CIS AND *TRANS* REGULATION OF FELINE IMMUNODEFICIENCY VIRUS

FIONA JANE THOMPSON.

A thesis submitted for the degree of
Doctor of Philosophy

Department of Veterinary Pathology,
Faculty of Veterinary Medicine,
Glasgow University

January 1994

To Mum and Dad

with love and thanks.

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Declaration.

The studies described in this thesis were carried out in the Department of Veterinary Pathology at the University of Glasgow Veterinary School between October 1988 and March 1992. The author was responsible for all the results except where it is stated otherwise.

No part of this thesis has been presented to any other University but it has been reproduced in parts in the following publication:

Thompson, F. J., J. Elder and J. C. Neil (1994) *Cis* and *trans* regulation of FIV: Identification of functional binding sites in the long terminal repeat. J. Gen. Virol. In Press.

Fiona J. Thompson

January 1994.

Acknowledgements.

I would like to thank my supervisor Prof. Jim Neil for his guidance, advice and encouragement throughout the course of this study, not forgetting his skill with 'Harvard Graphics' and the English language !

I would also like to thank Profs. D. Onions, O. Jarrett and W. Jarrett for their generous provision of resources and lab facilities within their department.

I am indebted to the MRC AIDS directed programme for continued funding and for providing a scientifically rewarding trip to the States.

A huge thanks must go to all the members of the MRC lab (past and present) for never failing to help, especially Dr. M. Hosie for providing virus samples and for countless ELISAS and western blots (and intrepid adventures), Dr. G. Reid for the protein expression work (and dreadful songs), Mrs. C. Cannon for her tissue culture skills (and dancing lessons), Dr. M. Harris for his rapid protocols (and terrible puns), Ms. S. Hislop for bench supervision (and her drawers!), Mr. T. McPherson for the smooth running of the laboratory (and his endless appetite), Dr. N. Spibey for transfection experience (and his whistling(not)), Mr. T. Dunsford for ELISAS and cell culture (and the Scotsman), Mr. M. McDonald for ELISAS (and cheap and cheerful car repairs), Mr. J. Callanan for virus samples (and the green cross code!) and finally special thanks to Dr. M. Rigby and Mrs. N. MacKay for all their help, support, experience and useful discussion (not to mention their messiness and tidiness respectively!).

Thanks also to everyone in the Department of Vet. Path. especially the 'tea room gang' (you know who you are!) for making it such a lively place to work.

More generally I would like to thank Dr. M. Plumb and Ms. S. Lowe for helping me with the nuclear protein preps and analysis of the footprinting assays, Drs. J. Elder, J. E. Clements and E. Holmes for fruitful collaboration, Dr. McGinlay for being the best 'asker' of biology questions ever, the O'Neil family for their computer, Mr. A. May for excellent photography work, Dr. J. McKeand for

Figure 1.5, Dr. Mark Rigby for his proof reading skills and everyone in the MOL lab for putting up with me while I was writing.

On a more personal note I would like to thank all my friends including Fiona, Sandra, Takis, Margaret, Brian and especially Shelagh for always being at the end of a phone! Thanks also to Diane for all her support, to Sean for brightening things up and to EDs lab for easing the transition to a new field.

Last but certainly not least I am most grateful to my long suffering family, Mum, Dad, John, Alan, Gran, Aunt Jean and Uncle Davy, for all their love, encouragement, stimulation and continued support (both emotional and financial) throughout my studies and finally thanks to Dodie for being there (well most of the time!).

Abbreviations.

ATP	adenosine triphosphate
bis	N, N'-methylenebisacrylamide
bp	base pairs
BSA	bovine serum albumin
CAEV	Caprine Arthritis-Encephalitis Virus
CAT	chloramphenicol acetyltransferase
$CaCl_2$	calcium chloride
Ci	Curie
CIP	calf intestinal alkaline phosphatase
cm	centimetre
cpm	counts per minute
CTP	cytidine triphosphate
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EIAV	Equine Infectious Anaemia Virus
EtBr	ethidium bromide
EtOH	ethanol
FeLV	Feline Leukaemia Virus
FIV	Feline Immunodeficiency Virus
g	gram
HCl	hydrochloric acid
HIV	Human Immunodeficiency Virus
KCl	potassium chloride
kb	kilobase
kDa	kilodalton

λ	lambda
l	litre
LTR	long terminal repeat
M	molar
mA	milliamp
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimole
mRNA	messenger RNA
MW	molecular weight
NaCl	sodium chloride
NaOAc	sodium acetate
nm	nanomole
^oC	degrees centigrade
OD A_n	optical density at wavelength n
PAGE	polyacrylamide gel electrophoresis
p	plasmid
pg	picogram
pm	picomole
PMSF	phenyl methyl sulphonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SIV	Simian Immunodeficiency Virus
TE	Tris-EDTA buffer
TEMED	N,N,N'n'-tetramethylethylene diamine

Tris	Tris (hydroxymethyl) methylamine
U	unit
µCi	micro Curie
µg	microgram
µl	microlitre
µm	micromolar
UV	ultra violet light
V	volts
V:V	volume:volume ratio
W:V	weight:volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-2-galactopyranoside

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Chapter 1 Introduction

Chapter 3 Sequence Analysis of the FIV LTR

Summary.

The DNA sequence of the long terminal repeat (LTR) of several U.K. isolates of feline immunodeficiency virus (FIV) were determined and compared with those of other world-wide strains. These studies revealed a highly conserved structure with > 80% sequence identity between the different isolates.

Nuclear protein binding sites in the LTR of FIV were identified by the method of DNase I footprinting. Using nuclear protein extracts from a feline T-lymphoma cell line, several discrete footprints were generated upstream of the transcriptional initiation site (-50 to -150). The specificity of protein binding was examined by competition with oligonucleotides representing consensus DNA binding sites for known transcription factors. Binding to AP-1 (-124) and ATF (-58) motifs was observed, with cross competition between these sites. A strong footprint was also detected over a tandemly repeated C/EBP motif (-94 to -86) while an adjacent weaker footprint was found to be specific for a NF-1 motif (-72 to -63).

The effect on FIV LTR promoter activity of progressively deleting these nuclear factor binding sites was examined by linking LTR deletion mutants to the chloramphenicol acetyl transferase gene. Deletion of the AP-1 site caused a 10-25 fold loss of CAT activity while deletion past the ATF site reduced activity virtually to background levels. The effects of deleting the C/EBP and NF-1 sites were less marked and varied according to cell type.

Trans-activation of the LTR was assayed using constructs linked to a CAT reporter gene. The full length FIV LTR was not significantly *trans*-activated. However the expression of a deleted LTR construct lacking the AP-1/AP-4 site but retaining the C/EBP and ATF sites was partially restored by co-infection with FIV or by co-transfection with an infectious molecular clone of FIV (PPR).

The ORF-2 gene of FIV-G8 was cloned and sequenced and used in co-transfection studies to try to analyse viral *trans*-activation. This region of the

genome is highly conserved among FIV isolates (~80%) and it has been suggested that it may be involved in *trans*-activation however the co-transfection experiments which were carried out during this study did not show any evidence of such a function.

These results show that host transcription factors responsive to cellular activation have a major role in regulating FIV expression and suggest that virus-coded *trans*-activators may play a cell-type specific accessory role via U3 sequences.

CHAPTER 1

Chapter 1 Introduction.

1.1 Retroviridae.

The virus family Retroviridae includes all the viruses which have been found to contain an RNA-dependent DNA polymerase (reverse transcriptase) enzymatic activity. Although this is the primary criterion for retroviral classification there are a number of additional features which characterise this group. All retroviruses have common morphological, biochemical and physical properties that further justify their inclusion in a single virus family. A detailed review of the taxonomy of retroviruses is given by Weiss *et al* (1) and is summarised here in Figure 1.1. In the past, attempts to classify retroviruses relied on the ultrastructure of the virions and the antigenic relationships between viruses. More recently classification has been based on the nucleotide sequence of conserved viral genes and this more detailed analysis has helped to further subdivide the family into 7 genera (Figure 1.2B) (2, 385). However, for the purposes of this thesis the previous classification, which divided the retroviruses into three major sub-families, will be used (Figure 1.2A) (1).

1.1.1 Oncovirinae.

As the name indicates the prototype members of this group are oncogenic. Oncoviruses can be classified into 4 morphological groups, types A, B, C, and D. The largest group are the type C retroviruses. Exogenous oncoviruses can spread between individuals by contact or congenitally and are known to cause important diseases in both humans and animals. Many, if not all, mammalian species contain endogenous oncoviruses integrated into the host genome. These are transmitted vertically as proviruses in the germ line. These viruses are often only partially expressed but can, in some cases, be

Figure 1.1 Physical Properties of Retroviridae.

(1) Morphology		Spherical enveloped virions 80-150nm diameter variable surface projections dense nucleoprotein core
(2) Composition		
Nucleic acid	2% by weight	2 linear ssRNA subunits positive polarity 5' methylated cap 3' polyadenylation R region tRNA complexed
Protein	60% by weight	Internal virion proteins encoded by *gag* Virion enzymatic functions encoded by *pol* Envelope proteins encoded by *env*
Lipid	35% by weight	derived from cell membrane
Carbohydrate	3% by weight	associated with *env* proteins
(3) Physical Properties		
Density		in sucrose 1.16-1.18g/ml
Sensitive to		lipid solvents, detergents and heat (56°C, 30 min)
Resistant to		UV and X-irradiation

Abbreviations: ss, single stranded; tRNA, transfer RNA molecule; R, repeat; RNA, ribonucleic acid; UV, ultra-violet; min, minutes.

Adapted from Weiss *et al* (1).

fully activated to produce pathogenic virus. Alternatively they can remain silent. Exogenous and endogenous oncoviruses can recombine *in vivo* and this process can generate viruses with increased pathogenicity. Furthermore viruses with remarkable neoplastic properties can be generated by recombination between viral and cellular DNA sequences (3).

1.1.2 Lentivirinae.

Slow, persistent infections are characteristic of Lentiviruses. They cause chronic, progressive often fatal diseases of their hosts. They are responsible for a number of important diseases in several species of animals. To establish a persistent infection these viruses must evade destruction by the host immune response. Lentiviruses appear to have established a number of special strategies enabling them to achieve this. One of the most important methods appears to be antigenic variation which is a common feature of lentiviruses (3, 10). Furthermore if conditions required for a productive infection are not available lentiviruses have the ability to set up latent or slow persistent infections in the host (46). This appears to require viral regulatory factors which are expressed from small ORFs found in the lentiviral genomes. In the absence of these factors the levels of viral gene expression can be greatly reduced. Under more favourable cellular conditions both cellular and viral factors can act to increase transcription and it is considered that this may cause a switch from latent to lytic infection *in vivo* (47, 48). However the exact nature of this process is still poorly understood.

1.1.3 Spumavirinae.

Spumaviruses have been isolated from a number of species. They can establish persistent infections but have so far proved to be apathogenic. They are often found as contaminants of primary cell cultures where they can

Figure 1.2 Retroviral Classification.

A Old Classification

Virus Group	Type		Prototype	Other examples
Oncoviruses	A		Intracisternal particles	
	B		MMTV	
	C	1)	RSV	ALV
		2)	MoMLV	FeLV, MuLV, BaEV
		3)	HTLV-I	HTLV-II, BLV
	D		SRV	SPA
Lentiviruses			VV	HIV, SIV, FIV, BIV CAEV, EIAV
Spumaviruses			HFV	SFV, FeSFV, BSV

B New Classification

Genera	Species
Mammalian type B oncovirus group	MMTV
MLV-related viruses (Mammalian type C retrovirus group)	MoMLV
Type D retrovirus group	MPMV
Avian type C retrovirus group (ALV-related viruses)	ALV
Foamy virus group (Spumavirus)	HFV
HTLV-BLV group	HTLV-I
Lentivirus group	HIV

Listed above are the main retrovirus groups characterised by shared genetic and biological features. '**A**' indicates the previous retroviral classification scheme as outlined in (1, 3 and 16). '**B**' indicates the recent classification of retroviruses, adapted from (2, 385).

Abbreviations: MMTV, Mouse Mammary Tumour Virus; RSV, Rous Sarcoma Virus; ALV, Avian Leukosis Virus; MoMLV, Moloney Murine Leukaemia Virus; FeLV, Feline Leukaemia Virus; MuLV, Murine Leukaemia Virus; BaEV, Baboon Endogenous Virus; HTLV-I and II, Human T-cell Leukaemia Virus; BLV, Bovine Leukaemia Virus; SRV, Simian Acquired Immunodeficiency Syndrome (SAIDS) Retrovirus; SPA, Sheep Pulmonary Adenomatosis; HIV, Human Immunodeficiency Virus; VV, Visna Virus; SIV, Simian Immunodeficiency Virus; FIV Feline Immunodeficiency Virus; BIV, Bovine Immunodeficiency Virus; CAEV, Caprine Arthritis-Encephalitis Virus; EIAV, Equine Infectious Anaemia Virus; HFV, Human Foamy Virus; SFV, Simian Foamy Virus; FeSFV Feline Syncytium Forming Virus; BSV, Bovine Syncytial Virus; MPMV, Mason-Pfizer Monkey Virus.

cause a pronounced and characteristic cytopathic effect (CPE) (3).

1.2 Lentivirinae.

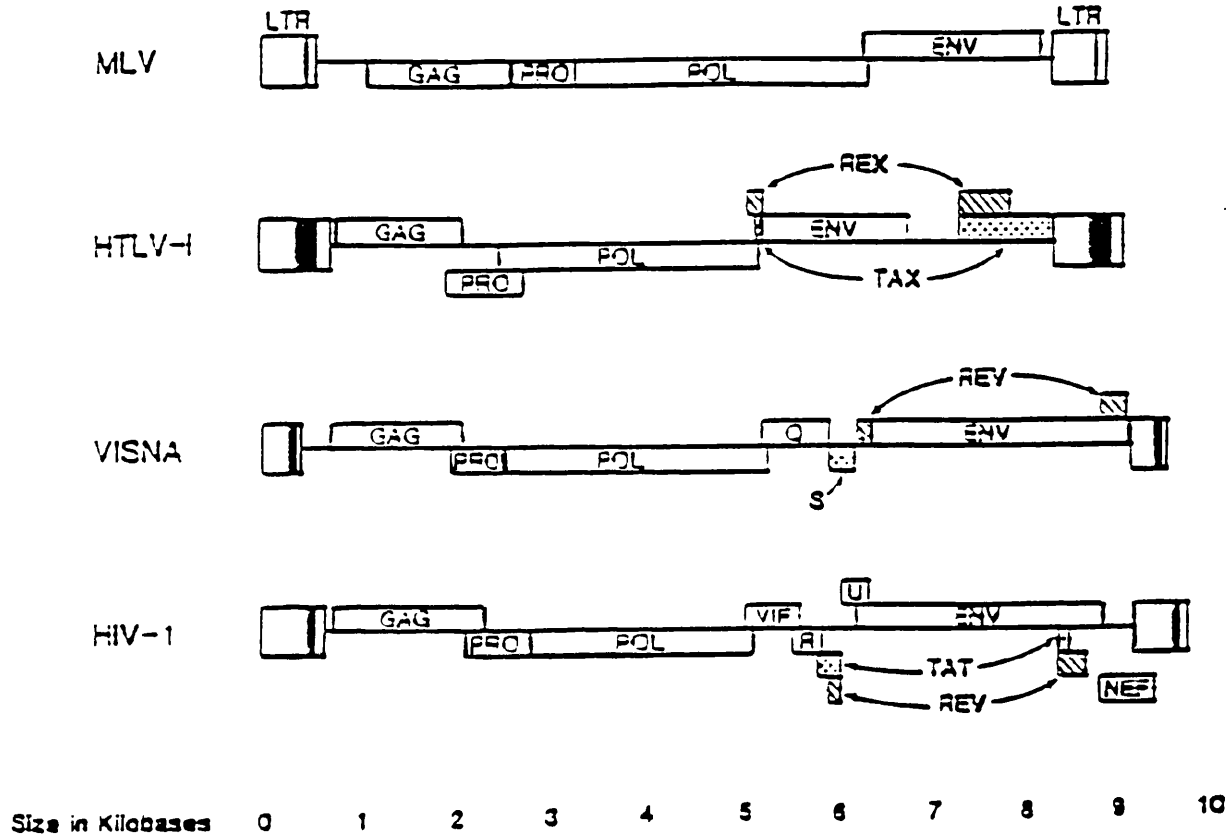
1.2.1 Morphology.

Extracellular, mature, lentivirus virions are spherical particles 100-150nm in diameter with a cone-shaped electron-dense nucleoprotein core, wrapped in an envelope that is acquired by budding from the cell surface. Viral surface projections are clearly seen on immature and/or budding particles but these spikes are progressively lost during maturation and are rarely seen in older cultures (4).

