-Burman and his co-workers in California, 1983 (Soe *et al.*1983). It was therefore considered that this strong conservation of sequence may be the result of a positive selection on these sequences arising from some beneficial effect to the host.

8.5. Conservation of the enFeLV loci in feline DNA.

Those enFeLV loci containing both 5' and 3' *env* sequences do not appear to be particularly well conserved with regards to chromosomal position in the feline genome. Only two constant loci were found in every sample analysed, from a total of over 16 unique proviral loci in the population sampled. There is, however, a significant conservation of loci containing only 5' *env* sequences. Two such loci were identified and found in every cell line and tissue tested with one possible exception (T20) where the presence of the larger band was obscured by the excess of DNA present in that sample. However, it was concluded that each sample contained at least one unique locus containing 5' *env* sequences only, which might therefore be suspected to be the source of enFeLV. The observed conservation may be due to the diploid nature of the locus, thereby favouring inheritance. In general, because of their insertional origins, ERVs tend to be haploid, but genetic mutations may have resulted in a diploid phenotype. Alternatively, it may be that conservation of chromosomal position represents the conservation of integration at a site favourable to enFeLV expression and therefore represent a positive selection of the product(s) of this locus. Nevertheless, these results do not suggest that individual proviruses are highly conserved. The genetic fingerprinting technique looks at the polymorphisms present in the 3' half of the genome encompassing the *env* gene, the 3' LTR and adjoining

cellular DNA. Therefore, although the fingerprints suggest that there is a conservation of the *env* gene sequences in a specific chromosomal position, the expression of different sizes of genomic (larger) transcripts from different cell lines and tissues (e.g. 3201B and MCC cells) indicated that the 5' half of these genomes may be different. It is possible that these expressing loci represent the integration of an enFeLV provirus into a site favourable for expression in an ancestor of the modern cat, which has since been subject to autonomous genetic drift in individual animals. The conservation of the *env* gene sequences may have been positively selected for, while mutations and deletions in the rest of the provirus were tolerated. The 5' virus -cell junctions could be used to examine this hypothesis, but would be complicated by the greater heterogeneity of the *gag* and *pol* regions of the truncated enFeLV loci seen in Soe's studies. (Soe *et al.*1983 Soe *et al.*1985). Therefore the use of *gag* or *pol* probes may not be suitable and the use of the enU3 probe is prohibited because of the presence in feline DNA of multiple (>150) solo LTR elements. (O'Brien, 1986) Perhaps the best way to compare if the two truncated *env* containing bands are from the same or different sized loci in individual cats would be to digest the feline DNA with EcoRI, an enzyme that generally fails to cut within the provirus, and probing the resulting Southern blot with an *env* specific probe. If the two conserved *env* containing bands represent the same locus in each sample, they would also appear conserved after EcoRI digest. If, however, they are different proviruses, with conserved *env* genes and integration sites, they may not be conserved in size.

8.6. Recombination of enFeLV loci.

Because recombination of retroviral sequences is considered be a consequence of the co-packaging of two RNA species in a virion, (see Chapter 1/4.2.1.) it seemed likely that the expressed enFeLV loci would be more favourably involved in recombination to produce FeLV-B viruses. The sequence comparison of the SU coding regions of both exogenous and endogenous viruses has indicated that, in most cases, the recombination process must involve proviruses with more extensive

enFeLV *env* sequences than contained in the expressed, truncated species. Sequence analysis of FeLV-B/GM1 virus identified a novel recombinant which may have arisen from a truncated enFeLV locus. However, although the highly expressed enFeLV transcripts can be packaged into virions, recombination resulting in functional FeLV-B does not appear to follow this course, in most cases. The generation of recombinants from these sequences appear to be the exception rather than the rule. More often, it seems that recombination involves the enFeLV sequences whose expression has not been detected in this study. The reasons for this and a possible alternative mechanism of recombination have not been addressed, but may simply reflect the greater probability of creating a functional FeLV-B *env* gene by recombination with low expressed but full length enFeLV proviruses. Expression of full length *env* genes may occur in a tissue which has not yet been examined, or at a level too low for detection by Northern analysis, but sufficient for recombination. This expression may occur in a subset of cells that is highly susceptible to infection by FeLV-A. The frequency of recombination leading to the generation of FeLV-B viruses in natural feline populations cannot be accurately estimated, as subgroup B viruses could arise as novel recombinants or due to horizontal transmission in the cat population. The detection of recombinants may be further complicated by the restricted replication of FeLV-B viruses in cats. (See section 8.10) A recently published study involving the PCR amplification of DNA derived from 3201B cells transfected with infectious FeLV subgroup C DNA have identified recombinant virus sequences using polymerase chain reaction and specific oligonucleotide amplimers. (Pandey *et al.*1991) Analysis of the PCR amplimers used in this study against the sequences of FeLV-C and enFeLV *env* suggest that the major size of recombinant generated in this study could come only from recombination with an enFeLV locus containing a full length *env* gene. However, there is some evidence of a minor species whose size may be compatible with recombination involving a truncated envelope sequence. This observation is in line with the results of Overbaugh *et al* (1988) who recovered FeLV-B like viruses from AH927 feline fibroblast cells

transfected with FeLV-A DNA at a high frequency and in a short time. From the results of this thesis, these cells appear to be free from detectable enFeLV expression. Whether the method of recombination involved in transfected cells is representative of recombination *in vivo* is not known and recombination following exogenous virus infection has not been studied. The identification of FeLV-B like recombinants from FeLV-A infected cells may therefore be a useful system in which to test the parameters affecting enFeLV sequence involvement in recombination.

8.7. Sequence requirements for FeLV-B specificity

Sequence analysis of all FeLV-B viruses isolated prior to this study suggested that some portion of enFeLV derived variable regions common to all FeLV-B gp70 coding sequences would be responsible for the specific binding of FeLV-B gp70 to its receptor. The common variable regions concerned were Vr Ia, II, III, IV and V. However, GM1-B gp70 does not contain Vr V. This indicates that the Vr V region of FeLV-B viruses is not necessary for FeLV-B subgroup specificity. The requirements for Vr Ia-IV could be examined further by the construction of viruses with hybrid gp70 coding sequences containing different proportions of FeLV-A and B sequences. The results noted here are consistent with the recent observation that the first 120 amino acids of MuLV SU carries the determinants which allow the SU protein to recognise its specific receptor. This domain encompasses the first of two variable regions in the amino-terminal domain, identified by alignment of a broad range of mammalian type-C retroviruses (Battini *et al.*1992) and corresponds to enFeLV Vr II and III. The second variable region identified in this amino-terminal domain, corresponds to enFeLV Vr IV, and is proposed to play a role in the stabilisation of the receptor specific structure of amphotropic MuLV SU. (Battini *et al.*1992) Therefore, although recombination of both MuLV and FeLV usually involves the replacement of both the amino-terminal and proline rich domains, it is the specific sequence variations in the amino-terminus which are implicated in receptor specificity. For FeLV-B, the proline-rich region may have to be exchanged to stabilise the very

different amino terminal domain, or may confer some further functional necessity to the FeLV SU. This could be investigated further by constructing FeLV SU chimeric viruses and analysis of virus subgroup and efficiency of replication.

8.8. Possible role of enFeLV expression in resistance to virus infection.

The major beneficial role in vertebrates with which ERVs have been associated is cellular resistance to infection by exogenous viruses which use the same receptor as the endogenous *env* gene. This is generally considered to follow from the expression of an endogenous *env* protein product and its blockade of the cell receptors. It has been reported that 3201B cells are resistant to infection by FeLV-B (J. Rojko, Ohio State University. pers. commun.) It is possible that this restriction may simply be due to the lack of specific receptor expression in these cells, (the tissue distribution of receptors for FeLV-B has not been established as yet) but the evidence of *env* gene expression leads to the suggestion that resistance is a function of the expressed *env* gene protein product. Sequence analysis of the truncated *env* gene has shown that this gene encodes a truncated protein of approximately half the length of normal FeLV SU (calculated at 33.5kD excluding glycosylation) Chapter 6 described the identification of a protein of 35kD that has properties consistent with the translational product of the expressed *env*-orf. The $p35^{env}$ encodes all of the FeLV-B-like amino acid sequences found in the GM1-B virus. Therefore, despite the fact that only half the SU protein is present, it appears to carry information sufficient for receptor specificity. It is therefore proposed that $p35^{env}$ contains sufficient sequence information for specific receptor binding, although this has yet to be formally tested. Recent work on MuLV has shown that a similarly truncated MuLV SU, containing the 5' 245 amino acids of SU (encoding a 30kD protein) was capable of generating resistance to exogenous MuLV, indicating that this fragment was capable of interacting with the cellular receptor. (Heard & Danos, 1991) This implied that the amino-terminal of MuLV could adopt a functional conformation and recognise its receptor even without the carboxy-terminal domain. The truncated MuLV SU protein

still exhibited resistance when artificially retained in the endoplasmic reticulum by a KDEL sequence, indicating that the interaction between the SU and the cellular receptor resulting in blockade could occur within the ER. Given the overall structural and functional similarities of retroviral SU proteins, it is highly likely that the truncated feline *env* protein has similar properties.

The same group that found 3201B cells to be resistant to FeLV-B infection also reported that MCC cells were permissive for FeLV-B, albeit at a reduced efficiency compared to fibroblast cells. (J. Rojko, Ohio State University. pers. commun.) One possible explanation of this apparent discrepancy might have been a sequence difference between the expressed enFeLV *env* genes. However, no such differences were apparent. A more likely explanation follows the fact that the MCC cells express less of enFeLV *env* protein and hence, display a lower level of receptor blockade and consequently, lower cellular resistance than 3201B cells. These two cell lines expressed similar amounts of enFeLV RNA but there was a significant and consistent difference in the relative proportions of small and large transcripts. In 3201B cells, the smaller transcript was generally more abundant than the larger; this was also the case for T3 and F422 cells, and for many expressing tissues *in vivo*. However in MCC cells, the larger transcript was consistently more abundant than the smaller. This differential transcript level may be a consequence of splicing controls, resulting in the reduced levels of enFeLV *env* mRNA and of protein. Immune-precipitation studies of 3201B and MCC cells confirmed that $p35^{env}$ content, as a percentage of the total cell protein in the precipitate, was lower in MCC cells than in 3201B cells. The small amount of protein produced in MCC cells may be functional but insufficient to titrate out free receptors for exogenous virus completely. This would explain the reduced infectibility of MCC cells as compared to fibroblast cells. As the larger transcript of MCC enFeLV RNA is clearly smaller than that in 3201B cells, and so presumably more extensively deleted, the decrease in splicing may be a consequence of the deletion of some *cis*-acting sequences important to the control of splicing.

The results therefore suggested that the expression of $p35^{env}$ was proportional to its relative resistance to infection by FeLV-B. In order to confirm that $p35^{env}$ can bind to the cell receptors for FeLV-B and thereby cause resistance to exogenous infection, it will be necessary express the *env*-orf in cells that are permissive for FeLV-B but do not express enFeLV RNA to see if the resulting cell line is resistant to subsequent infection by FeLV-B. Feline fibroblast cells (such as AH927 or FEA) or canine cell lines would be suitable. It may be interesting to test if cats are homo- or heterozygous for the enFeLV expressing loci, and if this affects the level of expression of enFeLV *env* expression and correlates with resistance to infection by FeLV-B.

The presence of expressed enFeLV sequences in the cat does not appear to present an increased risk of viral recombination and disease (unlike the murine ERVs). It may be that the more ancient feline ERVs have been modified through evolutionary selection, in order to provide an effective method of resistance to virus infection but reducing the risk of virus recombination.

8.9. Possible effects of enFeLV expression on the feline immune response.

As well as causing resistance to FeLV-B infection by receptor blockade, the expression of enFeLV *env* may have an influence on the host immune response to exogenous virus infection. It has been suggested that the expression of enALV SU protects chickens from lethal inflammatory responses frequently seen in chf(-) chickens by establishing a partial tolerance to ALV *env* antigens. (Halpern & Friis, 1978, Crittenden *et al.*1982) Central to this theory is that the T-cell epitopes of the endogenous ALV *env* proteins expressed during thymic education would be recognised as self antigens. These would include epitopes common to all ALV SUs regardless of subgroup. Infection of exogenous ALV may therefore stimulate an immune response only to a proportion of epitopes on SU that are specific for the exogenous virus. This is suggested to reduce the amount of cell killing by the immune system and so limit deleterious inflammatory responses. The expression of

enFeLV elements has been shown in foetal thymic cells and hence occurs early in development. It is therefore likely that these are seen as self antigens. Whether exogenous FeLV infection has the same effect on the host immune system as has been suggested for ALV is not known, but the influence of enFeLV *env* gene expression may be more complex. Exogenous lymphomacytic choriomeningitis virus (LCMV) infection of mice carrying viral transgenes targeted to pancreatic cells resulted in the breakdown of tolerance to the endogenously expressed antigens and a destructive immune response leading to diabetes. (Oldstone *et al.*1991, Ohashi *et al.*1991) This suggested that the T-cells specific for these endogenous virus proteins were not deleted during thymic differentiation of self and non-self, but were inactivated by another means. Reactivity to self antigens by previously inactivate immune cells has also been implicated in the development of other autoimmune diseases. (Kronenberg, 1991) It is possible that in the feline system, the loss of tolerance to endogenous antigens could result in the destruction of those cells expressing enFeLV *env* proteins, as well as exogenous virus infected cells. Depending on the extent of cell destruction, this could result in severe immune suppression due to the massive destruction of lymphoid cells, or the clearing of the infection from the animal via the destruction of virally infected cells. FeLV-infected cats frequently present with thymic atrophy (Anderson *et al.*1971, Jarrett, 1984) despite apparently low levels of FeLV infection in the thymus (Rojko *et al.*1981) and it is interesting to speculate that this may be a consequence of a host immune response to uninfected feline tissue expressing enFeLV.

The immune system could also be influenced by the processing of $p35^{env}$. The stable insertion of viral envelope proteins into the cell membrane is dependent on the anchoring function of TM. As the enFeLV *env*-orf expressed does not contain TM coding sequences, it is unlikely that in uninfected cells, these proteins would be retained in the plasma membrane. However, it is not known whether the protein is transported from the ER into the secretory pathway and released from the cell, or

whether it is retained in the ER and degraded. This could significantly affect the exposure of the immune system to $p35^{env}$ epitopes.

It may be further speculated that the infecting virus can alter the cellular location of $p35^{env}$. A number of studies have indicated that the *env* proteins of retroviruses associate as oligomers within the ER (Einfeld & Hunter, 1988, Thomas *et al.*1991). Further, it has been demonstrated that this association is necessary for the efficient transport of *env* proteins from the ER to the cell surface, (Einfeld & Hunter, 1988) and that the oligomerisation can occur with surface proteins alone, although the interaction is stabilised by TM. The truncated MuLV gp70 that had been shown to mediate resistance in cells was shown to be efficiently excreted from the cells. (Heard & Danos, 1991) In contrast, a RSV *env* gene lacking most of TM was unable to be transported from the ER. (Delwart & Panganiban, 1989) For FeLV, exogenous virus infection could result in $p35^{env}$ reaching the cell surface in association with exogenous virus *env* oligomers. This may lead to a *de novo* immune response to $p35^{env}$.

The controversial FeLV-associated tumour specific antigen, FOCMA, was demonstrated on the surface of FL74 lymphoma cells and on the surface of virally transformed cells. (Essex *et al.*1971a, Snyder *et al.*1978) The presence of FOCMA was tested by looking for a FOCMA specific antibody response in cats. The sera were tested by immune fluorescence against FL74 cells, and a positive reaction of feline sera to the epitopes on the surface of these cells was considered a positive indication of the expression of the tumour antigen in the animal. (Mathes *et al.*1976) It was also reported that a FOCMA antiserum response indicated a good prognosis for the development of leukemia. (Essex *et al.*1971b, Schaller *et al.*1975) A number of theories were advanced regarding the genetic origin and function of this antigen. The observation that monoclonal antibodies reactive against FOCMA expressing cells were also reactive against cells infected with FeLV-C led one group to propose that FOCMA was encoded by an FeLV-C virus infecting the cell. (Vedbrat *et al.*1983) Further studies led to the conclusion that the FOCMA antigen was structurally similar

but distinct from FeLV-C. (Snyder *et al.*1983) It has also been suggested that the expression of FOCMA is the result of the generation of a recombinant FeLV containing sequences not found in standard replication competent FeLV strains. (Ruscetti *et al.*1980, Snyder *et al.*1983) The true nature of FOCMA is still unresolved. From the results of this study it cannot be conclusively shown that enFeLV *env* expression accounts for all the phenomena attributed to the FOCMA antigen but it demonstrates a rational basis for an immune response to enFeLV *env* genes and its cross reaction with FL74 cells.

It will be important to follow these observations further by examining the cellular localisation of $p35^{env}$, before and after virus infection, and to look at the host immune response to $p35^{env}$ during the course of viral infection. In this way it may be established whether the $p35^{env}$ is normally regarded as a self or a foreign antigen.

REFERENCES

Albritton, L.M., L. Tseng, D. Scadden, and J.M. Cunningham. 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell **57**:659-666.

Amanuma, H., A. Katori, M. Obata, N. Sagata, and Y. Ikawa. 1983. Complete nucleotide sequence of the gene for the specific glycoprotein (gp55) of Friend spleen focus-forming virus. Proc. Natl. Acad. Sci. USA **80**:3913-3917.

Anderson, L.J., W.F. Jarrett, O. Jarrett, and H.M. Laird. 1971. Feline leukaemia-virus infection of kittens: mortality associated with atrophy of the thymus and lymphoid depletion. J. Natl. Cancer Inst. **47**:807-817.

Astrin, S.M. 1978. Endogenous viral genes of the White Leghorn chicken: common site of residence and sites associated with specific phenotypes of viral gene expression. Proc. Natl. Acad. Sci. USA **75**:5941-5945.

Astrin, S.M., E.G. Buss, and W.S. Hayward. 1979. Endogenous viral genes are non-essential in the chicken. Nature **282**:339-340.