1.2.2 Genomic organisation.

Lentiviral genomes are diploid and contain 2 identical RNA subunits of around 9kb in length. Each genomic subunit is polyadenylated, capped and has a positive polarity, similar to that of eukaryotic mRNA. In its simplest form retroviral gene replication involves only three distinct virus encoded genes. These are the *gag* gene, which encodes the virion structural proteins, the *pol* gene which encodes the virion associated enzymes and *env* which encodes the envelope glycoproteins. In genomic RNA and in the integrated DNA provirus, these 3 genes are invariably arranged in the same order (5'-*gag-pol-env*-3'). In addition to the standard retroviral genes the lentiviruses have unique small open reading frames (ORFs) located between *pol* and *env* and at the 3' terminus. Once the provirus has integrated into the cellular DNA these genes are flanked by the characteristic long terminal repeats (LTRs) generated during the process of reverse transcription. The LTRs contain enhancer/promoter regulatory elements required for efficient transcription of the retroviral genome.

Figure 1.3 Retroviral Genomic Organisation.



Shown above is a comparison of the proviral genomic organisation between a representative simple retrovirus, MLV, with that seen in HIV-1 and other complex retroviruses. All known viral genes are named and drawn to scale, known transcriptional activator genes are marked by stippling and known post-transcriptional regulatory genes are indicated by hatching. LTRs are indicated by large terminal boxes with the 'R' region in black. Abbreviations: R, Vpr; U, Vpu; MLV, murine leukaemia virus; HTLV-1, human T-cell leukaemia virus; HIV-1, human immunodeficiency virus. Adapted from (6, 8).

Studies on lentiviruses continue to reveal increasingly complex transcription patterns. Most is known about the Human Immunodeficiency Virus Type 1 (HIV-1), the aetiological agent of acquired immunodeficiency syndrome. As well as the prototypic retroviral genes, the HIV-1 genome encodes at least six additional gene products (48, 49 and Figure 1.3).

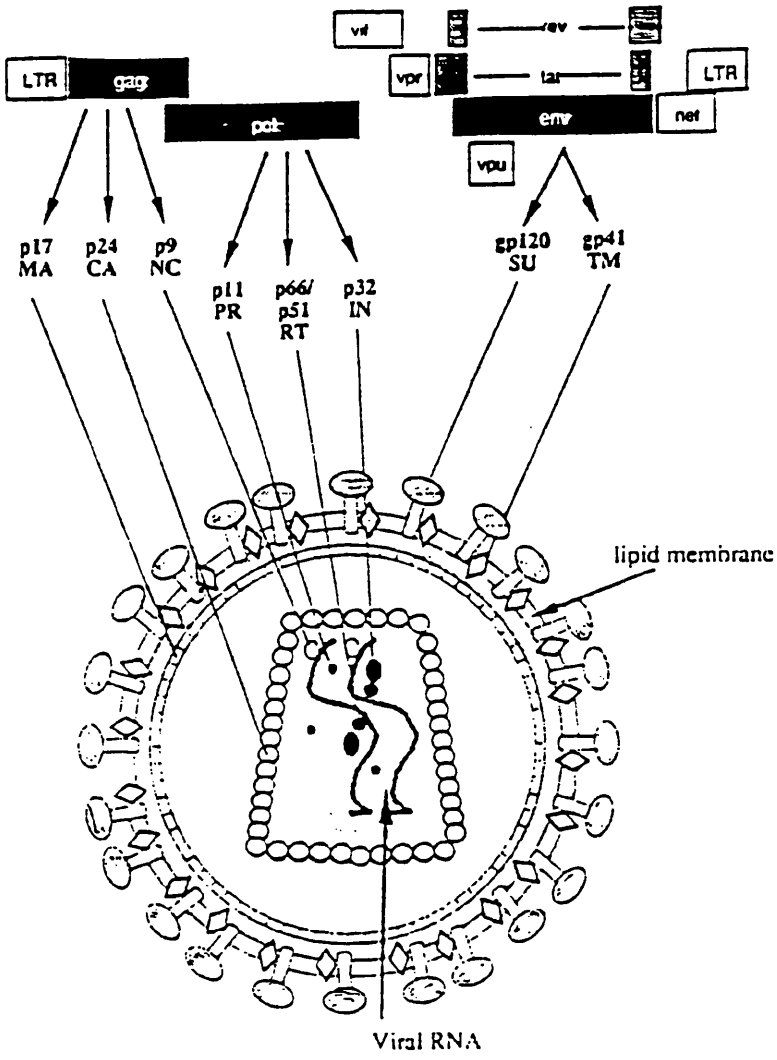
1.2.2.1 HIV-1 genomic structure.

HIV-1 has been extensively studied and as a result is one of the best characterised retroviruses (Figure 1.4, 16). This will serve as the standard example in the following review, but it is becoming increasingly apparent that all lentiviruses have properties in common (3).

1.2.2.1.1 The *Gag-pol* region.

The *gag-pol* region of the HIV-1 genome is transcribed as a single mRNA molecule. This encodes several proteins beginning with a myristylated product of 17kDa, known as the matrix protein (MA), which is found mainly in the matrix between the nucleocapsid and the viral envelope. This is followed by the region which encodes the 24kDa CA (capsid protein), which is the major structural component of the viral capsid, and the NC (nucleocapsid) which is a small protein of 9kDa with strong affinity for nucleic acid. This protein has been implicated in RNA packaging and dimer formation (50). An aspartate protease (PR) of ~11kDa is encoded entirely by the *pol* frame. This protein is responsible for cleaving the primary translation products of the *gag-pol* region into components found in mature HIV-1 virions. The following region encodes RT (reverse transcriptase) which has a RNA or DNA directed DNA polymerase activity and an RNase H function. This protein is 66kDa and is responsible for the synthesis of viral DNA. The final product encoded by this region is IN (integrase), a protein of 32kDa that

Figure 1.4 Structure of Human Immunodeficiency Virus.



The above diagram shows the position in HIV-1 virions of the protein products expressed by the HIV-1 genome. Like other retroviruses HIV-1 has two copies of 35S single stranded genomic RNA with the polarity of mRNA. The genomic RNA supposedly exists in the form of a ribonucleoprotein complex containing reverse transcriptase (p66), endonuclease (p32) and retroviral RNA-binding protein (p9). The viral core is composed of p24 as a major capsid protein. The myristylated Gag protein (p17) forms the outer shell and its amino terminal end is inserted into the lipid membrane envelope derived from the infected cell membrane. The extracellular (gp120) and transmembrane (gp41) glycoproteins exist in the form of a noncovalent complex on the envelope. See text (1.2.2.1) for a discussion of the possible functions of *tat*, *rev*, *vpu*, *vpr* and *vif*. Adapted from (9).

has been implicated in the integration mechanism by genetic and biochemical experiments (51).

1.2.2.1.2 The *env* region.

The other major domain, encoding viral structural proteins, in the HIV genome is the *env* gene. This gene is translated via a spliced subgenomic mRNA. The primary product of *env* is processed by multiple N-linked glycosylations and cleaved to yield two proteins both of which are found in the virus particle. The first is SU (surface protein), a large 120kDa, highly glycosylated protein on the surface of the virion. This protein interacts with host receptors that mediate virus entry and is the major target for neutralising antibodies. In HIV this protein is linked by non-covalent interactions to a 41kDa transmembrane protein (TM). As well as a transmembrane portion, TM has a cytoplasmic carboxy-terminal tail and is thought to mediate fusion of viral and host membranes during virus entry (16).

1.2.2.1.3 Additional open reading frames.

The additional ORFs found within the HIV genome encode a number of regulatory genes. The *tat* and *rev* genes of HIV are expressed early in infection and are essential for HIV-1 replication *in vitro* (5, 49).

1.2.2.1.3.1 *Tat.*

The *tat* gene of HIV-1 is essential for viral replication in culture (6, 30, 113, 114) and is known to consist of two coding exons that together encode a protein of 86 amino acids (As reviewed in 6). Analysis of expressing cells has shown that Tat is localised within the cell nucleus where it is further

concentrated in the nucleoli (6, 52). The mechanism of action of Tat is discussed in a later section (1.5.2). Genes encoding viral *trans*-activators have been found in other retroviruses, namely HTLV, EIAV, SIV, Visna and BIV (25, 53-56).

1.2.2.1.3.2 *Rev.*

Considerable evidence now exists to show that the viral *rev* gene product is responsible for the shift, during HIV replication, from spliced mRNA production to the predominant expression of incompletely spliced viral transcripts (47, 49). *Rev* has been found to be essential for a productive HIV infection as *rev*-defective viruses cannot progress to the late structural phase of viral gene expression (57). Rev is a phosphorylated protein of 116 amino acids which is localised to the nucleolus of infected cells (6) and it is considered to function by binding to a Rev response element (RRE) located within the *env* gene (58). A Rev like function has been found in visna, HTLV, SIV, EIAV, BIV and FIV (59, 60, 67, 273, 320, 372-375) and overall these data suggest that a gene product functionally equivalent to HIV-1 Rev will be a general characteristic of lentiviruses.

1.2.2.1.3.3 *Nef.*

HIV-1 Nef is a 27kDa, myristylated protein that is associated with the cytoplasmic membrane structures in expressing cells. Nef is not essential for *in vitro* replication and the role of HIV-1 Nef *in vivo* remains unclear. Studies using SIV_{mac239} have established that there are strong selective forces for open functional forms of SIV *nef in vivo*, although deletion of *nef* had no detectable effect *in vitro* (74). Furthermore the fact that the *nef* ORF is reasonably conserved between all primate lentiviruses suggests that this protein is likely to play a significant role in the viral life cycle within the

infected host (49). Recent reports have begun to suggest that *in vivo* HIV-1 Nef may down-regulate CD4 expression on human T-cells and this in turn may facilitate the efficient release of infectious progeny virions and hence viral spread *in vivo* (376-378).

1.2.2.1.3.4 *Vpr.*

The 96 amino acid *vpr* gene product is also dispensable for HIV-1 replication in culture. Vpr is detectable in the virion itself as a structural protein and it is thought that *in vivo* Vpr increases the rate of viral replication (6, 61). Experiments have suggested that HIV-1 Vpr activates transcription of several cellular and viral promoters (379) and may influence cellular differentiation (380) however the relevance of these activities to HIV-1 replication in vivo remains unclear.

1.2.2.1.3.5 *Vif.*

The *vif* ORF encodes a 23kDa protein which is required for efficient transmission of cell free virus in culture (62, 63). It is postulated that Vif may play a role in the enhancement of viral infectivity (341) and/or in the late stages of virion maturation and morphogenesis (342). Vif was found to be essential for the spread of HIV-1 in a number of cell types (381) and it was found that Vif was required at the stage of viral particle formation for cell to cell as well as cell free transmission of HIV-1 (341, 381) Furthermore Vif mutants of HIV-1 were severely impaired in their ability to complete the synthesis of proviral DNA which suggests that Vif may play a role in the processing and/or the transport of the internalised HIV core (381). It is interesting to note that this protein is well conserved among the primate lentiviruses and also appears to be retained in some ungulate lentiviruses (49) and in FIV (73). Although the molecular basis for the activity of Vif is as

yet unknown its wide distribution in lentiviral genomes may be an indication of its biological importance (73).

1.2.2.1.3.6 *Vpu.*

In contrast Vpu has no known equivalent in other viral systems. The translated gene product is a 16kDa transmembrane phosphoprotein. It has been proposed that Vpu enhances the maturation and release of infectious HIV-1 virion particles (6, 49, 72). Several reports have indicated that Vpu may function by disrupting CD4-gp160 complexes in the endoplasmic reticulum of infected cells which in turn allows increased processing of the viral gp160 and a decrease in the stability of intracellular CD4 (382-384). However conclusive evidence of the mechanism of action of Vpu has not yet been established .

1.2.2.1.4 *Cis*-acting control regions.

All retroviral genomes contain share *cis*-acting control elements, clustered mainly near the RNA termini, that are required for the virus life cycle (1).

1.2.2.1.4.1 The R region.

A terminal repeat of varying length, called R, is found at the 5' and 3' ends of the viral RNA. The R region is necessary for DNA strand transfer during reverse transcription and this region of the genome contains the cap site and the signal sequences for the polyadenylation and processing of viral RNA transcripts (1, 16).

1.2.2.1.4.2 Primer binding site.

Two short sequences are required for the priming of DNA synthesis. At the 5' end the primer binding site (PBS) binds the complementary tRNA primer and a polypurine tract near the 3' end serves as the plus strand primer (1, 16).

1.2.2.1.4.3 Long terminal repeat (LTR).

Two longer sequences between the PBS and R at the 5' end (U5) and between the polypurine tract and R at the 3' end (U3) are duplicated during the synthesis of viral DNA. These unique regions are copied to produce long terminal repeats (LTR), which are found at both ends of the DNA provirus. The LTRs have the structure 5'-U3-R-U5-3' and contain signals for the synthesis and processing of viral RNA. (1, 16, 17).

1.2.2.1.4.4 *Att* site.

Sequences at the 3' end of U5 and at the 5' end of U3 include short inverted repeats (IR) which vary in length between the different retroviruses. During the process of integration the viral DNA is joined to the host DNA at positions 2 nucleotides from these ends. The regions required for this integration are often referred to as the retroviral *att* sites (16).

1.2.2.1.4.5 Packaging signals.

Packaging signals usually called Psi (ψ) are the *cis*-acting sequences that are required for the packaging of viral RNA into the retroviral virion. Analysis of MuLV mutants have localised the viral packaging signals primarily to the 5' end of the viral genome (343) however these signals seem to vary from

virus to virus (347-348). The packaging signal for HIV-1 seems to be far more complex and is not yet fully understood (241-245, 350, 351).

1.2.2.1.4.6 TAR.

All HIV-1 RNA transcripts contain a region extending from +1 to +60 which can form a stable stem loop structure which is critical for activation by *tat* (119, 120, 122, 253, 254, 281-285) (1.5.2). Also, several studies have shown that the proviral DNA that corresponds to TAR can bind a number of cellular proteins (207-209, 253, 286) and mutation of these sites in TAR DNA results in a modest decrease in HIV-1 gene expression. Therefore extensive studies are currently underway in order to identify how these overlapping regulatory motifs in TAR DNA and RNA relate to the expression of HIV-1 *in vivo* (21).

1.2.2.1.4.7 *Rev* response element (RRE).

HIV-1 Rev is a regulatory protein that has a pivotal role in determining the shift from expression of regulatory (early) gene products to virion structural (late) proteins (See section 1.2.2.1.3.2 for more detail), (272). Rev mutants of HIV-1 are incapable of progressing to the synthesis of viral structural proteins (77, 344). It is known that Rev mediates its effect by binding to an RNA higher-order structural element designated the RRE (273-275). In all of the examined lentiviruses the RRE is located near the SU-TM junction of the *env* ORF (59, 273, 276-280). However a recent study has shown that the FIV RRE is in contrast found at the 3' end of the *env* ORF which reflects the position of *rev* in FIV. It is speculated (67) that the location of these elements in FIV may indicate the need for a certain spatial arrangement between the RRE and the splice acceptor site for *rev* exon 2 which is required for coordination of viral gene expression (67). However further work is required

before this interaction is fully understood. At the structural level all the RREs share a common feature: a long closing stem composed of several perfect helical elements interrupted by loops. The long stem structure may expose the active sites for intermolecular interactions or act as a scaffolding on which these reactions can occur (67).

1.2.3 Viral life cycle.

1.2.3.1 Replication.

The lentiviral replication cycle commences with the entry of extracellular particles into susceptible host cells. This process requires the attachment of virus to a host-encoded cell surface receptor and the reduction of the extracellular enveloped particle to a subviral intracytoplasmic nucleoprotein complex probably via receptor mediated endocytosis (352-355). Following this step the virion RNA is copied to double-stranded DNA by the virion associated reverse transcriptase. Therefore following infection DNA appears first, probably in the cytoplasm, and rapidly moves to the nucleus, where some of it circularises (47), however the relevance of this latter phenomenon to productive infection is not yet clear.

The exact kinetics of provirus integration in HIV-1 infected cells have not been established but it appears that the fraction of the DNA which integrates depends upon several factors including cell type, cell activation state and the availability of nucleotide pools (354, 356). Analysis of a one step growth cycle for HIV in human $CD4^+$ lymphocytes (H9) (47) showed that specific HIV-1 RNA appears 12 to 16 hours post infection and increases thereafter. However whether the viral DNA must integrate to provide a transcription template is not known. It is argued (47) that integration could be the limiting factor to the efficiency with which a virus can initiate a productive infection because within 8 hours after HIV-1 infection, sufficient linear DNA was

present to support transcription in every cell, if that form of the template was the active one (47). However it must be noted that in order for a provirus to integrate the host cell must undergo division therefore since HIV-1 can productively infect fully differentiated cells such as monocytes and macrophages, which no longer divide, integration cannot be an essential step in the viral life cycle in all cell types (357, 358).