Astrin, S.M. and H.L. Robinson. 1979. Gs, an allele of chickens for endogenous avian leukosis viral antigens, segregates with *ev-3*, a genetic locus that contains structural genes for virus. J. Virol. **31**:420-425.

Astrin, S.M., L.B. Crittenden, and E.G. Buss. 1980a. *ev 2*, a genetic locus containing structural genes for endogenous virus, coded for Rous-associated virus type 0 produced by line 72 chickens. J. Virol. **33**:250-255.

Astrin, S.M., H.L. Robinson, L.B. Crittenden, E.G. Buss, J. Wyban, and W.S. Hayward. 1980b. Ten genetic loci in the chicken that contain structural genes for endogenous avian leukosis viruses. Cold Spring Harbor Symp. Quant. Biol. **19**:1105-1109.

Baluda, M.A. and P. Roy-Burman. 1973. Partial characterisation of RD114 virus by DNA-RNA hybridisation studies. Nature [New Biol.] **244**:59-62.

Bassin, R.H., S. Ruscetti, I. Ali, D.K. Haapala, and A. Rein. 1982. Normal DBA/2 mouse cells synthesise a glycoprotein which interferes with MCF virus infection. Virology **123**:139-151.

Battini, J., J.M. Heard, and O. Danos. 1992. Receptor choice determinants in the envelope glycoproteins of amphotropic, xenotropic, and polytropic murine leukaemia viruses. J. Virol. **66**:1468-1475.

Benveniste, R.E. and G.J. Todaro. 1974. Evolution of C-type viral genes: inheritance of exogenously acquired viral genes. Nature **252**:456-459.

Benveniste, R.E., C.J. Sherr, and G.J. Todaro. 1975. Evolution of type C viral genes: origin of feline leukaemia virus. Science **190**:886-888.

Benveniste, R.E. and G.J. Todaro. 1975. Segregation of RD-114 AND FeLV-related sequences in crosses between domestic cat and leopard cat. Nature **257**:506-508.

Berger, S.A., N. Sanderson, A. Bernstein, and W.D. Hankins. 1985. Induction of the early stages of Friend erythroleukaemia with helper-free Friend spleen focus-forming virus. Proc. Natl. Acad. Sci. USA **82**:6913-6917.

Berry, B.T., A.K. Ghosh, D.V. Kumar, D.A. Spodick, and P. Roy-Burman. 1988. Structure and function of endogenous feline leukaemia virus long terminal repeats and adjoining regions. J. Virol. **62**:3631-3641.

Besmer, P. 1983. Acute transforming feline retroviruses. Curr. Topics Microbiol. Immunol. **107**:1-27.

Bestwick, R., B. Boswell, and D. Kabat. 1984. Molecular cloning of biologically active Rauscher spleen focus- forming virus and the sequences of its *env* gene and long terminal repeat. J. Virol. **51**:695-705.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. 7:1513-1523.

Bishop, J.M. and H. Varmus. 1985. Function and origins of retroviral transforming genes, p.249-356. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbor Laboratory, New York.

Blatt, C., K. Mileham, M. Haas, M.N. Nesbitt, M.E. Harper, and M.I. Simon. 1983. Chromosomal mapping of the mink cell focus-inducing and xenotropic *env* gene family in the mouse. Proc. Natl. Acad. Sci. USA **80**:6298-6302.

Bosselman, R.A., F. van Straaten, C. Van Beveren, I.M. Verma, and M. Vogt. 1982. Analysis of the *env* gene of a molecularly cloned and biologically active Moloney mink cell focus-forming proviral DNA. J. Virol. **44**:19-31.

Bova, C.A., J.C. Olsen, and R. Swanstrom. 1988. The avian retrovirus *env* gene family: molecular analysis of host range and antigenic variants. J. Virol **62**:75-83.

Brightman, B.K., Q. Li, D.J. Trepp, and H. Fan. 1991. Differential disease restriction of Moloney and Friend murine viruses by the mouse *Rmcf* gene is governed by the viral long terminal repeat. J. Exp. Med. **174**:389-396.

Buckler, C.E., S.P. Staal, W.P. Rowe, and M.A. Martin. 1982. Variation in the number of copies and in the genomic organisation of ecotropic murine leukaemia virus proviral sequences in sublines of AKR mice. J. Virol. **43**:629-640.

Buller, R.S., A. Ahmed, and J.L. Portis. 1987. Identification of two forms of an endogenous murine retroviral *env* gene linked to the *Rmcf* locus. J. Virol. **61**:29-34.

Buller, R.S., M. Sitbon, and J.L. Portis. 1988. The endogenous mink cell focus-forming (MCF) gp70 linked to the *Rmcf* gene restricts MCF virus replication *in vivo* and provides partial resistance to erythroleukaemia induced by Friend murine leukaemia virus. J. Exp. Med **167**:1535-1546.

Buller, R.S., G. Van Zant, P.W. Eldridge, and J.L. Portis. 1989. A population of murine haematopoetic progenitors expresses an endogenous retroviral gp70 linked to the *Rmcf* gene and associated with resistance to erythroleukaemia. J. Exp. Med. **169**:865-880.

Busch, M.P., B.G. Devi, L.H. Soe, B. Perbal, M.A. Baluda, and P. Roy-Burman. 1983. Characterisation of the expression of cellular retrovirus genes and oncogenes in feline cells. Hematol. Oncol. **1**:61-75.

Casey, J.W., A. Roach, J.I. Mullins, K.B. Burck, M.O. Nicolson, M.B. Gardner, and N. Davidson. 1981. The U3 portion of feline leukaemia virus DNA identifies horizontally acquired proviruses in leukemic cats. Proc. Natl. Acad. Sci. USA **77**:7778-7782.

Chatis, P.A., C.A. Holland, J.E. Silver, T.N. Frederickson, N. Hopkins, and J.W. Hartley. 1984. A 3' end fragment encompassing the transcriptional enhancers of nondefective Friend virus confers erythroleukemogenicity on Moloney leukaemia virus. J. Virol. **52**:248-254.

Chattopadhyay, S.K., M.R. Lander, E. Rands, and D.R. Lowy. 1980. Structure of endogenous murine leukaemia virus DNA in mouse genomes. Proc. Natl. Acad. Sci. USA **77**:5774-5778.

Chattopadhyay, S.K., M.W. Cloyd, D.L. Linemeyer, M.R. Lander, E. Rands, and D.R. Lowy. 1982. Cellular origin and role of mink cell focus-forming viruses in murine thymic lymphomas. Nature **295**:25-31.

Cheney, C.M., J.L. Rojko, G.J. Kociba, M.L. Wellman, S.P. DiBartola, L. Rezanka, L. Forman, and L.E. Mathes. 1990. A feline large granular lymphoma and its derived cell line. In Vitro Cell Dev. Biol. **26**:455-463.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156-159.

Clark, S. and T. Mak. 1983. Complete nucleotide sequence of an infectious clone of Friend spleen focus-forming provirus: gp55 is an envelope fusion glycoprotein. Proc. Natl. Acad. Sci. USA **80**:5037-5041.

Cloyd, M.W. 1983. Characterisation of target cells for MCF viruses in AKR mice. Cell **32**:217-225.

Cloyd, M.W. and S.K. Chattopadhyay. 1986. A new class of retrovirus present in many murine leukaemia systems. Virology **151**:31-40.

Coffin, J.M. 1979. Structure, replication, and recombination of retrovirus genomes: some unifying hypotheses. J. Gen. Virol. **42**:1-26.

Coffin, J. 1982. Endogenous Viruses, p.1109-1204. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbor Laboratory, New York.

Coffin, J.M., P.N. Tsichlis, K.F. Conklin, A. Senior, and H.L. Robinson. 1983. Genomes of endogenous and exogenous avian retroviruses. Virology. **126**:51-72.

Coffin, J.M. 1990. Retroviridae and their replication, p.1437. In B.N. Fields and D.M. Knipe (eds.), Virology. Raven Press, New York.

Coffin, J.M., J.P. Stoye, and W.N. Frankel. 1991. Endogenous murine retroviruses and leukaemia, p.175-191. In H.Y. Fan, I.S.Y. Chen, N. Rosenberg, and W. Sugden (eds.), Viruses that affect the immune system. American Society for Microbiology, Washington.

Conklin, K.F., J.M. Coffin, H.L. Robinson, M. Groudine, and R. Eisenman. 1982. Role of methylation in the induced and spontaneous expression of the avian endogenous virus *ev-1*: DNA structure and gene products. Mol. Cell. Biol. **2**:638-652.

Copeland, N.G., K.W. Hutchison, and N.A. Jenkins. 1983. Excision of the DBA Ecotropic provirus in dilute coat-color revertants of mice occurs by homologous recombination involving the viral LTRs. Cell **33**:379-387.

Crittenden, L.B., A.M. Fadly, and E.J. Smith. 1982. Effect of endogenous leukaemia virus genes on response to infection with avian leukosis and reticuloendotheliosis viruses. Avian. Dis. **26**:279-264.

Cullen, B.R., A.M. Skalka, and G. Ju. 1983. Endogenous avian retroviruses contain deficient promoter and leader sequences. Proc. Natl. Acad. Sci. USA **80**:2946-2950.

Dandekar, S., P. Rossitto, S. Pickett, G. Mockli, H. Bradshaw, R. Cardiff, and M.B. Gardner. 1987. Molecular characterisation of the *Akvr-1* restriction gene: a defective endogenous retrovirus-borne gene identical to *Fv-4^r*. J. Virol. **61**:308-314.

Delwart, E.L. and A.T. Panganiban. 1989. Role of Reticuloendotheliosis virus envelope glycoprotein in superinfection interference. J. Virol **63**:273-280.

Donahue, P.R., E.A. Hoover, G.A. Beltz, N. Riedel, V. Hirsch, J. Overbaugh, and J.I. Mullins. 1988. Strong sequence conservation among horizontally transmissible, minimally pathogenic feline leukaemia viruses. J. Virol. **62**:722-731.

Dorner, A.J., J.P. Stoye, and J.M. Coffin. 1985. Molecular basis of host range variation in avian retroviruses. J. Virol. **53**:32-39.

Dorner, A.J. and J.M. Coffin. 1986. Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85. Cell **45**:365-374.

Dunwiddie, C. and A. Faras. 1985. Presence of retrovirus reverse transcriptase-related gene sequences in avian cells lacking endogenous avian leukosis viruses. Proc. Natl. Acad. Sci. USA **82**:5097-5101.

Einfeld, D. and E. Hunter. 1988. Oligomeric structure of a prototype retrovirus glycoprotein. Proc. Natl. Acad. Sci. USA **85**:8688-8692.

Elder, J.H. and J.I. Mullins. 1983. Nucleotide sequence of the envelope gene of Gardner-Arnstein feline leukaemia virus B reveals unique sequence homologies with a murine mink cell focus-forming virus. J. Virol. **46**:871-880.

Elder, J.H. and J.I. Mullins. 1985. Nucleotide sequence of the envelope genes and LTR of the subgroup B, Rickard strain of feline leukaemia virus, p.1005-1010. In R.. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbor Laboratory , New York.

Essex, M., G. Klein, S.P. Snyder, and J.B. Harrold. 1971a. Antibody to feline oncornavirus-associated cell membrane antigen in neonatal cats. Int. J. Cancer **8**:384-390.

Essex, M., G. Klein, S.P. Snyder, and J.B. Harrold. 1971b. Correlation between humoral antibody and regression of tumours induced by feline sarcoma virus. Nature **233**:195-196.

Evans, L.H. and M.W. Cloyd. 1985. Friend and Moloney murine leukaemia viruses specifically recombine with different endogenous retroviral sequences to generate mink cell focus-forming viruses. Proc. Natl. Acad. Sci. USA **82**:459-463.

Evans, L.H. and F.G. Malik. 1987. Class II polytropic murine leukaemia viruses (MuLVs) of AKR/J mice: possible role in the generation of class I oncogenic polytropic MuLVs. J. Virol. **61**:1882-1892.

Evans, L.H. and J.D. Morrey. 1987. Tissue-specific replication of Friend and Moloney murine leukaemia viruses in infected mice. J. Virol. **61**:1350-1357.

Ferris, S.D., R.D. Sage, and A.C. Wilson. 1982. Evidence from mtDNA sequences that common laboratory strains of inbred mice are descended from a single female. Nature **295**:163-165.

Fischinger, P.J., S. Nomura, and D.P. Bolognesi. 1975. A novel murine oncornavirus with dual eco-xenotropic properties. Proc. Natl. Acad. Sci. USA **72**:5150-5155.

Frankel, W.N., J.P. Stoye, B.A. Taylor, and J.M. Coffin. 1989a. Genetic identification of endogenous polytropic viruses by using recombinant inbred mice. J. Virol. **63**:3810-3821.

Frankel, W.N., J.P. Stoye, B.A. Taylor, and J.M. Coffin. 1989b. Genetic analysis of endogenous xenotropic murine leukaemia viruses: association with two common mouse mutations and the viral restriction locus *Fv-1*. J. Virol. **63**:1763-1774.

Frankel, W.N., J.P. Stoye, B.A. Taylor, and J.M. Coffin. 1990. A linkage map of endogenous murine leukaemia proviruses. Genetics **24**:221-236.

Fulton, R., D. Forrest, R. McFarlane, D. Onions, and J.C. Neil. 1987. Retroviral transduction of T-cell antigen receptor ß-chain and myc genes. Nature **326**:190-194.

Gazdar, A.F., H. Oei, P. Lalley, W.W. Moss, and J.D. Minna. 1977. Identification of mouse chromosomes required for murine leukaemia virus replication. Cell **11**:949-956.

Gallaher, W.R. 1987. Detection of a fusion peptide sequence in the transmembrane protein of human immunodeficiency virus. Cell **50**:327-328.

Garcia, J.V., C. Jones, and A.D. Miller. 1991. Localisation of the amphotropic murine leukaemia virus receptor gene to the pericentromeric region of human chromosome 8. J. Virol. **65**:6316-6319.

Gardner, M.B., S. Rasheed, B.K. Pal, J.D. Estes, and S.J. O'Brien. 1980. *Akvr-1*, a dominant murine leukaemia virus restriction gene, is polymorphic in leukaemia-prone wild mice. Proc. Natl. Acad. Sci. USA **77**:513-535.

Gardner, M.B., C.A. Kozak, and S.J. O'Brien. 1991. The Lake Casitas wild mouse: evolving genetic resistance to retroviral disease. Trends in Genetics **7**:22-27.

Gojobori, T. and S. Yokoyama. 1985. Rates of evolution of the retroviral oncogene of Moloney murine sarcoma virus and its cellular homologues. Proc. Natl. Acad. Sci. USA **82**:4198-4201.

Goodrich, D.W. and P.H. Duesberg. 1990. Retroviral recombination during reverse transcription. Proc. Natl. Acad. Sci. USA **87**:2052-2056.

Green, N., H. Hiai, J.H. Elder, R.S. Schwartz, R.H. Khiroya, C.Y. Thomas, P.N. Tsichlis, and J.M. Coffin. 1980. Expression of leukemogenic recombinant viruses associated with a recessive gene in HRS/J mice. J. Exp. Med. **152**:249-264.

Groudine, M., R. Eisenman, and H. Weintraub. 1981. Chromatin structure of endogenous retroviral genes and activation by an inhibitor of DNA methylation. Nature **23**:311-317.

Halpern, M.S. and R.R. Friis. 1978. Immunogenicity of the envelope glycoprotein of avian sarcoma virus. Proc. Natl. Acad. Sci. USA **75**:1962-1966.

Hanafusa, H., T. Miyamoto, and T. Hanafusa. 1970a. A cell-associated factor essential for formation of an infectious form of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA **66**:314-321.

Hanafusa, T., H. Hanafusa, and T. Miyamoto. 1970b. Recovery of a virus from apparently normal chick cells by infection with avian tumour viruses. Proc. Natl. Acad. Sci. USA **67**:1797-1803.

Harbers, K., A. Schneike, H. Stuhlmann, D. Jahner, and R. Jaenisch. 1981. DNA methylation and gene expression: endogenous retroviral genome becomes infectious after molecular cloning. Proc. Natl. Acad. Sci. USA **78**:7609-7613.

Hartley, J.W. and W.P. Rowe. 1976. Naturally occurring murine leukaemia viruses in wild mice: characterisation of a new "amphotropic" class. J. Virol. **19**:19-25.

Hartley, J.W., N.K. Wolford, L.J. Old, and W.P. Rowe. 1977. A new class of murine leukaemia virus associated with developement of spontaneous lymphomas. Proc. Natl. Acad. Sci. USA **74**:789-792.

Hartley, J.W., R.A. Yetter, and H.C. Morse III.. 1983. A mouse gene on chromosome 5 that restricts infectivity of mink cell focus-forming recombinant murine leukaemia viruses. J. Exp. Med. **158**:16-24.

Hayward, W.S., S.B. Braveman, and S.M. Astrin. 1980. Transcriptional products and DNA structure of endogenous avian proviruses. Cold Spring Harbor Symp. Quant. Biol. **44**:1111-1122.

Heard, J.M. and O. Danos. 1991. An amino terminal fragment of the Friend murine leukaemia virus envelope glycoprotein binds the ecotropic receptor. J. Virol. **65**:4026-4032.

Hoggan, M.D., R.T. O'Neill, and C.A. Kozak. 1986. Nonecotropic murine leukaemia viruses in BALB/c and NFS/N mice: characterisation of the BALB/c Bxv-1 provirus and the single NFS endogenous xenotrope. J. Virol. **60**:980-986.

Holland, C.A., C.Y. Thomas, S.K. Chattopadhyay, C. Koehne, and P.V. O'Donnell. 1989. Influence of enhancer sequences on thymotropism and leukemogenicity of mink cell focus-forming viruses. J. Virol. **63**:1284-1292.

Hsiao, W.L.W., S. Gattoni-Celli, and I.B. Weinstein. 1986. Effects of 5-azacytidine on expression of endogenous retrovirus-related sequences in C3H 10T1/2 cells. J. Virol. **57**:1119-1126.

Hu, S., N. Davidson, M.O. Nicolson, and R.M. McAllister. 1977. Heteroduplex study of the sequence relations between RD-114 and baboon viral RNAs. J. Virol. **23**:345-352.