Copy numbers of unintegrated viral DNA in infected cells vary among retroviruses. Even the same virus can show variation depending on the host cell. For example spleen necrosis virus accumulates 200 copies/cell in chicken cells early after infection whereas only 2 copies are made in rat kidney cells, and these figures are unaffected by variations in the multiplicity of infection (66). On average 20 copies of the HIV-1 provirus are produced in H9 cells (47) but more HIV DNA is made in other cell types (75). It is postulated that the build up of retroviral DNA may result from multiple rounds of superinfection in cells where there is not sufficient synthesis of viral glycoprotein to block all of the receptor molecules on the cell surface and consequently very high numbers of reverse transcripts can accumulate (47, 64-66). This is in contrast to cells where a superinfection barrier has been established and in this case linear DNA content is seen to fall presumably due to degradation of unintegrated material and low level production of new viral DNA (47, 64).

1.2.3.2 Transcription.

Before the recent growth of retrovirology the best examples of differential gene expression in viruses involved DNA viruses such as simian virus 40 (SV40), polyomavirus, adenovirus and herpesvirus (76). For these viruses certain groups of genes are expressed early after infection and others are expressed late. This differential gene expression is controlled mainly through transcriptional initiation at stage specific promoters. Several reports have

indicated that there are early and late transcriptional phases in HIV infection (5, 8).

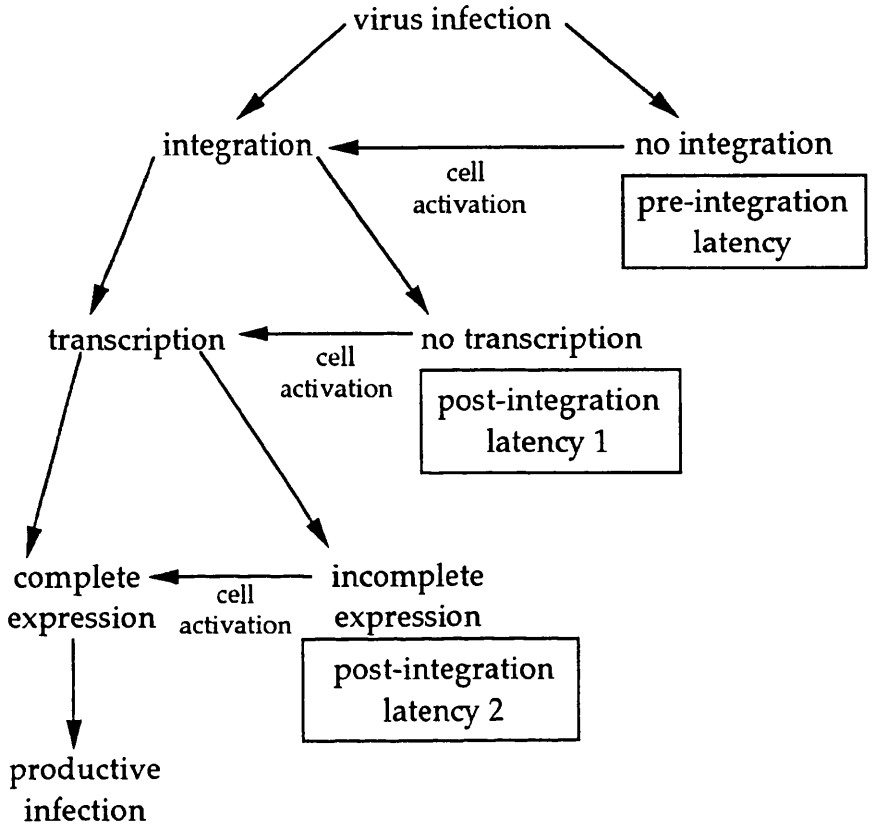
In HIV infection early mRNA transcripts are predominantly the smaller (2kb) spliced species that code for the low molecular weight regulatory proteins (Tat, Rev) and the 4.3kb spliced RNA which codes for the envelope proteins and possibly other proteins such as Vif and Vpr. Because HIV transcripts are derived from one major promoter in the LTR understanding the regulation of gene expression in HIV requires a different perspective than that outlined above for the DNA viruses. It has been shown that HIV-1 Rev mutants accumulate small transcripts and can not progress to late-structural gene expression, whereas in the presence of the Rev protein cytoplasmic accumulation of the 9.2kb RNA over spliced products is seen and this indicates that this regulatory gene, *rev*, plays an essential role in determining differential expression in HIV (57, 77-80).

This sequential transcription pattern has now been shown in other retroviral systems; spliced mRNA species selectively appear early in infection in visna virus (46) and one of these early mRNAs appears to transactivate viral transcription (81).

1.2.3.3 Latency.

Understanding viral latency is crucial to interpreting the pathogenesis of lentiviral infections. The life cycle of retroviruses can be separated into pre and post integration stages (Figure 1.5). In the case of HIV-1, integration of the DNA provirus into the host cell genome depends on the activated state of the host cell (68) and this integration step must occur before a productive virus infection can proceed (68, 82, 354). Thus although HIV-1 retains the capacity to bind and infect quiescent thymocytes *in vitro* (68, 82, 85, 86) inefficient reverse transcription (82) and/or a block to integration of full length HIV-1 DNA (68) restricts the viral life cycle, in these cells, to

Figure 1.5 Potential Stages of Retroviral Latency in T-cells.



The above diagram shows the alternative pathways which may be a taken by a retrovirus (eg HIV-1) following infection of T-cells (68, 82). See text (1.2.3.3) for further discussion.

preintegration stages. In quiescent T-cells, *in vivo*, there appears to be a reservoir of unintegrated extrachromosomal HIV-1 DNA (87). Because T-cell activation is a prerequisite for viral DNA integration it becomes apparent that the contribution of factors affecting T-cell activation such as mitogenic agents or opportunistic infections may in turn affect the reactivation of extrachromosomal HIV-1 DNA forms (68). However the exact stage in the HIV-1 replication cycle where this integration block occurs and the mechanisms involved in such a process are as yet unknown.

The pattern of HIV DNA, RNA and protein synthesis in human T-cells during a one step growth cycle *in vitro* provides some alternative insights into how latency may occur (47). In this case synthesis of HIV-1 DNA occurred rapidly after infection however, in contrast, HIV RNA synthesis showed a complex temporal pattern of expression (47). In particular there was a significant delay in the production of the full length 9.2kb mRNA and this delay may be accounted for by a combined effect of viral and cellular factors. Because the assembly of infectious virus would require the expression of 9.2 and 4.3kb *gag-pol* and *env* mRNAs, the early transcriptional phase, when the 2kb mRNA predominates could represent the *in vitro* equivalent of a latent stage (47). This hypothesis suggests that a controlled shift from the early to the late transcriptional phase may represent the transition from latent to acute infection *in vivo* (47). Whatever the mechanism(s) of latency the slow progress of clinical symptoms characteristic of HIV infection are not due to HIV being an intrinsically slow growing retrovirus but is due instead to the complex nature of viral gene regulation and virus-host interactions (5, 8, 47, 87).

It is also known that HIV-1 can replicate in non-dividing cells (359-361) and it is thought that this may be due to nuclear entry of the preintegration complex before mitosis (360, 361). Recent evidence suggests that the determinants which govern nuclear import of HIV-1 DNA are contained within the virus core (gag) (358) and this may explain the distribution of

HIV-1 in non-T-cell compartments of the infected host such as terminally differentiated and non-proliferating cells such as macrophages and microglial cells (362-365).

Despite an intense effort to understand the molecular mechanisms involved in the control of retroviral growth the exact functions of many regulatory events still remain to be clarified. Furthermore since most analyses have been done by using heterologous systems or sub-genomic fragments of viral DNA in transient assays or during a steady-state viral infection we must be wary of over interpreting these results. Care has to be taken so that we do not draw the wrong conclusions about the *in vivo* replication of these viruses from the *in vitro* data that is generated. It is clear that much more work is required before we learn the full details of the lentiviral life cycle.

1.3 Eukaryotic gene expression.

Since retroviruses rely on the host cell transcription machinery to effect their gene expression, a full understanding of their replication requires a knowledge of the host factors involved in this process and their modes of action.

1.3.1 RNA Polymerase II transcription.

Initiation of transcription of eukaryotic mRNA is a complex process requiring an interplay between regulatory DNA sequences, site-specific DNA binding proteins and protein-protein interactions. The enzyme RNA polymerase II (Pol II) as well as a number of other auxiliary DNA binding proteins known as transcription factors constitutes the basic transcriptional apparatus that is needed to initiate transcription at a specific sequence of DNA defined as the promoter. Mutational analysis has shown that promoters are modular arrays of protein binding sites which can determine

the rate and position of transcriptional initiation (69).

From functional analysis of a wide variety of promoter elements and attempts to reconstitute transcription *in vitro*, it is clear that there are general factors required for the transcription of all Pol II genes, and sequence specific factors that are required for the optimum transcription of only a subset of these genes. A basal level of transcription can be achieved when the general factors bind to core promoter elements (70).

The proximal elements of most RNA pol II promoters contain a TATA box, which is usually located 20-30bp upstream from the transcription start site. This sequence plays a role in determining the exact site of initiation since the TATA box acts in a position and orientation-dependent manner (13). Factors which bind to the TATA box have been identified. They are thought to form a pre-initiation complex which then binds Pol II to give an active transcription complex (90).

DNA sequence elements located proximal and distal to the core promoter elements can dramatically enhance or repress transcription. These *cis*-acting control elements are DNA binding sites for sequence-specific transcription factors. Complex protein-protein interactions are clearly involved in transcriptional control and much recent work has been aimed at elucidating these intricate processes (as reviewed by 7, 88-91).

1.3.2 Enhancers and silencers.

Distal elements which are known to increase the expression of various genes or impose tissue specific expression on adjacent promoters, are known as enhancers. Enhancers act independently of orientation and in some instances can affect gene expression at distances of up to several thousand base pairs (92-94). It has been proposed that enhancers bind activators which interact with some component of the transcriptional machinery, bringing it to the DNA or changing its conformation on the DNA in such a way that levels of

gene expression are controlled according to the stage of cell division and differentiation (89, 92-94).

Much of the regulation of gene expression occurs at the level of transcription with a hierarchy of coarse and fine controls that together determine the transcriptional activity of each gene. Early work on eukaryotic transcriptional regulation concentrated principally on enhancers but recently there have been a number of reports on negative regulatory elements. There are several mechanisms which are thought to be involved in negative control of gene expression (15). Extensive work on chromosomal rearrangements in Drosophila (96) led to the identification in both plants and animals (97) of higher order chromatin structures which must be interrupted for transcription to occur and this appears to be brought about, at least in part, by modifications of the histone proteins (95, 98). Several modes of action have been identified for proteins that down-regulate transcription. These include: competition for transactivator binding sites (99, 100) and/or competition for protein dimer formation (101-103); sequestering of transactivator proteins (104, 105) and masking of transactivator activation domains (106, 107). Furthermore, silencer elements which work, like enhancer elements, in a position and orientation-independent manner have been found and multimeric arrays of these elements have been shown to intensify the negative effect which they exert. (108, 109).

1.3.3 Transcription factors: structural features.

Eukaryotic genes are regulated differentially in response to a complex set of environmental and developmental stimuli. It is clear that these distinct transcriptional regulatory patterns of individual genes are determined primarily by the specific protein-DNA interactions occurring at the promoters (91-94).

Structural studies and sequence comparisons allow DNA-binding proteins to

be grouped into classes that use related structural motifs for DNA recognition (7, 110). Some families of DNA binding proteins were first recognised due to structural similarities between the protein whereas other families were first identified by amino acid sequence comparisons. Several comprehensive reviews of the large well established families which include the helix-turn-helix (HTH) proteins, the homeodomains, zinc finger proteins, the steroid receptors, leucine zipper proteins and the helix-loop-helix proteins have been published (7, 12, 71, 90, 110, 112). Two smaller families have been identified that use β-sheets for DNA binding (111). Sequence comparisons indicate that there are a number of additional families of DNA binding proteins, but less structural data are available for the families that have been characterised most recently (110).

To date several distinct structural motifs including helix-turn-helix (H-T-H) and zinc fingers have been proposed for the DNA binding domains of eukaryotic regulatory proteins (12, 71). Furthermore structural motifs such as the leucine zipper have been found in several proteins; including the yeast GCN4 transcriptional activator, the fos, jun, and myc oncoproteins and the enhancer binding protein (C/EBP) (83). It is thought that the 'zipper' region provides the structural basis for dimer formation but it is likely that other sequence motifs outside this region are involved in DNA interactions (83). Within each structural motif there are often families of related proteins that recognise similar DNA sequences and these motifs are conserved throughout the eukaryotic kingdom. However the processes of transcriptional activation and repression appear not to be related in a simple fashion to the class of DNA binding protein (12, 71).

1.4 Control of lentiviral gene expression.

Once integrated into the host chromosome, the provirus serves as both a repository of viral genetic information which can be replicated along with

cellular DNA and as a template for the synthesis of progeny viral RNA. In the 5' long terminal repeat (LTR) the U3 region lies upstream of the initiation site for viral RNA transcription. Sequences within this region can have a major influence upon the transcription of viral genes, even though the RNA Pol II and cofactors are supplied by the host cell (16).

The central role in viral replication of this host-dependent transcription step and the broad spectrum of host cell-specific transcription patterns means that many biological properties of retroviruses will be determined by sequences within the LTR (17).

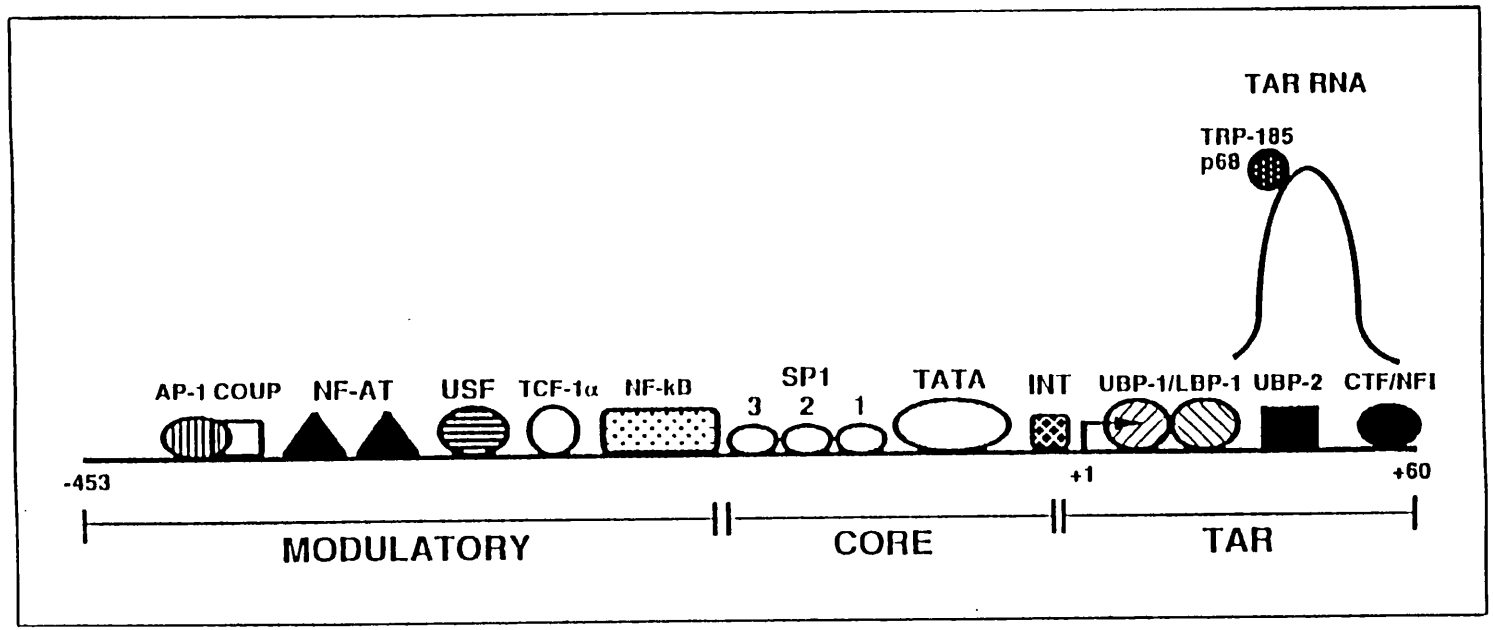
The LTR elements of all lentiviruses so far discovered contain canonical TATA promoter sequences and usually a CCAAT-like motif (17).

Transcriptional regulatory sequences in the LTR of certain C-type retroviruses have been shown to be potent determinants of their pathogenicity (18, 19). However despite striking differences in the regulation of viral gene expression between C-type oncoviruses and lentiviruses, it is likely that transcriptional signals also play an important role in regulating viral replication and hence pathogenesis by immunosuppressive lentiviruses (20).

1.4.1 Control of HIV-1 gene expression.

The HIV-1 LTR contains several *cis*-acting regulatory elements which have been shown to be important in transcription and post-transcriptional regulation of viral gene expression. Although there is some strain variation in precise composition the general features of the HIV LTR include a *tat* responsive region (TAR), a TATA box, an upstream enhancer region (which includes 2 NF-κB sites), 3 Sp1 binding sites and a 5' negative regulatory element (Figure 1.6). For a comprehensive review of the factors that control HIV gene expression see (21).