Hu, W-S. and H.M. Temin. 1990a. Genetic consequences of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genetic recombination. Proc. Natl. Acad. Sci. USA **87**:1556-1560.

Hu, W-S. and H.M. Temin. 1990b. Retroviral recombination and reverse transcription. Science **250**:1227-1233.

Hughes, S.H., K. Toyoshima, J.M. Bishop, and H.E. Varmus. 1981. Organisation of the endogenous proviruses of chickens: implications for origin and expression. Virology **108**:189-207.

Hughes, S.H. 1982. Sequence of the long terminal repeat and adjacent segments of the endogenous avian virus Rous-associated virus 0. J. Virol. **43**:191-200.

Humphries, E.H., C. Glover, R.A. Weiss, and J.R. Arrand. 1979. Differences between the endogenous and exogenous DNA sequences of Rous-associated virus-0. Cell **18**:803-815.

Humphries, E.H., R. Allen, and C. Glover. 1981. Clonal analysis of the integration and expression of endogenous avian retroviral DNA acquired by exogenous viral infection. J. Virol. **39**:584-596.

Hunter, E. 1978. The mechanism for genetic recombination in the avian retroviruses. Curr. Top. Microbiol. Immunol. **79**:295-309.

Hunter, E. and R. Swanstrom. 1990. Retrovirus envelope glycoproteins. Curr. Top. Microbiol. Immunol. **157**:187-253.

Ikeda, H. and T. Odaka. 1983. Cellular expression of murine leukaemia virus gp70 related antigen on thymocytes of uninfected mice correlated with *Fv-4* gene-controlled resistance to Friend leukaewmia virus infection. Virology **133**:65-76.

Ikeda, H. and T. Odaka. 1984. A cell membrane "gp70" associated with *Fv-4* gene: immunological characterisation, and tissue and strain distribution. Virology **133**:65-76.

Ikeda, H., F. Laigret, M.A. Martin, and R. Repaske. 1985. Characterisation of a molecularly cloned retroviral sequence associated with *Fv-4* resistance. J. Virol. **55**:768-777.

Ikeda, H. and H. Sugimura. 1989. *Fv-4* resistance gene: a truncated endogenous murine leukaemia virus with ecotropic interference properties. J. Virol. **63**:5405-5412.

Jarrett, W.F.H., W.B. Martin, G.W. Crighton, R.G. Dalton, and M.F. Stewart. 1964. Leukaemia in the cat. Transmission experiments with leukaemia (lymphosarcoma). Nature **202**:566.

Jarrett, W.F.H., E. Crawford, W.B. Martin, and F. Davie. 1964. Leukaemia in the cat. A virus-like particle associated with leukaemia (lymphosarcoma). Nature **202**:567.

Jarrett, O. and P.H. Russell. 1978. Differential growth and transmission in cats of feline leukaemia viruses of subgroups A and B. Int. J. Cancer **21**:466-472.

Jarrett, O., W.D.Jr. Hardy, M.C. Golder, and D. Hay. 1978a. The frequency of occurence of feline leukaemia virus subgroups in cats. Int. J. Cancer **21**:334-337.

Jarrett, O., P.H. Russell, and W.D.Jr. Hardy. 1978b. The influence of virus subgroup on the epidemiology of feline leukaemia virus, p.25-28. In P. Bentveltzen (ed.), Advances in comparative leukaemia research. Elsevier, Amsterdam.

Jarrett, O. 1984. Pathogenesis of feline leukaemia virus-related diseases, p.135-154. In J.M. Goldman and O. Jarrett (eds.), Mechanisms of viral leukaemogenesis. Churchill-Livingstone, Edinburgh.

Jenkins, N.A. and G.M. Cooper. 1980. Integration, expression, and infectivity of exogenously acquired proviruses of Rous-associated virus-0. J. Virol. **36**:684-691.

Jenkins, N.A., N.G. Copeland, B.A. Taylor, and B.K. Lee. 1981. Dilute (d) coat colour mutation of DBA/2J mice is associated with the site of integration of an ecotropic MuLV genome. Nature **293**:370-374.

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

Junghans, R.P., L.R. Boone, and A.M. Skalka. 1982. Retroviral DNA H structures: displacement-assimilation model of recombination. Cell **30**:53-62.

Kai, K., H. Ikeda, Y. Yuasa, S. Suzuki, and T. Odaka. 1976. Mouse strain resistant to N-, B-, and NB-tropic murine leukaemia viruses. J. Virol. **20**:436-440.

Katz, R.A. and A.M. Skalka. 1990. Generation of diversity in retroviruses. Ann. Rev. Genet. **24**:409-445.

Kawashima, K., H. Ikeda, J.W. Hartley, E. Stockert, W.P. Rowe, and L.J. Old. 1976. Changes in expression of murine leukaemia virus antigens and production of xenotropic virus in the late preleukemic period in AKR mice. Proc. Natl. Acad. Sci. USA **73**:4680-4684.

Khan, A.S., W.P. Rowe, and M.A. Martin. 1982. Cloning of endogenous murine leukaemia virus-related sequences from chromosomal DNA of BALB/c and AKR/J mice: identification of an *env* progenitor of AKR-247 mink cell focus-forming proviral DNA. J. Virol. **44**:625-636.

Khan, A.S. 1984. Nucleotide sequence analysis establishes the role of endogenous murine leukaemia virus DNA segments in formation of recombinant mink cell focus-forming murine leukaemia viruses. J. Virol. **50**:864-871.

Kim, J.W., E.I. Closs, L.M. Albritton, and J. Cunningham. 1990. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. Nature **352**:725-728.

Koshy, R., R.C. Gallo, and F. Wong-Staal. 1980. Characterisation of the endogenous feline leukaemia virus-related DNA sequences in cats and attempts to identify exogenous viral sequences in tissues of virus-negative leukemic animals. Virology **103**:434-445.

Kozak, C.A. and W.P. Rowe. 1980. Genetic mapping of xenotropic murine leukaemia virus-inducing loci in five mouse strains. J. Exp. Med. **142**:219-229.

Kozak, C.A. and W.P. Rowe. 1982. Genetic mapping of ecotropic murine leukaemia virus-inducing loci in six inbred strains. J. Exp. Med. **155**:524-534.

Kozak, C.A. 1983. Genetic mapping of a mouse chromosomal locus required for mink cell focus-forming virus replication. J. Virol. **48**:300-303.

Kozak, C.A. and R.R. O'Neill. 1987. Diverse wild mouse origins of xenotropic, mink cell focus-forming, and two types of ecotropic proviral genes. J. Virol. **61**:3082-3088.

Kozak, C.A., C.J. Villar, and P. Voytek. 1989. Genetic aspects of oncogenesis by murine leukaemia viruses in wild mice. Crit. Rev. Oncogenesis **1**:127-145.

Kozak, C.A., L.M. Albritton, and J. Cunningham. 1990. Genetic mapping of a cloned sequence responsible for susceptibility to ecotropic murine leukaemia viruses. J. Virol. **64**:3119-3121.

Kronenberg, M. 1991. Self-tolerance and autoimmunity. Cell **65**:537-542.

Kumar, D.V., B.T. Berry, and P. Roy-Burman. 1989. Nucleotide sequence and distinctive characteristics of the env gene of endogenous feline leukaemia provirus. J. Virol. **63**:2379-2384.

Levy, D.E., R.A.Lerner, and M.C.Wilson. 1982. A genetic locus regulates the expression of tissue specific mRNAs from multiple transcription units. Proc.Natl.Acad.Sci. USA **79**:5823-5827.

Laigret, F., R. Repaske, K. Boulukos, A.B. Rabson, and A.S. Khan. 1988. Potential progenitor sequences of mink cell focus-forming (MCF) murine leukaemia viruses: ecotropic, xenotropic, and MCF-related viral RNAs are detected concurrently in thymus tissues of AKR mice. J. Virol. **62**:376-386.

Leis, J., D. Baltimore, J.M. Bishop, J. Coffin, E. Fleissner, S.P. Goff, S. Oroszlan, H. Robinson, A.M. Skalka, H.M. Temin, and V. Vogt. 1988. Standardised and simplified nomenclature for proteins common to all retroviruses. J. Virol. **62**:1808-1809.

Levy, L.S., M.B. Gardner, and J.W. Casey. 1984. Isolation of a feline leukaemia provirus containing the oncogene myc from a feline lymphosarcoma. Nature **308**:853-856.

Levy, D.E., R.A. Lerner, and M.C. Wilson. 1985a. Normal expression of polymorphic endogenous retroviral RNA containing segments identical to mink cell focus-forming virus. J. Virol. **56**:691-700.

Levy, D.E., R.A. Lerner, and M.C. Wilson. 1985b. The Gv-1 locus coordinately regulates the expression of multiple endogenous murine retroviruses. Cell **41**:289-299.

Li, J.-P., R.K. Bestwick, C. Machida, and D. Kabat. 1986. Role of a membrane glycoprotein in Friend virus erythroleukaemia: nucleotide sequences of nonleukemogenic mutant and spontaneous revertant viruses. J. Virol **57**:534-538.