Figure 1.6 Organisation of the HIV-1 long terminal repeat (LTR).



The above diagram shows a schematic of the HIV-1 LTR, indicating potential sites of interaction for DNA and RNA binding proteins. These include binding sites for the chicken ovalbumin upstream promoter (COUP) binding protein, activator protein 1 (AP-1), nuclear factor of activated T-cells (NF-AT), upstream stimulatory factor (USF), T-cell factor-1α (TCF-1α), nuclear factor (NF-κB), SP-1, TATA, initiator, untranslated binding protein 1 (UBP-1) or leader binding protein 1 (LBP-1), UBP-2 and CTF/NF-1. The LTR can be divided into 3 major regulatory regions (modulatory, core and tar) based on the overall effects of each region on HIV-1 gene expression. RNA produced from the TAR element forms as stable stem-loop structure as shown. This region is critical for *tat* activation and both the *tat* protein and cellular factors such as TRP-185 and p68 bind to the TAR RNA. The sequence is numbered with respect to the cap site. For further discussion see text (1.4.1 and 1.5) Adapted from (21).

1.4.2 Control of visna virus gene expression.

Cells of the monocyte/macrophage lineage are targets for visna virus infection *in vivo*. Gene expression of visna virus is highly restricted in monocytes, but is activated when monocytes differentiate into macrophages. Macrophage activation induces gene expression directed by the visna virus LTR (22). Furthermore studies in transgenic mice carrying the visna virus LTR indicate that this element is at least partly responsible for the cell and tissue tropism of the virus (23).

The visna virus LTR has been found to contain several *cis*-acting regulatory elements important for basal levels of transcription and inducible expression. These include a TATA box and a 43bp tandem repeat which includes an enhancer domain with a single AP-4 binding site and several sequences that are similar to the recognition site for a cellular transcription factor, AP-1 (24). It appears that both of these binding sites are important for regulating the basal level of visna virus transcription (24). Furthermore the AP-1 binding site immediately proximal to the TATA box has been shown to be a target for viral *trans*-activation (24). These binding motifs have been found in a similar array in the LTR of the closely related CAEV where they are thought to play a similar role (24).

1.5 *Trans*-activation.

In addition to the cellular factors that are involved in viral gene expression several virally encoded *trans*-acting proteins which also regulate viral gene expression have been identified.

1.5.1 *Trans*-activation of HTLV.

Early experiments in which the LTRs of HTLV-I and II were linked to

reporter genes showed that these elements were much more active in virus infected cells than in uninfected cells (25).

The phenomenon which required viral protein expression in *trans* was termed *trans*-activation. A 40kDa protein (Tax) was found to be expressed from the 3' end of the genome and was shown to be responsible for this *trans*-activation effect. Further studies have shown that a 21bp sequence present in 3 tandem copies in the U3 region of the HTLV-I LTR is a major target for regulation by Tax. This 21bp sequence acts like an enhancer element but the manner in which the Tax protein interacts with the DNA has not yet been defined. Tax itself does not appear to bind to the DNA sequence (26). This protein is essential for viral replication and can also augment the transcription of other viral promoters and certain cellular genes (27).

1.5.2 *Trans*-activation of HIV-1.

In contrast to viral transactivator proteins (eg, HTLV-I Tax and adenovirus E-1A) whose activity is relatively promiscuous (28, 29), activation by the Tat protein is HIV-1 specific. Both the functional expression of the viral *tat* gene product and an intact copy of the *cis*-acting target sequence for Tat, designated the *trans*-activation response element (TAR) are essential for HIV-1 replication *in vitro* (30, 113, 114). HIV-1 Tat can have a 100 to a 1000 fold effect upon the expression of viral genes or genes into which the Tat response element (TAR) has been incorporated (31, 115).

Extensive mutagenesis of the 86 amino acid Tat protein indicates that it possesses at least three functional domains (116-118). These include a highly conserved cysteine rich domain which may function by binding metal ions and may mediate protein-protein interactions *in vivo*, a C-terminal basic domain which is critical for nuclear localisation and RNA binding and a core domain which has been proposed to function as the activation domain of Tat (6, 116-118).

TAR is a 59 nucleotide stem-loop structure located at the 5' end of all HIV-1 transcripts (114, 119-121) (Figure 1.6) and both the localisation and orientation are critical for TAR function (114, 119, 122). Mutational analysis of TAR led to the hypothesis that the double stranded segments present in TAR serve primarily a structural role in the appropriate presentation of essential primary sequence information located in and immediately adjacent to the terminal loop and 3 nucleotide bulge of TAR (6). The use of chimeric proteins consisting of Tat fused to heterologous RNA binding domains provided direct evidence that TAR functions at the RNA level (123, 124). Following fusion to these alternative RNA-binding domains the novel sequence specificity conferred on Tat permitted transcriptional *trans*-activation of the HIV-1 LTR via the appropriate target sequence (123, 124) indicating that the only essential role for TAR is to act as the RNA target sequence for Tat (6).

Tat binding assays and extensive mutational analysis of TAR indicates that *in vitro* Tat has the ability to specifically bind TAR (as reviewed in 6) and this interaction is mediated by the basic region of Tat (125, 126). However it appears that cellular factor(s) are also involved in mediating the Tat-TAR interaction *in vivo* (127). Therefore the exact nature of this protein and an analysis of its normal role in the regulation of cellular gene expression is required before a complete understanding of Tat function *in vivo* is known (6).

In many cases Tat has been shown to increase the steady-state level of transcripts expressed from genes linked to the HIV-1 LTR (128, 129), and this may be due to enhancement of transcription rather than stabilisation of mRNAs containing TAR (52). However, the mechanism by which Tat enhances the rate of transcription has been more controversial. One idea is that Tat acts to increase the rate of transcription initiation, suggesting that TAR acts as the RNA equivalent of a DNA enhancer sequence (131-133). An alternative hypothesis was originally derived from the observation that Tat

had little effect on the level of RNA polymerase density adjacent to the site of transcription initiation but could dramatically increase the rate of transcription of sequences distal to the HIV-1 LTR (135). Consequently, it has been proposed that Tat may function by preventing premature termination of transcripts initiated within the HIV-1 LTR (131, 134-136). Therefore it appears that transcription from the HIV-1 LTR is similar to a phenomenon previously observed in some cellular proto-oncogenes (137) in that transcription can terminate prior to complete synthesis of functional mRNA species.

Studies have now confirmed that Tat can function, at least in part, by promoting transcription elongation both *in vivo* and *in vitro* (138-140). Evidence seems to indicate that Tat acts by increasing processivity rather than by preventing a specific termination event since, in the absence of Tat, transcription termination of transcripts occurs at multiple possibly random sites (141).

Since evidence has also been presented suggesting that Tat can act to significantly increase the level of transcription initiation (131, 133, 139) it appears possible that the increased processivity of transcription complexes formed in the presence of Tat may be correlated with an increased ability to assemble a functional transcription complex at the HIV-1 LTR promoter (131). Therefore it remains to be fully resolved whether or not these two phenomena are indeed functionally linked (131, 133, 139).

Finally a number of reports have shown that Tat can increase protein expression from genes linked to the viral LTR, and this can be a significantly greater effect than that observed at the level of steady-state mRNA (57, 129, 142, 143). The molecular basis for this post transcriptional action of Tat remains unclear however it has been suggested that Tat could alter the cytoplasmic compartmentalisation of TAR containing transcripts which in turn could influence the cytoplasmic fate of TAR containing mRNA (144, 145).

Therefore Tat may have a direct effect on transcription, an antitermination function or a post-transcriptional effect upon RNA abundance, indicating that Tat functions, together with cellular factors, at multiple steps in transcriptional activation (21).

1.6 Feline Immunodeficiency Virus.

Feline Immunodeficiency Virus (FIV) is one of the most recently identified lentiviruses. It was isolated in 1986 from a multi-cat household where animals were dying with chronic opportunistic infections which in retrospect were almost certainly due to immunodeficiency (32). Further studies distinguished FIV from the other major pathogenic feline retrovirus feline leukaemia virus (FeLV) which often shows a similar disease profile (33).

FIV is associated with a severe immunodeficiency syndrome in its natural host, the domestic cat. It was initially classified as a lentivirus on the basis of virion morphology and its possession of a magnesium dependent reverse transcriptase activity. Like the human and simian lentiviruses, FIV was found to be T-lymphotropic (33). However it appears that the FIV receptor is not the feline homologue of the HIV-1 receptor, human CD4 (292). Studies are presently underway to try to define the cellular receptor for FIV (292).

Sero-epidemiologic studies have shown that FIV infection occurs throughout the world, however the prevalence varies from country to country. Surveys have shown that in the UK, France and the USA 10 to 19% of sick cats and 1 to 6% of healthy cats are FIV sero-positive (34-38). In Japan the incidence of infection is much higher (39) but in the Netherlands, Germany and Switzerland infection is rare (35, 40). However, it should be noted that these figures may not represent a true epidemiological profile of virus distribution due to the differences in the sample populations that were tested, i.e. 'street cats' in Tokyo versus 'apartment cats' in Zurich. On the whole, male cats were found to be more commonly infected than female cats and transmission

may occur primarily by biting (34).

The first infectious molecular clone of FIV was isolated in 1989 (41) and the sequence data confirmed the inclusion of FIV in the lentiviral family. While FIV has no close relative there is a significant degree of genetic relatedness between FIV and other lentiviruses especially in the *gag-pol* region and common antigenic determinants are shared by the major core protein of FIV, CAEV and visna virus (41, 42). Furthermore in common with two other non-primate lentiviruses (visna and EIAV) FIV carries an additional stretch of coding sequence between the reverse transcriptase and integrase domains of *pol* (42, 43, 198). Further studies have shown that this region encodes a gene product that possesses dUTPase activity and that it is packaged into virions implying that it performs a role in the early stages of the virus life cycle (366). FIV has a genome length of 9.4kb, and the genomic organisation is similar to that of other lentiviruses, especially visna virus. In addition to the structural genes common to all retroviruses (42, 43, 199) FIV contains at least three short open reading frames (ORFs) (Figure 6.6 and 6.7) *vif* (73), ORF-2 (or ORF-A) (67, 368) and *rev* (67, 367). However, the cell tropism and pathogenesis of FIV infection are more similar to those of HIV and SIV (43, 44).

Although FIV is in itself a virus of important veterinary significance its discovery presented an excellent opportunity, due to the similarity in the dynamics of and the response to infection, for the development of a small animal model for AIDS (84). It is hoped that this animal model will help in the development of safe and effective strategies for the prevention and therapy of HIV infection in humans (84).

1.7 Aims of this study.

This project details part of a study which is underway to characterise the molecular structure and function of the UK isolates of FIV.

Since the lentiviruses share a common replication strategy, whereby virus replication is limited to cellular activation and differentiation (45), and yet they show little sequence relatedness between families, it is of interest to compare and contrast the virus and host regulatory mechanisms involved. This study on feline immunodeficiency virus and its regulation was initiated with this general aim.

CHAPTER 2

Chapter 2 Materials and Methods.

2.1 Materials.

Reagents and equipment used routinely are listed below. Details of specific purchases are given in the appropriate section.

2.1.1 Chemicals.

All were of Analar quality and were obtained from Sigma Chemical Company, BDH chemicals, Boehringer Corporation, GIBCO BRL or Pharmacia Ltd., unless stated otherwise.

2.1.2 Radiochemicals.

All were from Amersham UK Ltd., except [γ-^{32}P] ATP, which was from ICN Flow Biomedicals.

2.1.3 Enzymes.

All were supplied with appropriate buffers from BRL.

RNase A and T1 (Sigma) were dissolved in 10mM Tris.HCl pH7.5, 15mM NaCl in ddH_2O as a 10mg/ml stock. Solutions were then heat treated at 100^{o}C for 15 minutes and allowed to cool slowly to room temperature, solutions were stored at -20^{o}C.

2.1.4 Cloning vectors.

pIC19R/20H: *lacZ*$^+$/*amp*R$^+$ pBr322-derived plasmid vector with multiple cloning sites (146).

pCAT12: *amp*R$^+$ 0.9kb fragment containing the transposon-9 (TN-9) chloramphenicol acetyl-transferase (CAT) gene (147) from pLW4 (148) cloned into pUC12 (149, 150).

pLW2: *amp*R$^+$ pUC based plasmid (150) containing the herpes simplex virus (HSV) immediate early (IE) gene promoter and terminator sequences on either side of the TN-9 structural CAT gene (147, 148).

pVisna-LTRCAT: *amp*R$^+$ was a gift from Dr. G. Harkiss, Edinburgh University. It is based on the pMSG-CAT (Pharmacia) backbone from which the mouse mammary tumour virus (MMTV) LTR was removed and replaced with the visna virus LTR.

pFIV-34TF10: *amp*R$^+$ full length infectious molecular clone of FIV. This was isolated from a λEMBL-4 library of genomic DNA from CRFK cells chronically infected with FIV-Petaluma strain, and sub-cloned into the plasmid vector pUC119 (Gift from Dr. J. Elder, Scripps Clinic, La Jolla, California) (43).

pFIV-PPR: *amp*R$^+$ full length infectious molecular clone of FIV. This was isolated from a λEMBL-4 genomic DNA library prepared with DNA isolated from peripheral blood leucocyte of a cat from the San Diego area, USA, and sub-cloned into the plasmid vector pUC119 (Gift from Dr. J. Elder, Scripps Clinic, La Jolla, California) (43, 198).

pHFBG2: *amp*R$^+$ plasmid containing the HSV-1 thymidine kinase (TK) promoter upstream of the β-galactosidase (β-gal) structural gene. The polyadenylation signal is derived from Simian Virus 40 (SV40) (151).

pGEX: *lac*I^q/*amp*R$^+$ pBr322-derived plasmid in which insert DNA is fused to Glutathione S-transferase (GST) (Pharmacia) (152).

pBabe: *amp*R$^+$ /*puromycin*R$^+$ retroviral expression vector based on the Moloney murine leukaemia virus (MoMuLV) (153).

pCMV-IE: *amp*R$^+$ contains the human cytomegalovirus (HCMV) immediate early (IE) promoter and terminator regions in pUC7 (150). A gift from Dr. A. Akrigg, Porton Down.

pVisna-LTR*tat*: *amp*R$^+$ was a gift from Dr. J. E. Clements, John Hopkins University, Baltimore, Maryland. It contains the visna virus *tat* gene under the control of the visna virus LTR as described in (24).

RC-P1: was obtained from Dr. R. Jarrett, Glasgow University. This plasmid expresses the wild-type HIV-1 *tat* gene under the control of the HIV-1 LTR (154).

pHIV-LTR-CAT: was kindly provided by Dr. M. Harris, Glasgow University. The HIV-1 LTR was amplified from the plasmid RC-P1 by PCR and cloned into the plasmid pCAT12 (see above), orientation was confirmed by DNA sequencing.

2.1.5 Cell lines.

Normal feline T-cells: were isolated from peripheral blood by Dr. M. Hosie and Mr. T. Dunsford, Glasgow University. Cells could be maintained in culture, supplemented with recombinant IL-2, for several months.

Normal cat thymocytes: were kindly provided by Dr. M. Hosie and Mr. T. Dunsford, Glasgow University. The cells could be maintained in culture containing recombinant IL-2 for several months.

F422: This cell line was established from a thymic lymphosarcoma of a kitten inoculated with the second passage of the Rickard strain of FeLV (155).

Crandell feline kidney cells (CRFK): were derived from the renal cortical cells of a domestic kitten (14, 156). A clone of CRFK, designated ID10 (kindly provided by P. Andersen, Agritech Systems Inc., Portland, ME), had been shown to support the growth of the Petaluma strain of FIV (41).

AH927: This fibroblast cell line was derived from a feline embryo culture which underwent spontaneous transformation (157).

HeLa: These are human epithelial cells which were derived from a cervical carcinoma (158).

Jurkat: This is a human T-cell line which was cultured from a child with acute lymphoblastic leukaemia (159).

Vero: This cell line was kindly provided by Dr. J. E. Clements, John Hopkins University, Baltimore, Maryland. Vero cells were initially cultured from the kidney of an adult, African green monkey (160). The clone C4 was transfected

with pSV2-*neo* and the clone B9 was transfected with the pSV2-*neo*.pVLΔSx (161) which contains the visna virus *tat* gene under the control of the visna virus LTR (162).

3201: are suspension cells derived from a FeLV-negative feline lymphoma (163).

T3, T17, FL74: are FeLV-positive lymphoid tumour cell lines established from naturally occurring thymic lymphosarcomas (164, 165, 166).

2.1.6 Bacterial strains.

E.coli. DH5α: F^-, ϕ80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *thi*-1, *deo*R, *gyr*A96, *rel*A1, Δ(*lac*ZYA-*arg*F), U169, λ^- (167). (Supplied as competent cells by Life Technologies Inc., GIBCO BRL).

E.coli. DS941: *strep*R^+, AB 1157, *rec*F143, *lac*ZΔM15, *lac*I^q. Obtained from Prof. D. Sherratt. Institute of Genetics, Glasgow University.