Li, J.P., R.K. Bestwick, C. Spiro, and D. Kabat. 1987. The membrane glycoprotein of Friend spleen focus forming virus: evidence that the cell surface component is required for pathogenesis and that it binds to a receptor. J. Virol. **61**:2782-2792.

Li, J.P., A.D. D-Andrea, H.F. Lodish, and D. Baltimore. 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature **343**:762-764.

Lilly, F., M.L. Duran-Reynals, and W.P. Rowe. 1975. Corrolation of early murine leukaemia virus titer and H-2 type with spontaneous leukaemia in mice of the BALB/c x AKR cross: a genetic analysis. J. Exp. Med. **141**:882-889.

Linemeyer, D.L., S.K. Ruscetti, E.M. Scolnick, L.H. Evans, and P.H. Duesberg. 1981. Biological activity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus DNA. Proc. Natl. Acad. Sci. USA **78**:1401-1405.

Livingston, D.M. and G.J. Todaro. 1973. Endogenous type C virus from a cat cell clone with properties distinct from previously described feline type C virus. Virology **53**:142-151.

Lung, M.L., C. Hering, J.W. Hartley, W.P. Rowe, and N. Hopkins. 1980. Analysis of the genomes of mink cell focus-inducing murine type-C viruses: a progress report. Cold Spring Harbor Symp. Quant. Biol. **44**:1269-1274.

Lung, M.L., J.W. Hartley, W.P. Rowe, and N.H. Hopkins. 1983. Large RNase T1-resistant oligonucleotides encoding p15E and the U3 region of the long terminal repeat distinguish two biological classes of mink cell focus-forming type C viruses of inbred mice. J. Virol. **45**:275-290.

Marsh, J.L., M. Erfle, and E.J. Wykes. 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481-485.

Mullins, J.I., J.W.Casey, M.O.Nicolson, K.B.Burck, and N.Davidson. 1981. Sequence arrangement and biological activity of cloned feline leukemia virus proviruses from a virus-productive human cell line. J. Virol. **38:**688-703.

Martinelli, S.C. and S.P. Goff. 1990. Rapid reversion of a deletion mutation in Moloney murine leukaemia virus by recombination with a closely related endogenous provirus. Virology **174**:135-144.

Masuda, M. and H. Yoshikura. 1990. Construction and characterisation of the recombinant Moloney murine leukaemia viruses bearing the mouse *Fv-4 env* gene. J. Virol. **64**:1033-1043.

Mathes, L.E., D.S. Yohn, E.A. Hoover, M. Essex, J.P. Schaller, and R.G. Olsen. 1976. Feline oncornavirus-associated cell membrane antigen. VI. Cytotoxic antibody in cats exposed to feline leukaemia virus. J. Natl. Cancer Inst. **56**:1197-1200.

McAllister, R.M., M. Nicolson, M.B. Gardner, R.W. Rongey, S. Rasheed, P.S. Sarma, R.J. Huebner, M. Hatanaka, S. Oroszlan, R.V. Gilden, A. Kabigting, and L. Vernon. 1972. C-Type virus released from cultured human rhabdomyosarcoma cells. Nature [New Biol.] **235**:3-6.

Meier, H., D.D. Myers, and R.J. Huebner. 1969. Genetic control by the hr-Locus of susceptibility and resistance of leukaemia. Proc. Natl. Acad. Sci. USA **63**:759-766.

Meruelo, D., A. Rossomando, M. Offer, J. Buxbaum, and A. Pellicer. 1983. Association of endogenous viral loci with genes encoding murine histocompatibility and lymphocyte differentiation antigens. Proc. Natl. Acad. Sci. USA **80**:5032-5036.

Moroni, C. and G. Schumann. 1975. Lipopolysaccharide induces C-type virus in short term cultures of BALB/c spleen cells. Nature **254**:60-61.

Moroni, C., G. Schumann, M. Robert-Guroff, E.R. Suter, and D. Martin. 1975. Induction of endogenous murine C-type virus in spleen cell cultures treted with mitogens and 5-bromo-2'deoxyuridine. Proc. Natl. Acad. Sci. USA **72**:535-538.

Morse III, H.C., C.A. Kozak, R.A. Yetter, and J.W. Hartley. 1982. Unique features of retrovirus expression in F/St mice. J. Virol. **43**:1-7.

Motta, J.V., L.B. Crittenden, H.G. Purchase, H.A. Stone, and R.L. Witter. 1975. Low oncogenic potential of avian endogenous RNA tumour virus infection or expression. J. Natl. Cancer Inst. **55**:685-689.

Mullins, J.I., D.S. Brody, R.C. Binari,Jr., and S.M. Cotter. 1984. Viral transduction of *c-myc* gene in naturally occurring feline leukaemias. Nature **308**:856-858.

Neil, J.C. 1984. Molecular aspects of feline leukaemia viruses and their related diseases, p.219-239. In P.W. Rigby and N.M. Wilkie (eds.), Viruses and Cancer. Cambridge University Press, Cambridge.

Neil, J.C., D. Hughes, R. McFarlane, N.M. Wilkie, D.E. Onions, G. Lees, and O. Jarrett. 1984. Transduction and rearrangement of the *myc* gene by feline leukaemia virus in naturally occurring T-cell leukaemias. Nature **308**:814-820.

Neil, J.C. and D.E. Onions. 1985. Feline leukaemia viruses: molecular biology and pathogenesis. Anticancer Res. **5**:49-63.

Neil, J.C. and D. Forrest. 1987. Mechanisms of retrovirus-induced leukaemia: selected aspects. Biochem. Biophys. Acta **907**:71-91.

Neil, J.C., D. Forrest, D.L. Doggett, and J.I. Mullins. 1987. The role of feline leukaemia virus in naturally-occurring leukaemias. Cancer Sur. **6**:117-137.

Niman, H.L., M.B. Gardner, J.R. Stephenson, and P. Roy-Burman. 1977a. Endogenous RD-114 virus genome expression in malignant tissues of domestic cats. J. Virol. **23**:578-586.

Niman, H.L., J.R. Stephenson, M.B. Gardner, and P. Roy-Burman. 1977b. RD-114 and feline leukaemia virus genome expression in natural lymphomas of domestic cats. Nature **266**:357-360.

Niman, H.L., M. Akhavi, M.B. Gardner, J.R. Stephenson, and P. Roy-Burman. 1980. Differential expression of two distinct endogenous retrovirus genomes in developing tissues of the domestic cat. J. Natl. Cancer Inst. **64**:587-594.

Nunberg, J.H., M.E. Williams, and M.A. Innis. 1984. Nucleotide sequences of the envelope genes of two isolates of feline leukaemia virus subgroup B. J. Virol. **49**:629-632.

O'Brien, S.J., E.J. Berman, J.D. Estes, and M.B. Gardner. 1983. Murine retroviral restriction genes *Fv-4* and *Akvr-1* are alleles of a single locus. J. Virol. **47**:649-651.

O'Brien, S.J. 1986. Molecular genetics in the domestic cat and its relatives. Trends in Genetics **2**:137-142.

Obata, Y., H. Ikeda, E. Stockert, and E.A. Boyse. 1975. Relation of G_{IX} antigen of thymocytes to envelope glycoprotein of murine leukaemia virus. J. Exp. Med. **141**:188-197.

Obata, Y., E. Stockert, M. Yamaguchi, and E.A. Boyse. 1978. Source and hormone dependance of G_{IX}-gp70 in mouse serum. J. Exp. Med. **148**:793-798.

Odaka, T., H. Ikeda, A. Moriwaki, M. Mizuno, and K. Kondo. 1978. Genetic resistance in Japanese wild mice (*Mus musculus molossinus*) to an NB-tropic Friend murine leukaemia virus. J. Natl. Cancer Inst. **61**:1301-1305.

Odaka, T., H. Ikeda, H. Yoshikura, K. Moriwaki, and S. Suzuki. 1981. *Fv-4*: gene controlling resistance to NB-tropic Friend murine leukaemia virus. Distribution in wild mice, introduction into genetic background of BALB/c mice, and mapping of chromosomes. J. Natl. Cancer Inst. **67**:1123-1127.

Ohashi, P.S., S. Oehen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odermatt, B. Malissen, R.M. Zinkernagel, and H. Hengartner. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in virus antigen transgenic mice. Cell **65**:305-317.

Oldstone, M.B.A., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitis in a transgenic model: role of anti-self (virus) immune response. Cell **65**:319-331.

Onions, D., O. Jarrett, N. Testa, F. Frassoni, and S. Toth. 1982. Selective effect of feline leukaemia virus on early erythroid precursors. Nature **296**:156-158.

Overbaugh, J., N. Riedel, E.A. Hoover, and J.I. Mullins. 1988. Transduction of endogenous envelope genes by feline leukaemia virus *in vitro*. Nature **332**:731-734.

Pandey, R., A.K. Ghosh, D.V. Kumar, B.A. Bachman, D. Shibata, and P. Roy-Burman. 1991. Recombination between feline leukaemia virus subgroup B or C and endogenous *env* elements alters the in vitro biological activities of the viruses. J. Virol. **65**:6495-6508.

Panganiban, A.T. and D. Fiore. 1988. Ordered interstrand and intrastrand DNA transfer during reverse transcription. Science **241**:1064-1069.

Pani, P.K. 1977. Evidence for complementary action of *tv-b* and *tv-e* genes that control susceptibility to subgroup E RNA tumour viruses in chickens. J. Gen. Virol. **37**:639-646.

Pathak, V.K. and H.M. Temin. 1990a. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. Proc. Natl. Acad. Sci. USA **87**:6019-6023.

Pathak, V.K. and H.M. Temin. 1990b. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single round of infection: deletions and deletions with insertions. Proc. Natl. Acad. Sci. USA **87**:6024-6028.

Pulsinelli, G.A. and H.M. Temin. 1991. Characterisation of large deletions occuring during a single round of retrovirus vector replication: novel deletion mechanism involving errors in strand transfer. J. Virol. **65**:4786-4797.

Quint, W., W. Boelens, P. van Wezenbeek, T. Cuypers, E. Robanus-Maandag, G. Selten, and A. Berns. 1984. Generation of AKR mink cell focus-forming viruses: a conserved single-copy xenotrope-like provirus provides recombinant long terminal repeat sequences. J. Virol. **50**:432-438.

Quintrell, N., H.E. Varmus, J.M. Bishop, M.O. Nicholson, and R.M. McAllister. 1974. Homologies among the nucleotide sequences of the genomes of C-type viruses. Virology **58**:568-575.

Rasheed, S., M.B. Gardner, and E. Chan. 1976. Amphotropic host range of naturally occurring wild mouse leukemia viruses. J. Virol. **19**:13-18.

Rasheed, S. and M.B. Gardner. 1980. Characterisation of cat cell cultures for expression of retrovirus, FOCMA and endogenous sarc genes, p.393-400. In M. Essex and A.J. McClelland (eds.), Proceedings of the Third International Feline Leukaemia Virus Meeting. Elsevier, North-Holland.

Rassart, E., L. Nelbach, and P. Jolicoeur. 1986. Cas-Br-E murine leukaemia virus: sequence of the paralytogenic region of its genome and derivation of specific probes to study its origin and the structure of its recombinant genomes in leukaemia tissues. J. Virol. **60**:910-919.

Reeves, R.H. and S.J. O'Brien. 1984. Molecular genetic characterisation of the RD114 gene family ofendogenous feline retroviral sequences. J. Virol. **52**:164-171.

Rein, A. 1982. Interference grouping of murine leukaemia viruses: a distinct receptor for the MCF-recombinant viruses in mouse cells. Virology **120**:251-257.

Rickard, D.G., J.E. Post, F. deNoronha, and L.M. Barry. 1969. A transmissible virus-induced lymphocytic leukaemia of the cat. J. Natl. Cancer Inst. **42**:987-1014.

Rigby, P.W.J., M. Dieckmann, C. Rhoquades, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity in vitro with DNA polymerase 1. J. Mol. Biol. **113**:521-560.

Roberts, J.D., B.D. Preston, L.A. Johnston, A. Soni, L.A. Loeb, and T.A. Kunkel. 1989. Fidelity of two retroviral reverse transcriptases during DNA-dependant DNA synthesis in vitro. Mol. Cell. Biol. **9**:469-476.

Robinson, H.L. 1976. Intracellular restriction on the growth of induced subgroup E avian type C viruses in chicken cells. J. Virol **18**:856-866.

Robinson, H.L., R. Eisenman, A. Senior, and S. Ripley. 1979. Low frequency production of recombinant subgroup E avian leukosis viruses by uninfected V-15b chicken cells. Virology **99**:21-30.

Robinson, H.L., S.M. Astrin, A.M. Senior, and F.H. Salazar. 1981. Host-susceptibility to endogenous viruses: defective, glycoprotein-expressing proviruses interfere with infections. J. Virol **40**:745-751.

Robinson, H.L., B.M. Blais, P.N. Tsichlis, and J.M. Collin. 1982. At least two regions of the viral genome determine the oncogenic potential of avian leukosis viruses. Proc. Natl. Acad. Sci. USA **79**:1225-1229.

Rohdewohlde, H., H. Weiher, W. Reik, R. Jaenisch, and M. Breindl. 1987. Retrovirus integration and chromatin structure: Moloney murine leukaemia proviral integration sites map near DNase I-hypersensitive sites. J. Virol. **61**:336-343.

Rojko, J.L., E.A. Hoover, B.L. Finn, and R.G. Olsen. 1981. Determinants of susceptibility and resistance to feline leukaemia virus infection. II. Susceptibility of feline lymphocytes to productive feline leukaemia virus infection. J. Natl. Cancer Inst. **67**:899-910.

Rosen, C.A., W.A. Haseltine, J. Lenz, R. Ruprecht, and M.W. Cloyd. 1985. Tissue selectivity of murine leukaemia virus infection is determined by long terminal repeat sequences. J. Virol. **55**:862-866.

Rovigatti, U.G. and S.M. Astrin. 1983. Avian endogenous viral genes. Curr. Top. Microbiol. Immunol. **103**:1-23.

Rowe, W.P. and T. Pincus. 1972. Quantitative studies of naturally-occurring murine leukaemia virus infection of AKR mice. J. Exp. Med. **135**:429-436.

Ruscetti, S., D. Linemeyer, J. Feild, D. Troxler, and E. Scolnick. 1979. Characterisation of a protein found in cells infected with the spleen focus-forming virus that shares immunological cross-reactivity with the gp70 found in mink cell focus-inducing virus particles. J. Virol. **30**:787-789.

Ruscetti, S.K., L. Donner, and C.J. Sherr. 1980. A model for FOCMA expression in cells transformed by feline leukaemia and sarcoma viruses. Animal Virus Genetics **46**:541-549.

Ruscetti, S., L. Davis, J. Feild, and A. Oliff. 1981. Friend murine leukaemia virus-induced leukaemia is associated with the formation of mink cell focus-inducing viruses and is blocked in mice expressing endogenous mink cell focus-inducing xenotropic viral envelope genes. J. Exp. Med. **154**:907-920.

Ruscetti, S., R. Matthai, and M. Potter. 1985. Susceptibility of BALB/c mice carrying various DBA/2 genes to development of Friend murine leukaemia virus-induced erythroleukaemia. J. Exp. Med. **162**:1579-1587.

Saiki, R.K., D.H. Gelfand, B. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487-491.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463-5467.

Sanger, F., A.R. Coulson, B.G. Barrell, A.J.H. Smith, and B. Roe. 1980. Cloning in single stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161-178.

Sarma, P.S. and T. Log. 1971. Viral interference in feline leukaemia-sarcoma complex. Virology **44**:352-358.

Sarma, P.S. and T. Log. 1973. Subgroup classification of feline leukaemia and sarcoma viruses by viral interference and neutralisation tests. Virology **54**:160-169.

Sarma, P.S., T. Log, D. Jain, P.R. Hill, and R.J. Huebner. 1975. Differential host range of viruses of feline leukaemia-sarcoma complex. Virology **64**:438-446.

Schaller, J.P., M. Essex, D.S. Yohn, and R.G. Olsen. 1975. Feline oncornavirus-associated cell membrane antigen. V. Humoral immune response to virus and cell membrane antigens in cats inoculated with Gardner-Arnstein feline sarcoma virus. J. Natl. Cancer Inst. **55**:1373-1378.

Schwartz, D.E., R. Tizard, and W. Gilbert. 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853-869.

Schwartzberg, P., J. Colicelli, and S.P. Goff. 1985. Recombination between a defective retrovirus and homologous sequences in host DNA: reversion by patch repair. J. Virol. **53**:719-726.

Shields, A., O.N. Witte, E. Rothenberg, and D. Baltimore. 1978. High frequency of abberant expression of Moloney murine leukaemia virus in clonal infections. Cell **14**:601-609.

Shih, C.C., J.P. Stoye, and J.M. Coffin. 1988. Highly preferred targets for retrovirus integration. Cell **53**:531-537.

Smith, D.B. and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-S-transferase. Gene **67**:31-40.

Snyder, H.W.Jr., W.D.Jr. Hardy, E.E. Zuckerman, and E. Fleissner. 1978. Characterisation of a tumour-specific antigen on the surface of feline lymphosarcoma cells. Nature **275**:656-658.

Snyder, H.W.Jr., M.C. Singhal, E.E. Zuckerman, F.R. Jones, and W.D.Jr. Hardy. 1983. The feline oncornavirus-associated cell membrane antigen (FOCMA) is related to, but distinguishable from, FeLV-C gp70. Virology **131**:315-327.

Soe, L.H., B.G. Devi, J.I. Mullins, and P. Roy-Burman. 1983. Molecular cloning and characterisation of endogenous feline leukaemia virus sequences from a cat genomic library. J. Virol. **46**:829-840.

Soe, L.H., R.W. Shimizu, J.R. Landolph, and P. Roy-Burman. 1985. Molecular analysis of several classes of endogenous feline leukaemia virus elements. J. Virol. **56**:701-710.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

Speck, N.A. and D. Baltimore. 1987. Six distinct nuclear factors interact with the 75-base-pair repeat of the Moloney leukaemia virus enhancer. Mol. Cell. Biol. **7**:1101-1110.

Spodick, D.A., L.H. Soe, and P. Roy-Burman. 1984. Genetic analysis of the feline RD114 retrovirus-related endogenous elements. Virus Research **1**:543-555.