2.1.7 Stock solutions and growth media.

Ammonium persulphate: 10% and 25% W:V stock, stored in aliquots at -20°C .

Ampicillin (500X): 25mg/ml in ddH_2O. Filtered through 0.22μm filter, aliquoted and stored at -20°C.

Bradford's (1X): 0.1mg/ml Coomassie Blue G, 1:20 V:V 95% EtOH, 1:10 V:V Orthophosphoric acid in ddH_2O. Stored in dark glass at room temperature.

Denaturation Buffer: 1.5M NaCl, 0.5M NaOH in ddH_2O. Stored at room temperature.

Denhardt's Solution (50X): 1% Bovine Serum Albumin, (BSA). 1% Ficoll, 1% polyvinyl pyrollidone in ddH_2O. Aliquoted and stored at -20°C.

DNA Size Markers: Phi(ϕ)X174 RF DNA digested with *Hae* III and Lambda (λ) DNA digested with *Hind* III. Both used at 1μg/10μl. Supplied by NBL.

Ethidium Bromide: 10mg/ml stock in ddH_2O, working concentration 0.5μg/ml. Stored protected from light at room temperature.

Gel Loading Buffer:

1) For protein gels (2X): 0.0625M Tris.HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue, pH6.8, in ddH_2O. Stored at room temperature.

2) For DNA gels (10X): 50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol, in 1X TBE. Stored at room temperature.

HBS 2X: 280mM NaCl, 50mM HEPES (N-2-hydroxyethyl-piperazine-N'-2'-ethanesulphonic acid), 1.5mM Na_2HPO_4, 1.5mM NaH_2PO_4, pH7.12 with NaOH. Filtered (0.22μm), stored in aliquots at -20°C, but once thawed stored at 4°C.

L-Broth: 1% Bacto tryptone, 0.5% yeast extract (both from, Oxoid, Unipath Ltd., England), 0.5% NaCl, W:V in ddH_2O. Autoclaved and stored at 4°C.

L-Broth Agar: as for L-Broth + 1.5g/100ml bacto-agar (Oxoid). Autoclaved then allowed to cool (antibiotics were added at this stage, if required) poured with sterile technique on to plastic plates, allowed to set, dried by inversion in 37°C oven and stored, for short periods, at 4°C.

2-Mercaptoethanol (100X): 5×10^{-3} M stock in sterile PBS, stored aliquoted at -20°C.

Neutralisation Buffer: 1.5M NaCl, 0.5M Tris.HCl pH8 in ddH_2O. Stored at room temperature.

Polyacrylamide solution with urea (8%): 39.9g acrylamide, 2.1g bis-acrylamide, 220.5g urea, 52.5ml 10X TBE, in a total of 525 ml ddH_2O. Mixed, filtered (0.45μm) and stored in dark glass at 4°C. 50ml of stock solution containing 50μl 25% ammonium persulphate and 50μl temed (Biorad) was used for each sequencing gel using a 21 x 50cm 'Bio-Rad' gel kit.

Polyacrylamide (30%): 29% Acrylamide, 1% Bis-acrylamide, in ddH_2O. Mixed, filtered (0.45μm) and stored in dark glass at 4°C.

Phosphate Buffered Saline (PBS) : 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4 adjusted to pH7.3. Autoclaved and stored at 4°C.

Pre-hybridisation Buffer:

1) For nylon membrane (Amersham, Hybond-N) at high temperatures: 5X SSPE, 5X Denhardt's, 0.5% SDS.

2) For nylon membrane at low temperatures: 5X SSPE or SSC, 50% deionised formamide, 1X Denhardt's.

Both solutions were used fresh or stored in aliquots at -20°C. 100-300μg/ml denatured salmon sperm DNA was added before use.

Salmon sperm DNA: 10mg/ml stock in ddH_2O, denatured by boiling for 10 minutes, and slow cooling on ice. Aliquoted and stored at -20°C.

SSC (20X): 3M NaCl, 0.3M Sodium citrate. pH7.0 with NaOH in ddH_2O. Stored at room temperature.

SSPE 20X: 3.6M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA pH8.3 with NaOH in ddH_2O. Stored at room temperature.

SOC: 2% Bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl to ddH_2O. Autoclaved and stored at 4°C. Before use add, with sterile technique, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM Glucose.

TE: 1mM EDTA, 10mM Tris.HCl, (pH as required). Autoclaved and stored at room temperature.

TBE (10X): 0.9M Tris.HCl, 0.9M Boric acid, 25mM EDTA pH8.3. Stored at room temperature.

TMS: 0.25M sucrose, 10mM Tris.HCl pH 7.5, 5mM $MgCl_2$. Autoclaved and stored at 4°C.

Versene (1X): PBS containing 1mM EDTA and 1:100 V:V phenol red. Autoclaved and stored at room temperature.

X-Gal (5-bromo-4-chloro-3-indolyl-B-galactoside) (1000X): 20% W:V in dimethyl formamide (DMF). Stored in aliquots protected from the light at -20°C.

2.2 Methods.

2.2.1 Cell and virus production.

2.2.1.1 Maintenance and storage of eukaryotic cells.

Eukaryotic cells were grown in plastic flasks (Nunc, Lab Tek) at 37°C in an atmosphere of 5% CO_2 in air. Suspension cells were grown in RPMI-1640 and adherent cells in Dulbecco's Modified Eagle's Medium (DMEM), both were supplemented with 10% foetal calf serum (FCS), 2mM glutamine, 100units/ml penicillin and 10μg/ml streptomycin (all Gibco, UK). Suspension cell media (except Jurkat cultures) required the addition of 2×10^{-5}moles/ml of 2-mercaptoethanol (Sigma). This medium was supplemented with 100units/ml of recombinant human IL-2 (kindly provided by Jack Nunberg, Cetus Corp., Emeryville, California, USA) for peripheral blood T-cells and thymocytes.

To maintain the stability of the Vero cell lines, B9 and C4, DMEM medium containing 10% foetal calf serum (FCS), 2mM glutamine, 100units/ml penicillin and 10μg/ml streptomycin (all Gibco, UK) was supplemented with 800μg/ml gentamycin (G-418, GIBCO).

Suspension lines (primary T-cells, thymocytes, 3201, F422 and Jurkat) were subcultured every 3 to 4 days and maintained at densities between 5×10^5 and 1.5×10^6 cells/ml. Primary T-cells and thymocytes were stimulated in medium containing 7.5μg/ml Concanavalin A (Sigma). After three days the cells were cultured in medium without Con A (Dr M. Hosie personal communication).

Monolayer cells (HeLa, CRFK, Vero and AH927) were subcultured at a ratio of 1:10 twice weekly. The spent culture fluid was removed and the adherent cell monolayer washed with 1X versene. The monolayers were dispersed by rinsing with a solution of 0.25% trypsin (Gibco BRL) in versene. The cells were diluted with fresh culture medium, centrifuged at 1000rpm for 5 minutes, then

resuspended and replated at the appropriate density. For long term storage, cells were resuspended at approximately $3x10^6$cells/ml in foetal calf serum + 10% dimethyl sulphoxide (DMSO, Koch-Light Laboratories Ltd.), frozen slowly to -70°C and then placed in liquid nitrogen.

2.2.1.2 Virus isolation.

2ml cultures of T-cells were set up at a concentration of 2×10^6 cells/ml in normal T-cell media. Samples of blood plasma from cats, known to have signs of immunodeficiency and that were FeLV negative, (Dr. M. Hosie, personal communication, 34), were diluted 1:5 in media, filtered through a 0.45μm filter and incubated with the cells overnight. The following day the cells were washed and the concentration of viable cells was adjusted to 5×10^5 cells/ml. Cultures were maintained at this level, throughout the experiment, by the addition of fresh cells. The cultures were examined daily for the appearance of a cytopathic effect (CPE). FIV production was monitored by Western blot (kindly performed by Dr. M. Hosie as described in 168). When a positive culture was identified the cell suspension was centrifuged at 1000rpm for 5 minutes and the supernatant was filtered through a 0.45μm filter and frozen in small aliquots at -70°C. The viruses isolated in this manner were designated, FIV-Glasgow 2 (FIV-G2) and FIV-Glasgow 5 (FIV-G5).

2.2.1.3 Production of virus infected cells.

Several UK isolates of FIV have now been isolated. These were designated FIV-G1 (kindly provided by J. Callanan, Glasgow University), FIV-G8, FIV-G14 (kindly provided by Dr. M. Hosie) FIV-G2 and FIV-G5. All isolates were propagated in primary feline thymocytes. Uninfected thymocytes were incubated overnight with 25ml of infected cell culture supernatant at a concentration of 2×10^6 cells/ml. The following day the cells were washed and

the concentration adjusted to 5 x 10^5 cells/ml. The cultures were supplemented with fresh cells as required and monitored for the appearance of a cytopathic effect. The production of FIV p24 antigen in the culture supernatant was followed using the antibody-based enzyme-linked immunosorbent assay (ELISA) kit (FIV antigen detection kit, Idexx). All the tests were kindly performed by Dr. M. Hosie. Once a productive FIV infection was apparent, usually around five days post infection, cells were harvested and viral DNA was isolated.

2.2.2 Isolation, amplification and purification of DNA.

2.2.2.1 Viral DNA isolation.

FIV DNA was isolated from infected cells by the Hirt method (169). In short, 10^7 cells were resuspended in lysis buffer (10mM Tris.HCl pH 7.5, 10mM EDTA and 1% SDS) for 10 minutes at room temperature. 25% V:V 5M NaCl was then added and left overnight at 4°C. This preparation was then spun at 15,000rpm, for 30 minutes and the supernatant treated with proteinase K (300μg/ml) for 4 hours at 37°C, for the final 30 minutes RNase A was added (100μg/ml). DNA was then precipitated with 2 volumes of 95% EtOH. Finally DNA was resuspended in TE pH8, the OD $A_{260/280}$ was recorded and DNA was stored at -20°C.

2.2.2.2 Oligonucleotide synthesis.

Oligonucleotides were generated on a Applied Biosystems 3818A Automated DNA Synthesiser (operated by Mr. T. McPherson, Glasgow University). DNA was removed from the columns using 2ml of concentrated ammonia and deprotected at 55°C, overnight. The ammonia was removed under vacuum and the resulting DNA pellet was resuspended in TE pH8, the OD $A_{260/280}$ was

recorded and DNA was stored at -20°C. If required double stranded oligonucleotides were annealed in TM buffer (10X 100mM Tris.HCl pH8.3, 50mM $MgCl_2$). Equimolar amounts of single stranded, purified oligonucleotides were incubated in 1X TM buffer at 80°C for 10 minutes and then cooled slowly to 4°C.

2.2.2.3 DNA amplification by polymerase chain reaction (PCR).

Polymerase Chain Reaction (PCR) (170) was used to generate large amounts of DNA derived from specific regions of the viral genome. The Perkin Elmer Cetus GeneAmp DNA Amplification Reagent Kit (Supplied by USB, Cambridge Bioscience) which employs a recombinant *Thermus aquaticus* (*Taq*) DNA polymerase, (*AmpliTaq*), was used, according to the manufacturers' specifications. In order to minimise the possibility of contamination the kit components were pre-mixed in ddH_2O. The final 100µl reaction mixture contained 10mM Tris.HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% W:V gelatin, 200µM of each nucleotide, 2.5 units of enzyme and either 1µg infected cell DNA or 10ng plasmid DNA, together with the 1µg of each primer. Reactions were carried out in 0.5ml reaction tubes and overlayed with 50µl of mineral oil to prevent evaporation. Reactions were then cycled 30 times on a Techne PHC-1 thermocycler. Programme: denaturation 1 minute 92°C, annealing 1 minute 45°C, and elongation 3 minutes 72°C. In order to identify the PCR product 10% of each PCR reaction was separated on an agarose gel (2.2.2.4), after the gel was stained and photographed it was transferred to nitrocellulose (2.2.6.1) and probed with an end-labelled oligonucleotide (2.2.5.3) homologous to the internal sequence of the desired product. After a positive identification the remainder of the DNA products were purified on agarose gels (2.2.2.4).

2.2.2.4 Agarose gel electrophoresis.

Gels containing 1% agarose W:V in 1X TBE were used to separate and analyse DNA molecules (171). 100 or 200ml gels were poured in perspex tanks and wells were made using a 10, 20 or 30 tooth comb. Gel loading buffer (2.1.7) was added to the samples (final concentration 1X) which were then subjected to electrophoresis in 1X TBE at approximately 5V/cm. Gel concentrations of 0.8-1.5% were used for optimum separation of appropriate bands and low melting point (LMP) agarose was used for preparative runs. A known concentration of DNA size markers were run alongside the samples in order to gauge product size and to allow estimation of sample DNA concentration. Gels were stained for 10-20 minutes in 1X TBE containing 0.5μg/ml ethidium bromide, viewed on a shortwave UV transilluminator and photographed using Polaroid high speed, black and white film, Type 667.

2.2.2.5 Purification of DNA from agarose gels.

When sufficient separation between bands had been achieved the DNA band of interest was excised from the gel using a clean scalpel. This gel slice was subjected to three rounds of freeze/thawing and then spun through a 0.45μm cellulose acetate filter ('Spin-X' ;Co-star Ltd.). A 1/10th volume of 3M NaOAc and 2 volumes of 95% EtOH was added to precipitate the DNA (-20°C overnight or -70°C, 30 minutes). The pellet was collected by centrifugation washed with 70% EtOH and dried under vacuum. Finally the DNA was resuspended in TE pH8, OD $A_{260/280}$ was recorded and DNA was stored at -20°C.

2.2.2.6 Enzymatic manipulations of nucleic acids.

Restriction endonucleases, T4 polynucleotide kinase, calf intestinal alkaline

phosphatase (CIP) and ribonucleases A and T1 (2.1.3) were all used, with the buffers supplied, in accordance with the manufacturers' recommendations. However, restriction enzyme digests of crudely purified miniprep plasmid DNA (2.2.3.4.1) was performed with an excess of enzyme (5units/100ng DNA) plus RNases A and T1 at 0.5units/μl at 37°C for 1 hour.

2.2.3 Production of recombinant clones.

2.2.3.1 Ligation of DNA molecules.

Ligations were carried out according to the protocols supplied with the T4 DNA ligase and 5X reaction buffer (BRL Ltd.). Where possible 50ng of vector DNA and a 10-20 fold molar excess of insert DNA was used, in a minimal volume (10-20μl). The ligation of blunt-ended molecules was found to work best on overnight incubation at 14°C while a minimum of 2 hours at room temperature was sufficient for the ligation of cohesive-ended DNA molecules.

2.2.3.2 Bacterial transformation.

20μl of competent DH5α cells (2.1.6) was added to 2μl of DNA solution, (either the products of a ligation reaction, containing 5-10ng of vector DNA or 0.05ng of control DNA). This mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 45 seconds and then replaced on ice for 2 minutes. 90μl of SOC (2.1.7) was added and the cells were incubated at 37°C, with shaking, for 1 hour. 30μl and 80μl aliquots of the transformed cells were then spread on to L-agar plates containing ampicillin (50μg/ml) (2.1.7) and if colour selection was required X-gal (0.02%, 2.1.7) was included. These plates were incubated at 37°C for a minimum of 12 hours.

2.2.3.3 Identification of recombinants.

For clones obtained directly by recombination with pUC or pIC vectors (2.1.4), recombinant colonies were initially identified by virtue of their colourless phenotype in the presence of X-gal (171). For constructions involving vectors in which the *lac*Z function was already disabled, other methods were necessary to identify recombinants, such as small scale plasmid preparation (2.2.3.4.1) and restriction analysis (2.2.2.6).

2.2.3.4 Preparation of plasmid DNA.

2.2.3.4.1 Small scale prep.

This procedure is based on the alkaline lysis method for DNA purification (172). 1.5ml overnight cultures of bacteria were pelleted and resuspended in 100µl of lysis buffer (50mM Glucose, 25mM Tris.HCl, 10mM EDTA pH8, + 2mg/ml lysozyme, added just before use) for 5 minutes at room temperature. 200µl of freshly made 0.2M NaOH/1% SDS was added. The mixture was inverted 3 times and left on ice for 5 minutes, it was then neutralised by vortexing with 150µl of 0.4M potassium acetate pH5. After a further 5 minutes on ice the lysate was cleared by centrifugation and the DNA precipitated with 2 volumes of 100% EtOH. This procedure yielded up to 1.5µg of crude DNA which was resuspended in 20µl of ddH_2O.

In order to identify recombinants miniprep DNA was subjected to restriction analysis (2.2.2.6) and the digested products were run on agarose gels (2.2.2.4). Once a recombinant had been identified the remainder of the overnight culture, grown for use in method (2.2.3.4.1), was adjusted to 25% glycerol. These stocks were stored at -20°C for several months and/or -70°C for long term storage.

2.2.3.4.2 Large scale prep.

This procedure is based on a detergent lysis method for plasmid DNA purification (173). A stationary phase 500ml culture of bacteria was spun at 5,000rpm for 5 minutes. The pellet was resuspended in 5ml 25% sucrose/50mM Tris.HCl pH8. 1ml of 20mg/ml lysozyme in 0.25M Tris.HCl pH8 was added and left to stand on ice for 5 minutes. The mixture was transferred to a polycarbonate tube and 1ml of 0.5M EDTA pH8 was added. After 5 minutes on ice, 8ml of 0.2% Triton-X100, 50mM Tris.HCl pH8, 62.5mM EDTA was added, rapidly, and the mixture was left on ice for 20 minutes. This mixture was then spun at 20,000rpm for 30 minutes at 0°C. 1g/ml of CsCl and 50µl of ethidium bromide stock solution (2.1.7) was added to the supernatant. This solution was spun at 48,000rpm for 36 hours at 20°C. The lower band was removed with a syringe and extracted three times with an equal volume of water-saturated butanol (top layer was discarded each time). When all the ethidium bromide had been removed the remaining clear solution was diluted 1:3 with TE pH8. 1/10th volume of 3M NaOAc and 2 volumes 100% EtOH were added and left to precipitate at 4°C for 30 minutes. The DNA pellet was collected by centrifugation, washed with 70% EtOH, dried, resuspended and the OD was measured, as above (2.2.2.1). By this method between 2-5mg/l of highly purified recombinant plasmid was prepared.

2.2.4 DNA sequence analysis.

2.2.4.1 Double-stranded sequencing reactions.

Double-stranded sequencing reactions were carried out by the method modified from that of Sanger *et al* (174), which employs base-specific, chain-terminating inhibitors. Double-stranded sequencing reactions were carried out using the 'Sequenase Version 2 DNA Sequencing' kit containing the

bacteriophage T7 DNA polymerase which is completely devoid of the 3'-5' exonuclease activity found in the native, wild-type enzyme, (supplied by USB, Cambridge Bioscience) and [α^{35}S]-dATP (1000Ci/mmol, Amersham). Routinely 'Universal primer', which was supplied with the kit, was used (Figure 5.1). Any specialised sequencing primers were generated and purified as outlined in (2.2.2.2). These primers were adjusted to concentrations of 0.5-1.0pmol per µl using the formula:

[C = 100 x A/N] where C = pmol/µl, A = OD_{260} and N = No. of primer bases.

Templates were denatured and sequencing performed according to the manufacturers' instructions.
Samples were run on 8% denaturing polyacrylamide gels, using 'Bio-Rad' gel kits (21cm x 50cm), for 3 and 5 hours at 2000V/30mA, fixed for 10 minutes (10% methanol, 10% acetic acid in ddH_2O) dried and exposed to X-ray film (Hyperfilm, Amersham) This enabled approximately 300-400bps of sequence to be read from each primer.

2.2.4.2 Maxam-Gilbert reactions.

The Maxam-Gilbert DNA sequencing system (Du Pont International, NEN Products) was used to sequence specific end-labelled LTR fragments (2.2.5.2). The limited DNA cleavage reactions, (G) and (G+A) were performed as outlined in the manufacturers' instructions. This method was adapted from that of (175).

2.2.5 Radiolabelling of nucleic acids.

2.2.5.1 Labelling of double-stranded fragments.

The 'Random Prime' DNA Labelling Kit, marketed by Boehringer Mannheim (176, 177) was used to generate labelled restriction fragments or PCR products. The kit components were used in accordance with the manufacturers' recommendations together with 50μCi [α^{32}P]-dCTP (3000 Ci/mmol, Amersham). Labelled products were purified on Sephadex G-50 beads ('Nick Column', Pharmacia). Typically, incorporations of 10^8-10^9 cpm/μg were achieved.

2.2.5.2 End-labelling of double-stranded fragments with T4 kinase.

This method is based on that of Berkner and Folk (178, 179). 50μg of plasmid was digested in a total volume of 100μl, using restriction enzymes which generated 5' overhangs. Digestions were performed at 37°C for 2 hours after which 20 units of calf intestinal phosphatase (CIP, Boehringer Mannheim) was added for a further 30 minutes. Reactions were stopped by incubation at 68°C for 5 minutes, after the addition of 10μl 10% SDS, 5μl 2M NaCl and 5μl 0.2M EDTA. The products were extracted twice with an equal volume of phenol:chloroform (V:V, 1:1) and the DNA was precipitated at -70°C with 2.5 volumes EtOH and 2M ammonium acetate. The pellet was collected by centrifugation, washed with 70% EtOH and dried under vacuum. The pellet was resuspended in TE, and stored at -20°C as stock DNA (1μg/ul). 1μg of this DNA was labelled using 12pmol [γ^{32}P]-ATP (3000Ci/mmol, ICN-Flow Biomedicals), in 40μl 1X kinase buffer (0.5M Tris.HCl pH9.2, 0.1M $MgCl_2$), with 15 units of T4 polynucleotide kinase. This reaction was incubated at 37°C for 30-60 minutes and then extracted, EtOH precipitated and pelleted as above. The pellet was resuspended in 32μl ddH_2O and digested with an appropriate

endonuclease for 90 minutes. The products of digestion were resolved on a 4% acrylamide gel. The gel was exposed to X-ray film for 30 seconds in order to identify the position of the end-labelled fragment. This region of the gel was excised, fragmented and incubated overnight at 37°C in 500µl of TE. The DNA solution was removed from the acrylamide by filtration through a 0.45µm filter ('Spin-X' ;Co-star Ltd.) and then precipitated with EtOH. The resulting labelled DNA fragment typically incorporated 10^7-10^8cpm/µg and was suitable for DNase I footprinting and Maxam-Gilbert sequencing reactions.

2.2.5.3 End-labelling of oligonucleotides with T4 kinase.

1µg of purified oligonucleotide (2.2.2.2) was labelled using 12pmol [γ^{32}P]-ATP (3000Ci/mmol) in 20µl of 1X kinase buffer (0.5M Tris.HCl pH9.2, 0.1M $MgCl_2$, 171), with 15 units of T4 polynucleotide kinase. This reaction was incubated at 37°C for 30-60 minutes and then extracted, EtOH precipitated and pelleted as above (2.2.5.2). The resulting labelled oligonucleotide typically incorporated 10^7-10^8cpm/µg and was used to probe PCR products (2.2.2.3).

2.2.6 Hybridisation analysis of nucleic acids.

2.2.6.1 Southern blot.

Nucleic acids were transferred from agarose gels to Hybond-N (Amersham) membranes essentially by the method of Southern (180). Transfer was confirmed by restaining the gel with EtBr. After transfer the membranes were baked for 2 hours in an 80°C oven.

2.2.6.2 Hybridisation of probes to filter-bound nucleic acid.

Detection of immobilised DNA on filters was achieved by hybridisation to

'random primed' DNA probes (2.2.5.1) or end-labelled oligonucleotides (2.2.5.3). Filters were preincubated for >4 hours at 42°C in a sealed polythene bag, or a hybridisation tube (Hybaid), containing 0.1ml/cm^2 prehybridisation solution. The freshly prepared DNA probe was denatured by boiling for 10 minutes and then cooled slowly on ice. The probe (10^6 cpm/cm^2) was added to the bag/tube and the hybridisation mixture was incubated at 42°C overnight. Filters were rinsed in 2X SSC and then washed twice, for 30 minutes, in either low (eg: 2X SSC/50°C) or high (eg: 0.1X SSC, 0.5% SDS/65°C) stringency buffers. Filters were air dried and then autoradiographed.

2.2.7 Nuclear protein preparation.

Crude nuclear protein extracts were prepared essentially as described (181, 182). In short, nuclei were prepared and extracted with 0.35M NaCl. The eluate was precipitated with ammonium sulphate and then redissolved and dialysed against storage buffer (50mM NaCl, 20mM Hepes pH7.9, 5mM $MgCl_2$, 0.1mM EDTA, 1mM dithiothreitol and 20% glycerol). Typically nuclei containing 100 to 150mg of DNA yielded 5ml (5 to 10 mg of protein/ml) of crude nuclear extract. All buffers contained 0.5mM phenylmethyl-sulphonyl fluoride (PMSF), 0.5mM benzamidine, 2mM levamisole, 10mM β-phosphoglycerate 50μM sodium orthovanadate, 10mM sodium butyrate and 0.1μg/ml each of pepstatin A, aprotinin, leupeptin and bestatin (all from Sigma).

2.2.8 DNase I footprint protection assays.

These assays were performed essentially as published procedures (183). Briefly, assay mixtures at a final volume of 100μl contained 300μg of crude nuclear extract in storage buffer (2.2.7), 5ng of end labelled DNA restriction fragment (2.2.5.2) and 1μg of poly dI-dC with or without 100ng of double stranded competitor oligonucleotide DNA. After limited DNase I digestion, nucleic acids

were purified and analysed by denaturing polyacrylamide gel electrophoresis and autoradiography.

2.2.9 Transient transfection of eukaryotic cells.

2.2.9.1 Calcium phosphate transfection.

This method was adapted from those of (184, 185). Typically 2.5-3.0 X 10^5 cells in 5ml of complete DMEM medium were plated on 60mm plastic Nunc dishes and left overnight at 37°C in a moist atmosphere with 5% CO_2. The following day transfections were set up; 5μg test DNA, 20μg carrier DNA (salmon sperm), 250μl 2XHBS, 30μl 2M $CaCl_2$ to a total of 500μl ddH_2O, (all the components were pre-mixed, except the $CaCl_2$, which was added dropwise while air was bubbled through the mixture with a pipette). This mixture was left to stand at room temperature for 30 minutes and then added to the cell culture medium. After another overnight incubation the spent medium was removed, cells were washed with 2mls of PBS containing 3mM EGTA for 60 seconds and then rinsed with 2ml fresh media, finally 5ml of complete DMEM was added to the plates which were incubated for a further 24 hours at 37°C. After a further wash with 2ml of PBS, 1ml of TEN (0.04M Tris.HCl pH7.4, 1mM EDTA, 0.15M NaCl in ddH_2O) was added to the plates. After 5 minutes at room temperature the cells could easily be scraped off the plates. Cells were removed from the TEN by gentle centrifugation (1 minute, 6,000rpm) and the pellet was well drained before being resuspended in 100μl 0.25M Tris.HCl pH7.8. Cell extracts were cleared by centrifugation (5 minutes, 13,000rpm) after 3 rounds of freeze/thawing. Supernatants containing the cell extract were harvested and stored at -20°C.

2.2.9.2 Electroporation of eukaryotic cells.

The protocol followed was essentially as outlined in (186, 187). In short, between 0.5 X 10^6 and 1.2 X 10^7 cells, pelleted from a healthy growing culture, were resuspended in 800µl of PBS. DNA was added in a small volume, typically 50µg at 1µg/ul in TE was sufficient, plus 50µg of carrier DNA (salmon sperm). The mixture was left on ice for 10 minutes and then placed in a 0.4cm electroporation chamber (Bio-Rad). Parameters were set at 0.5kV/cm with capacitance at 960 µFarrads, on a Bio-Rad Gene Pulser. After the cells were pulsed they were incubated for a further 10 minutes on ice before being added to fresh, prewarmed, medium (10^6cells/ml) and incubated for 48 hours at 37°C. Cells were harvested by centrifugation, washed with PBS, and then resuspended in a small volume (100-200µl) of 0.25M Tris.HCl pH7.8. Cell extracts were prepared and stored as above (2.2.9.1).

2.2.9.3 DEAE-Dextran transfection.

This method was first described almost 25 years ago (188), and the protocol has remained essentially the same. However transfection efficiencies can be increased by additional treatments such as dimethyl sulphoxide (DMSO) shock (189-191). Dead cells were removed from a culture of suspension cells using a Ficoll-Hypaque (Pharmacia) gradient. Suspension cultures were gently layered on top of the Ficoll-Hypaque, and then spun at 2000rpm for 10 minutes. The live cells, which collected in a band at the interface, were removed and washed several times with PBS. Finally, cells were resuspended at 1-2 X 10^7 cells/ml in PBS, containing 200µg/ml diethylaminoethyl ether-dextran (DEAE-Dextran, Pharmacia) and 15µg/ml of DNA. The reactions were incubated at 37°C for 30-90 minutes, with gentle agitation every 10 minutes. DMSO was added to a final concentration of 10%, after a 2-3 minutes incubation at room temperature the DMSO was diluted out by the addition of 10ml of PBS. Cells were pelleted,

washed with PBS, resuspended in 20ml of complete RPMI and incubated for 48 hours at 37°C. Cells were harvested as (2.2.9.2) and cell extracts were made and stored as outlined in (2.2.9.1).

2.2.10 Analysis of transfected cell extracts.

2.2.10.1 β-galactosidase assay.

These assays were carried out in 1ml reaction vials. 40µl of cell extract, prepared from any of the transfection methods above, was added to 0.5ml of Solution 1 (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgCl_2$ and 50mM 2-mercaptoethanol in ddH_2O) and 0.1ml of Solution 2 (60mM Na_2HPO_4, 40mM NaH_2PO_4, 2mg/ml ONPG (O-nitrophenyl-B-D-galactosidase) in ddH_2O). These reactions were left at 37°C for 30-90 minutes or until a colour change was apparent (clear -> yellow). Reactions were stopped by the addition of 0.25ml of 1M Na_2CO_3. 200µl of the reactions were transferred to microtitre plates and the ODs were read at 420nm using a Biotech microplate reader, EL-312.

2.2.10.2 Estimation of protein concentrations.

The Bradford method (192) was used to determine the protein concentration of transfected cell extracts (2.2.9.1). 10-20µl of sample were incubated with 1ml of Bradford's solution at room temperature for 15 minutes. The ODs of the samples were read at 595nm. These readings were used to estimate protein concentrations using a standard curve of known amounts of protein (BSA).

2.2.10.3 DNA preparation from transfected cells.

Transfected cells were lysed overnight in 10mM Tris.HCl pH8, 10mM EDTA and 1% SDS containing 300µg/ml proteinase K at 37°C. This solution was

extracted twice with an equal volume of phenol:chloroform 1:1 and the DNA was precipitated with ethanol. This DNA preparation was then analysed by agarose gel electrophoresis and Southern blot hybridisation (2.2.2.4., 2.2.6.1. and 2.2.6.2.).

2.2.10.4 Chloramphenicol acetyltransferase (CAT) assay.

The standard assay (193) was performed by adding the transfected cell extract (25-50µl) to a microcentrifuge tube, containing 10µl 1M Tris.HCl pH8, 4mM n-acetyl coenzyme A (Sigma) and 5µl of [^{14}C] chloramphenicol (50-60mCi/mmol, Amersham) made up to a final volume of 90µl with ddH_2O. The reactions were incubated at 37^{o}C for 2 hours which was known to be within the linear range of the assay (results not shown 171, 193, 302). The reactions were stopped by the addition of 300µl of ethyl acetate and vortexing. The liquid phases were separated by centrifugation, the top organic phase was transferred to a fresh tube and dried under vacuum. The residue was resuspended in 20µl of ethyl acetate and spotted on to thin layer chromatography plates coated with silica gel (TLC, Camlab). The products were chromatographed in a tank containing chloroform:methanol 95:5, until the solvent front had reached the top. The plates were then removed, dried and autoradiographed. CAT activity was quantified by excising the spots from the TLC plates and calculating the relative counts in the acetylated and non-acetylated forms by liquid scintillation counting.

2.2.11 Detection of FIV infection.

2.2.11.1 Immunofluorescence.

FIV-infected or transfected cells were resuspended at 2 X 10^6 cells/ml in PBS. 25µl of this cell suspension was placed on a chamber slide (Lab Tek, Nunc) and

the cells allowed to attach at 37°C for 60 minutes. The chambers were washed with PBS after which the cells were fixed with acetone for 10 minutes at room temperature. Dry slides were incubated with a mixture of sera from FIV-infected cats diluted 1:5 in PBS (kindly provided by Dr. M. Hosie) for 2 hours. The serum was washed off with PBS and the cells were then incubated with affinity purified goat anti-cat immunoglobulin conjugated to FITC, (1:1000), (kindly provided by T. Dunsford) for 1 hour. After a final wash in PBS the cells were examined under UV illumination.

2.2.11.2 Western immunoblotting.

The Western immunoblot procedure was a modification of that previously described by (194, 195). 10^6 cells were washed out of culture fluid and resuspended in 40µl lysis buffer (100mM Tris.HCl pH7.4, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate and 1% aprotinin). The lysate was spun briefly to remove cell debris and SDS-PAGE sample buffer (2.1.7) was added to the supernatant. Proteins were resolved on 10-20% gradient gels using the Bio-Rad Minigel apparatus and transferred to nitrocellulose using a Bio-Rad Trans-blot system. Non-specific antibody binding sites were blocked overnight by incubation in 0.5% non-fat milk powder (NFMP) in PBS.

The membrane was then incubated with serum diluted 1:10 in PBS containing 4% NFMP, 0.5% Tween-20 and 1mM EDTA for 2 hours. The membrane was thoroughly washed in PBS containing 0.05% Tween-20 and then incubated with biotinylated protein A (Amersham) diluted 1:250 in PBS containing 4% NFMP and 0.5% Tween-20 for an hour. After further washing, the membrane was incubated for 1 hour with streptavadin-alkaline phosphatase (Bio-Rad) diluted 1:500 in PBS containing 4% NFMP and 0.5% Tween-20. Membranes were washed again and then developed using a substrate containing 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (both Sigma as described

in 168, 194, 195).