Steffen, D.L., B.A. Taylor, and R.A. Weinberg. 1982. Continuing germ line integration of AKV proviruses during the breeding of AKR mice and derivative recombinant inbred strains. J. Virol. **42**:165-175.

Stewart, M.A., M. Warnock, A. Wheeler, N. Wilkie, J.I. Mullins, D.E. Onions, and J.C. Neil. 1986. Nucleotide sequences of a feline leukaemia virus subgroup A envelope gene and long terminal repeat and evidence for the recombinational origin of subgroup B viruses. J. Virol. **58**:825-834.

Stockert, E., L.J. Old, and E.A. Boyse. 1971. The G_{IX} system. A cell surface allo-antigen associated with murine leukaemia virus; implications regarding chromosomal integration of the viral genome. J. Exp. Med. **133**:1334-1355.

Stoye, J.P. and C. Moroni. 1983. Endogenous retrovirus expression in stimulated murine lymphocytes. Identification of a new locus controlling mitogen production of a defective virus. J. Exp. Med. **157**:1660-1674.

Stoye, J.P. and C. Moroni. 1984. Phenotypic mixing of retroviruses in mitogen-stimulated lymphocytes: analysis of xenotropic and defective endogenous mouse viruses. J. Gen. Virol. **65**:317-326.

Stoye, J. and J. Coffin. 1985. Endogenous viruses, p.357-404. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbor Laboratory, New York.

Stoye, J.P. and C. Moroni. 1985. Proliferation and differentiation requirements for the induction of two retroviral loci during B-cell activation. J. Gen. Virol. **66**:109-120.

Stoye, J.P. and J.M. Coffin. 1987. The four classes of endogenous murine leukaemia virus: structural relationships and potential for recombination. J. Virol. **61**:2659-2669.

Stoye, J.P., S. Fenner, G.E. Greenoak, C. Moran, and J.M. Coffin. 1988. Role of endogenous retroviruses as mutagens: the hairless mutation of mice. Cell **54**:383-391.

Stoye, J. and J.M. Coffin. 1988. Polymorphism of murine endogenous proviruses revealed by using virus class-specific oligonucleotide probes. J. Virol. **62**:168-175.

Stoye, J.P., C. Moroni, and J.M. Coffin. 1991. Virological events leading to spontaneous AKR lymphomas. J. Virol. **65**:1273-1285.

Takeuchi, Y., R.G. Vile, G. Simpson, B. O'Hara, M.K.L. Collins, and R.A. Weiss. 1992. Feline leukaemia virus subgroup B uses the same cell surface receptor as gibbon ape leukaemia virus. J. Virol. **66**:1219-1222.

Teich, N. 1982. Taxonomy of retroviruses, p.25-208. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbour Laboratory, New York.

Teich, N. 1985. Taxonomy of retroviruses, p.1-16. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbor Laboratory, New York.

Tereba, A. 1981. 5'-terminal deletions are a common feature of endogenous retrovirus loci located on chromosome 1 of White Leghorn chickens. J. Virol. **40**:920-926.

Tereba, A. and S.M. Astrin. 1982. Localisation of five endogenous retrovirus loci on chromosome 1 of White Leghorn chickens by in situ hybridisation. J. Virol. **43**:737-740.

Terry, A., R. Fulton, M. Stewart, D. Onions, and J.C. Neil. 1992. Pathogenesis of feline leukaemia virus T17: contrasting fates of helper, *v-myc* and *v-tcr* proviruses. J. Virol. in press.

Theilen, G.H., D.L. Dungworth, T.G. Kawakami, R.J. Munn, J.M. Ward, and J.B. Harrold. 1970. Experimental induction of lymphosarcoma in the cat with C-type virus. Cancer Res. **30**:401-408.

Thomas, D.J., J.S. Wall, J.F. Hainfeld, M. Kaczorek, F.P. Booy, B.L. Trus, F.A. Eiserling, and A.C. Steven. 1991. gp160, the envelope glycoprotein of Human Immunodeficiency Virus type 1, is a dimer of 125-kilodalton subunits stabalised through interractions between their gp41 domains. J. Virol. **65**:3797-3803.

Tindal, K.R. and T.A. Kunkel. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. Biochemistry **27**:6008-6013.

Troxler, D., D. Lowy, R. Howk, H. Young, and E. Scolnick. 1977. Friend strain of spleen focus-forming virus is a recombinant between ecotropic type-C virus and the *env* region of xenotropic type C virus. Proc. Natl. Acad. Sci. USA **74**:4671-4675.

Tsichlis, P.N., K.F. Conklin, and J.M. Coffin. 1980. Mutant and recombinant avian retroviruses with extended host range. Proc. Natl. Acad. Sci. USA **77**:536-540.

Tsichlis, P.N. and J.M. Coffin. 1980. Recombinants between endogenous and exogenous avian tumour viruses: role of the C region and other portions of the genome in the control of replication and transformation. J. Virol **33**:238-249.

Tsichlis, P.N. and S.E. Bear. 1991. Infection by mink cell focus-forming viruses confers interleukin 2 (IL-2) independance to an IL-2-dependant rat T-cell lymphoma line. Proc. Natl. Acad. Sci. USA **88**:4611-4615.

Tzavaras, T., M. Stewart, A. McDougall, R. Fulton, N. Testa, D.E. Onions, and J.C. Neil. 1990. Molecular cloning and characterisation of a defective recombinant feline leukaemia virus associated with myeloid leukaemia. J. Gen. Virol. **71**:343-354.

Varmus, H. and R. Swanstrom. 1982. Replication of retroviruses, p.369-512. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbor Laboratory, New York,

Varmus, H. and R. Swanstrom. 1985. Replication of retroviruses, p.75-134. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbor Laboratory, New York.

Varmus, H. 1988. Regulation of HIV and HTLV gene expression. Genes Dev. **2**:1055-1062.

Vedbrat, S.S., S. Rasheed, H. Lutz, M.A. Gonda, S. Ruscetti, M.B. Gardner, and W. Prensky. 1983. Feline oncornavirus-associated cell membrane antigen: a viral and not a cellularly coded transformation-specific antigen of cat lymphomas. Virology **124**:445-461.

Vijaya, S., D.L. Steffen, and H.L. Robinson. 1986. Acceptor sites for retroviral integrations map near DNase I-hypersensitive sites in chromatin. J. Virol. **60**:683-692.

Villar, C.J., T.N. Fredrickson, and C.A. Kozak. 1988. Effect of the *Gv-1* locus on Moloney ecotropic murine leukaemia virus induced disease in inbred wild mice. Curr. Top. Microbiol. Immunol. **137**:250-255.

Vogt, P.K. 1969. Envelope classification of avian RNA tumour viruses, p.153-167. In R.M. Dutcher (ed.), Comparative Leukaemia Research. Karger, Basel.

Vogt, P.K. and R.R. Friis. 1971. An avian leukosis virus related to RSV(0): Properties and evidence for helper activity. Virology **43**:223-234.

Vogt, P.K. 1977. Genetics of RNA tumour viruses, p.341-455. In H. Fraenkel-Conrat and R.R. Wagner (eds.), Comprehensive Virology Vol 9. Plenum, New York.

Wang, H., M.P. Kavanaugh, R.A. North, and D. Kabat. 1991. Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. Nature **352**:729-731.

Weiss, R.A. 1969. The host range of Bryan strain Rous sarcoma virus synthesised in the absence of helper virus. J. Gen. Virol. **5**:511-528.

Weiss, R.A., W.S. Mason, and P.K. Vogt. 1973. Genetic recombinants and heterozygotes derived from endogenous and exogenous avian RNA tumour viruses. Virology **52**:535-552.

Weiss, R. 1982. Experimental biology and assay of RNA tumour viruses, p.209-260. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.), RNA Tumor Viruses. Cold Spring Harbor Laboratory, New York.

Wejman, J.C., B.A. Taylor, N.A. Jenkins, and N.G. Copeland. 1984. Endogenous xenotropic murine leukaemia virus-related sequences map to chromosomal regions encoding mouse lymphocyte antigens. J. Virol. **50**:237-247.

White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. Quarterly Reviews of Biophysics **16**:151-195.

White, T.J., N. Arnheim, and H.A. Erlich. 1989. The polymerase chain reaction. Trends in Genetics **5**:185-189.

Wolff, L., E. Scolnick, and S. Ruscetti. 1983. Envelope gene of the Friend spleen focus-forming virus: deletion and insertions in the 3' gp70/p15E-encoding region have resulted in unique features in the primary structure of the protein product. Proc. Natl. Acad. Sci. USA **80**:4718-4722.

Wunsch, M., A.S. Schulz, W. Koch, R. Friedrich, and G. Hunsmann. 1983. Sequence analysis of Gardner-Arnstein feline leukaemia virus envelope gene reveals common structural properties of mammalian retroviral envelope genes. EMBO J. **2**:2239-2246.

Zemiecki, A., G. Kromer, R.G. Mueller, K. H'ala, and G. Wick. 1988. *ev-22*, a new endogenous avian leukosis virus found in chickens with spontaneous autoimmune thyroiditis. Arch. Virol **100**:267-271.