2.2.12 Prokaryotic expression vectors.

2.2.12.1 Construction of prokaryotic expression vectors.

The plasmid vector pGEX (2.1.4) was used to express recombinant FIV open reading frames. Vector and inserts were restricted (2.2.2.6) to produce compatible sticky ends and ligated as in (2.2.3.1).

Cultures of *E.coli* (DS 941, 2.1.6) cells were grown in L-broth containing streptomycin (25μg/ml) to an OD_{600} of 0.5-0.7. Cells were pelleted and resuspended in half their original culture volume with ice-cold 50mM $CaCl_2$. After 15 minutes on ice, cells were pelleted at 4°C, resuspended to one tenth their original culture volume with ice-cold 50mM $CaCl_2$ and stored at 4°C for 2-3 days.

200μl of competent *E.coli* (DS 941) cells were added to 25% of the ligation reaction containing the recombinant pGEX constructs. This mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 2 minutes and then incubated for 5 minutes at 37°C. 1ml of L-broth (2.1.7) was added and the cells were incubated at 37°C, with shaking, for 1 hour. Cells were gently spun out and resuspended in 100μl of fresh L-broth and plated on to L-agar plates containing ampicillin (100μg/ml) and streptomycin (25μg/ml). These plates were incubated at 37°C for a minimum of 12 hours (152).

2.2.12.2 Identification of positive recombinants.

Putative recombinant colonies were grown overnight in L-broth containing ampicillin (100μg/ml) and streptomycin (25μg/ml). 200μl of the overnight culture was diluted 1:10 with fresh L-broth containing ampicillin (100μg/ml) and streptomycin (25μg/ml) and grown, with shaking, for a further 2 hours.

Induction was achieved by the addition of isopropyl-β-D-thio-galactoside (IPTG) to a final concentration of 0.2mM. After a further 2 hours the cells were pelleted and resuspended in 200μl of sample buffer (2.1.7). Samples were boiled for 5 minutes and then separated on a 12% SDS-polyacrylamide gel along side Dalton Mark VII-L size markers (Sigma) and a sample prepared from a non-recombinant control culture (152).

CHAPTER 3

Chapter 3 Sequence Analysis of the FIV LTR. **Page**

Chapter 3 Sequence Analysis of the FIV LTR.

3.1 Introduction.

The lentiviral lifestyle is characterised by a regulatory cascade through which quiescent viral genomes can be rapidly activated to high levels of expression (142).

This process is mediated by a complex interplay between *trans*-acting cellular and viral factors and their *cis*-acting viral target sequences. The primary target for retroviral transcriptional control is the proviral long terminal repeat (LTR), which also carries signals for the correct processing of viral RNA transcripts, the faithful reverse transcription of the genome and the integration of proviral DNA into the host cell genome (196).

As such the LTRs are indispensable elements of the retroviral genome. However, despite their common functional roles LTRs from viruses outwith a close family often display little obvious sequence conservation. This is probably due to the nature of these elements, which are modular arrays of binding sites for factors which bind in a sequence dependent manner to short DNA target sequences (13, 21, 197).

Therefore, as an initial step towards understanding the regulation of FIV gene expression a detailed characterisation of the LTR of FIV was undertaken.

3.2 Methods.

3.2.1 Isolation of infected cell DNA.

To examine the extent of sequence diversity in natural isolates of FIV, five strains from distinct locations in the U. K. (FIV-G1, -G2, -G5, -G8, -G14) were selected (Figure 3.1). These viral strains were isolated (2.2.1.2) and propagated in feline T-cells (2.2.1.3). Typically, cells were harvested at five days post

Figure 3.1 Sources of FIV Isolates.

Isolate	Country of Origin	Region	Reference
Glasgow-1	Scotland	Glasgow	(34)*
Glasgow-2	Scotland	Perth	(34)
Glasgow-5	England	London	(34)
Glasgow-8	England	Portsmouth	(34)
Glasgow-14	Wales	Colwyn Bay	(34)
Petaluma (PET)	USA	Petaluma, CA.	(32,41,42)
34TF10	USA	Petaluma, CA.	(32,43)
PPR	USA	San Diego, CA.	(198)
TM-1	Japan	Tokyo	(199, 215)
Z-2	Switzerland	Zurich	(200)

Key

(*) Blood samples submitted to the University of Glasgow, Feline Virus Unit were used for a nationwide survey of FIV seroprevelance (34). Virus was isolated from positive samples as outlined in (2.2.1.2).

CA., California.

This figure shows the source and reference for each isolate of FIV detailed in this study.

infection and extrachromosomal viral DNA was purified by the method of Hirt (2.2.2.1, 169).

3.2.2 Generation of LTR fragments.

In the absence of molecular clones of the U.K. isolates of FIV it was decided to use PCR to generate a library of LTR sequences. PCR amplification of FeLV *env* regions had previously established some of the requirements for successful amplification from infected cell DNA (Dr. M. Rigby personal communication), circumventing the need for preliminary testing of reaction components and titration of template DNA. Primers based on the published nucleotide sequence of FIV-Petaluma (41) were generated and purified as above (2.2.2.2). Primers were made with the following sequences and are shown as asterisks (*) in Figure 3.3 and 3.8.

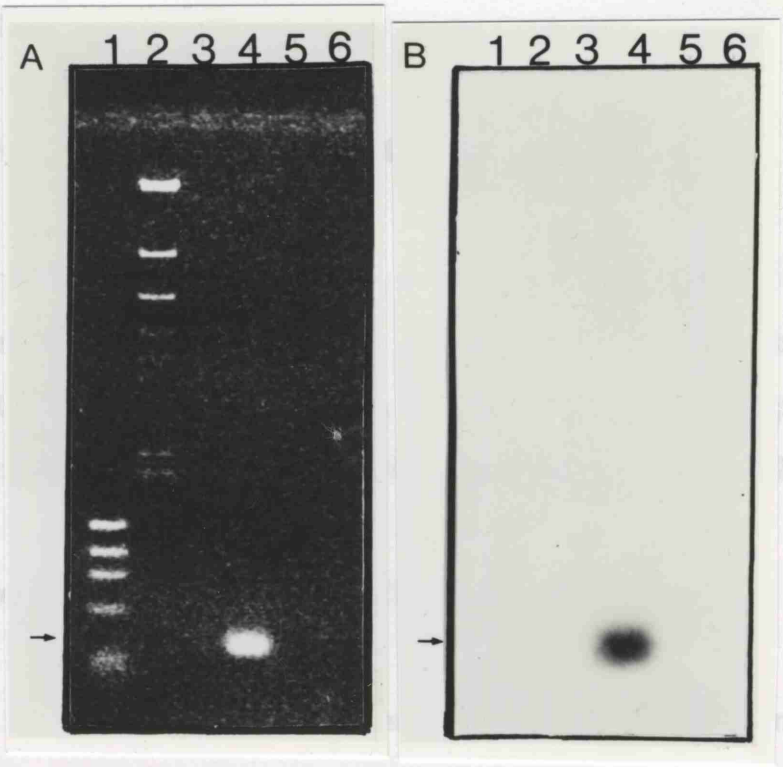
Primer 5	5'-TGGGATGAGTATTGGAACCCTGAAGA-3'
Primer 6	5'-TGCGAAGTTCTCGGCCCGGATTCC-3'

Primers 5 and 6 were used in PCR reactions with genomic DNAs from various infected cell preparations under standard conditions (2.2.2.3). 10% of each crude reaction product was analysed by agarose gel electrophoresis (2.2.2.4) and a typical gel is shown in Figure 3.2A. The product generated from infected cell DNA was of the expected size (~350bp). The same reaction performed with uninfected cell DNA showed no specific amplification and in the absence of added template no product was observed.

The close homology of the amplified products to the prototype LTR sequence was demonstrated by hybridisation analysis at high stringency (2.2.6). An oligonucleotide complimentary to internal LTR sequence was generated (Oligonucleotide 3: 5'-TAGCTGAGCATGACTCATAG-3') and end labelled for use as

Figure 3.2

Agarose Gel and Southern Blot Analysis of PCR Products.



A: Photograph of an ethidium bromide stained agarose gel containing;

lane 1 ΦX174 *Hae* III cut markers.
lane 2 λ *Hind* III cut markers.
lane 3 FIV infected cell DNA.
lane 4 FIV infected cell DNA + FIV specific primers 5 and 6 (3.2.2).
lane 5 primers 5+6.
lane 6 uninfected cell DNA + primers 5 and 6.

B: The above gel (A) was blotted onto nitrocellulose and probed with a ^{32}P labelled oligonucleotide complimentary to internal FIV LTR sequence (Oligonucleotide 3) see text (3.2.2) for details.

a probe (2.2.5.3). A typical blot is shown in Figure 3.2B. PCR reactions with infected cell DNA from all the available FIV strains generated a product which hybridised to the probe, even under stringent conditions (65°C 0.1X SSC, 0.5% SDS, 2.2.6.2).

3.2.3 Characterisation of amplified products.

Where positive amplification was detected the remainder of the PCR product was purified on an agarose gel. The products were extracted and ethanol precipitated as described (2.2.2.5). Estimation of yield was performed by gel electrophoresis against standard amounts of DNA markers (2.2.2.4).

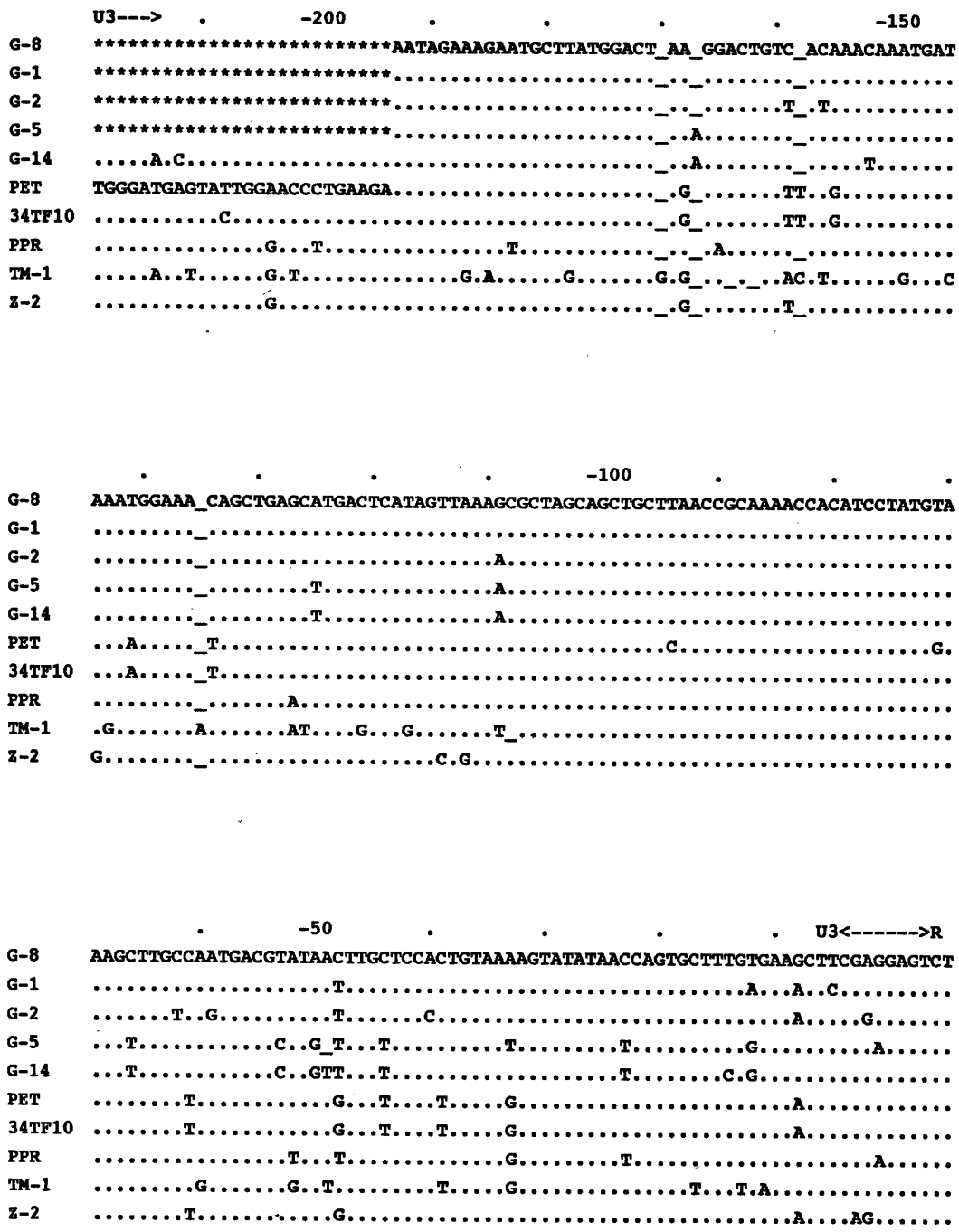
3.2.4 Production of recombinant LTR clones.

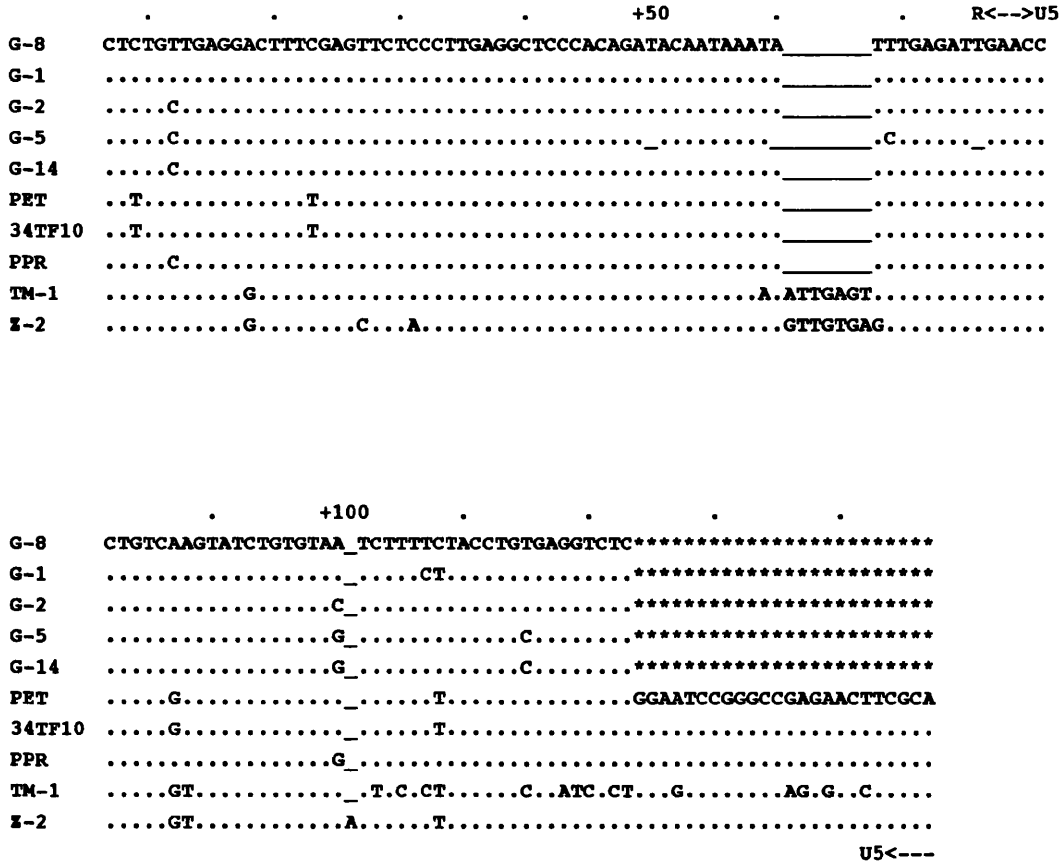
Others in the MRC Retrovirus Research Laboratory had found that blunt-end ligation of PCR products was inefficient and a number of precautions were followed in an attempt to increase efficiency (Dr. M. Rigby personal communication). Therefore, following purification products were ligated to *Hinc*II linearised pIC19R (2.2.3.1) at a high insert to vector ratio (500ng: 50ng insert:vector) in a large ligation volume (40µl) at 14°C, overnight. 2µl of a ligation reaction was used to transform competent *E.coli* (DH5α, 2.1.6) and recombinants were selected on agar containing X-gal (2.2.3.2). Positive recombinants were analysed as in (2.2.3.4.1) and a preparation of pure plasmid was made (2.2.3.4.2).

3.2.5 Sequence analysis of cloned LTRs.

The DNA sequence of the LTRs was determined by the dideoxy chain termination technique (2.2.4.1). Complete double stranded sequences were obtained and aligned (Figure 3.3) with other published FIV LTR sequences from

FIGURE 3.3 Sequence Alignment of FIV LTRs.





This figure shows the complete sequence alignment between the 5 UK isolates (G-8, G-1, G-2, G-5 and G-14), 3 US isolates PET (41,42), 34TF10 (43), PPR (198), a Japanese isolate TM-1 (199, 215) and the Swiss isolate Z-2 (200).

(*) indicates the oligonucleotides that were synthesised based on the published sequence of FIV Petaluma (41). These were used as primers in PCR reactions to generate full length LTRs (2.2.2.3). (.) indicates similarities between sequences and (_) indicate gaps that were introduced to give optimal alignment of the sequences. The sequences are numbered with respect to the cap site at the beginning of R. Figure 3.4 shows the percentage similarities between the LTRs and Figures 3.5 and 3.6 show their relatedness as unrooted tress. Potential protein binding sites are shown in Figure 3.8.

the USA, Japan and Switzerland (42, 198-200).

3.3 Results.

3.3.1 Comparison of FIV LTR sequences.

The comparison of all available FIV LTR sequences (3.2.5) revealed a highly conserved structure, with 90-98% sequence identity between the UK, US and Swiss isolates. The Japanese isolate (TM-1) is clearly more divergent with only 83-86% sequence identity to the UK, US and Swiss sequences (Figure 3.4). Furthermore the Japanese and Swiss isolates are the only sequences that have an insertion of 7 base pairs in the 'R' region of the LTR at position +60. The functional significance, if any, of these insertions is still not clear. It must be noted however that these sequence comparisons include the 5' and 3' primer sequences, which were complimentary to the Petaluma LTR sequence (41, 3.2.2). This PCR strategy reflects the fact that only the LTR sequence of FIV had been communicated at the outset of this study (41). Since all the UK strains were generated by PCR using these primers (3.2.2) it follows that they all contain identical 5' and 3' termini (shown as * in Figures 3.3 and 3.8) and that the percentage similarities between the different viral strains may vary slightly when these sequences are known. However it should be noted that this region of the genome has been sequenced for the G-14 FIV strain (Dr. M. Rigby unpublished results) and it contains only two base changes from the original FIV-PET sequence (Figure 3.3) (41). Therefore it does not seem that these sequences will alter the percentage similarities between the FIV strains to any great extent. In retrospect these regions of the genome could have been generated using inverse PCR (201, 202). Amplifications with internal FIV primers and infected cell DNA would have allowed the sequence of these regions to be defined and therefore compared between the different strains.

Figure 3.4 Sequence Divergence between FIV LTRs.

%	G-8	G-1	G-2	G-5	G-14	PET	34TF10	PPR	TM-1	Z-2
G-8		98	96	95	99	95	95	96	86	94
G-1			96	94	97	94	95	95	85	94
G-2				94	97	93	93	94	84	93
G-5					95	90	90	95	83	90
G-14						95	95	95	86	95
PET							99	92	84	94
34TF10								92	84	94
PPR									84	92
TM-1										83
Z-2										

This figure shows the percentage similarity between the LTRs of the different viral strains. These sequences were compared using the GCG Sequence Analysis programme on the Glasgow Universities VAX computer. See Figure 3.1 for isolate reference.

The LTR sequence is numbered with respect to the cap site/RNA initiation site, which corresponds to the beginning of the repeat region 'R' and base +1. Sequences upstream of this site, in U3 are given a negative value and sequences downstream, in R and U5, are positively numbered. At either end of the LTR there is a two base pair inverted repeat 'IR' (Figure 3.8) and these bases are perfectly conserved between the US, Swiss and Japanese isolates of FIV. Since these sequences are likely to be important for the integration of viral DNA into the host cell genome this may constrain sequence divergence here (1, 17, 196). The exact sequence of 'IR' in most of the UK isolates is not yet known due to the PCR strategy mentioned above. However in FIV G-14 the IR sequence is identical to the US, Swiss and Japanese isolates, which favours the idea that these regions are indeed highly conserved. See Appendix A for discussion.

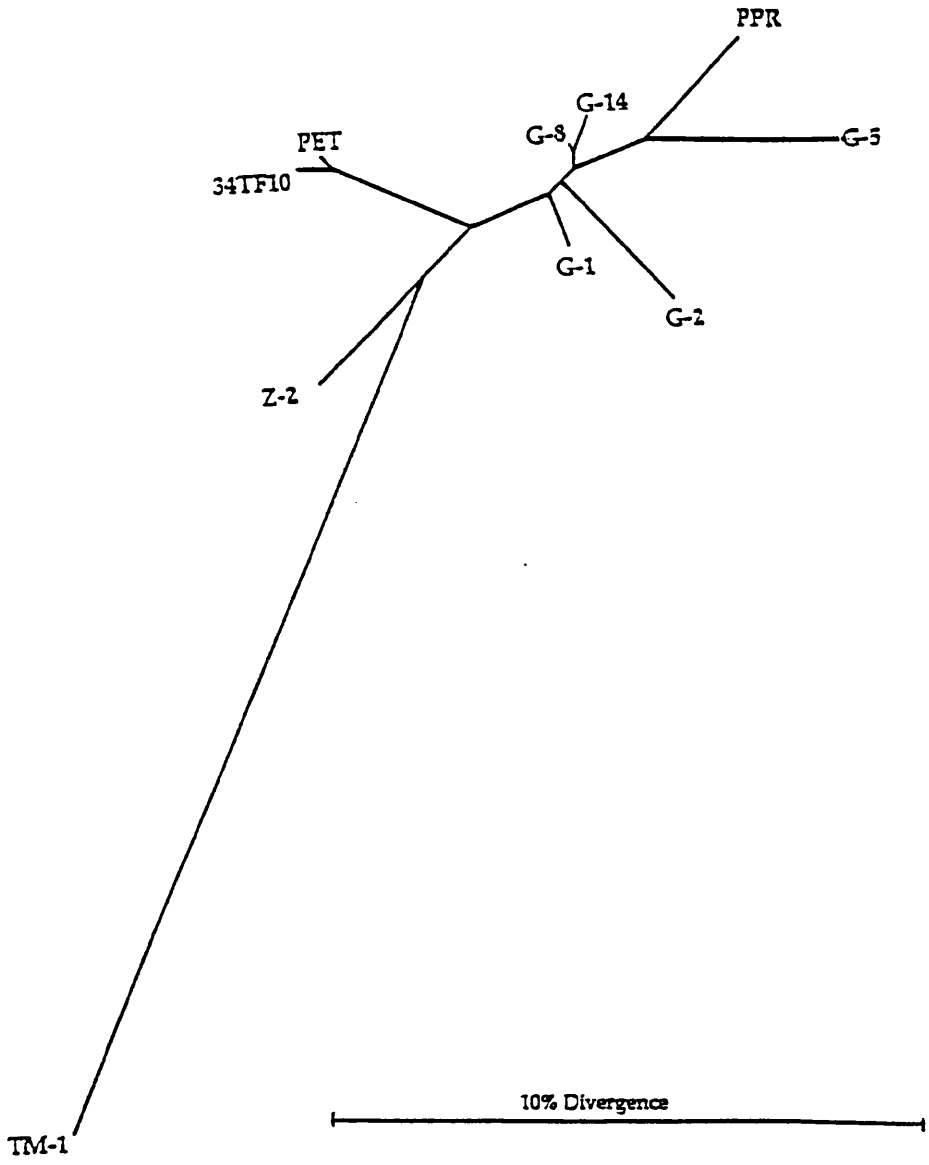
3.3.2 Phylogenetic analysis of the FIV LTRs.

These phylogenetic analyses were undertaken by Dr. E. Holmes (Centre for HIV Research, Edinburgh University) as detailed in (203). Nucleotide distances were estimated for the dataset using the program DNADIST from the PHYLIP package (204). This program was set to use an evolutionary model that allows different relative rates of change for transitions and transversions. Two different methods of phylogenetic analysis from the PHYLIP package were used in order to make a more reliable inference of phylogeny. DNAML implements a maximum likelihood (ML) method of phylogenetic inference with a transition:transversion ratio of 3:1 which made the underlying evolutionary model as realistic as possible. A tree was also constructed using NEIGHBOR, which clusters nucleotide distances using the neighbor-joining algorithm of (205).

Genetic distances between pairs of sequences for the FIV LTR are presented in Figure 3.6A. These figures indicated that the Japanese isolate (TM-1) is clearly the most divergent sequence. The maximum likelihood (ML) phylogenetic

Figure 3.5

Unrooted Maximum Likelihood Tree of Ten FIV LTR Sequences.



All branch lengths are drawn to scale.

See Figure 3.1 for isolate reference and text (3.3.2) for further discussion.

analysis of the ten LTR sequences compared above (3.3.1) is shown in Figure 3.5. Because there is no information as to the direction of evolutionary change (ie. there is no outgroup sequence) the trees presented here are unrooted. ML tree shows that the Japanese isolate TM-1 is quite distinct from the other sequences. The remaining UK, US and Swiss sequences appear to have diverged at approximately the same time in that they are almost equally related to each other approximating a 'star phylogeny' (206). A star phylogeny was also apparent from the analysis using the neighbour-joining (NJ) method (Figure 3.6B). However some of the UK isolates exchanged places between methods, as might be expected when there is little phylogenetic structure to the data. The results from the analysis of the LTR sequences are in close agreement with similar analyses of the *gag* and *env* sequences of FIV (203). Results of the *gag/env* study also showed that a Japanese isolate (TM-2) represents a distinct sequence subgroup which is clearly distinct from the North American and European FIV sequences. Further analysis of *gag* and *env* sequences provided evidence of an evolutionary radiation of FIV, with many isolates diverging at approximately the same time and although isolates from similar geographical sources often cluster together there is evidence of more than one origin for FIV in the UK, Holland and Italy (203). In parallel with what has been found in the *env* study (203) nucleotide variation seems to be clustered in the FIV LTR. In the FIV *env* gene the rate of nucleotide variation is relatively high but this is confined to clustered regions throughout the sequence (203). It is thought that this may reflect positive selective pressure by the host immune system (203). Although the relative rate of nucleotide variation within the FIV LTR is lower than *env* it also appears to be clustered. This may be due to the nature of the LTR which contains specific sequences that are important for the control of viral gene expression and therefore if the sequences change too drastically their function may be affected. Therefore sequence variation will be limited to the regions outwith these protein binding sites due to the selective pressure for maintenance of function.

Figure 3.6A

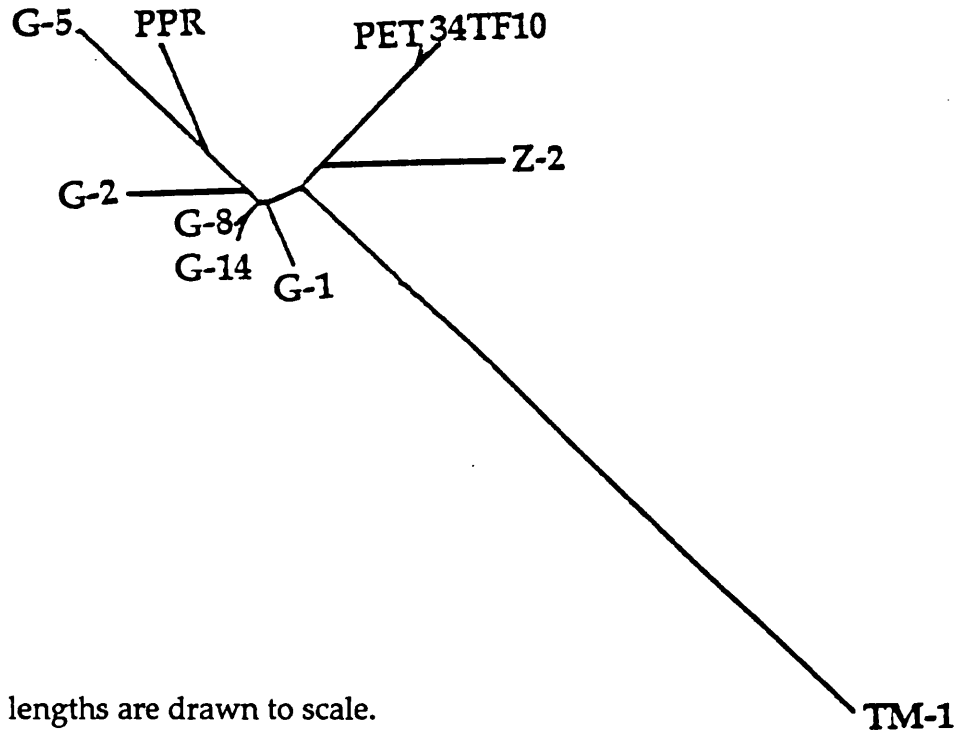
Matrix of Pairwise Nucleotide Sequence Distances between the LTRs of Various FIV Isolates.

G-1									
G-14	0.0234								
G-2	0.0378	0.0294							
G-5	0.0562	0.0540	0.0579						
G-8	0.0172	0.0058	0.0138	0.0471					
Pet	0.0499	0.0466	0.0653	0.0849	0.0468				
34TF10	0.0530	0.0477	0.0686	0.0822	0.0499	0.0085			
PPR	0.0499	0.0445	0.0561	0.0535	0.0378	0.0778	0.0811		
Z-2	0.0561	0.0539	0.0655	0.1011	0.0529	0.0591	0.0622	0.0811	
TM-1	0.1579	0.1527	0.1762	0.1918	0.1504	0.1723	0.1763	0.1728	0.1726

These nucleotide distances were determined by DNADIST with a Transition:Transversion Ratio of 3:1 (203, 204). See Figure 3.1 for isolate reference and text (3.3.2) for further discussion.

Figure 3.6B

Unrooted Neighbor-Joining Tree of FIV LTR Sequences.



All branch lengths are drawn to scale.

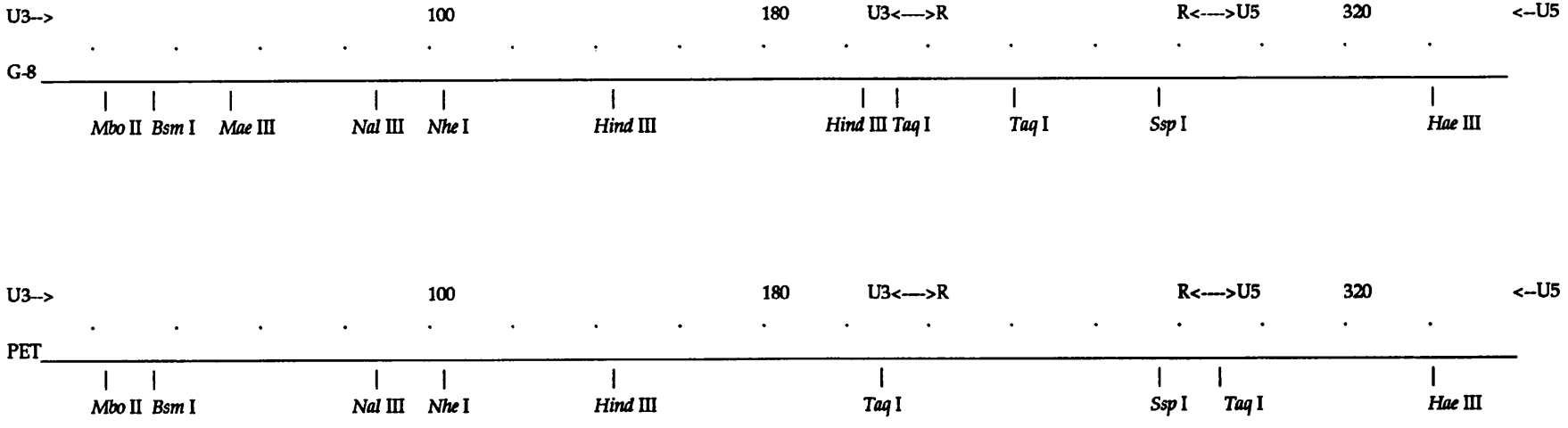
See Figure 3.1 for isolate reference and text (3.3.2) for further discussion.

Until recently it was considered that since the *env* gene had the highest rate of divergence it was the best tool to establish viral phylogeny. However work on HIV infected individuals has revealed that the variable *env* region V3 undergoes a 'resetting' phenomenon prior to sero-conversion in the infected host. In other words *env* undergoes an evolutionary pattern which may help to select a transmissible variant of HIV and this phenomenon may also be important for viral tropism and/or replication rate *in vivo* (333). It is conceivable that the LTR may also change in a similar fashion under selective pressure for the optimal sequence for expression in certain cell types. However throughout this same study (333) the *gag* gene of HIV continued to display diversity. Sequence variation in the *gag* gene is normally much lower than in *env* due to structural and functional constraints on the gene products, and differences in the selective forces of the host immune system which operate on the different gene products (333). Therefore in this case the *gag* gene is a more reliable measure of viral phylogeny (333). So it is evident that a clear picture of the evolutionary divergence of FIV may emerge if all the accumulated data, for *gag*, *env* and LTR, is compared and contrasted. However much more data from infected cats will have to be analysed before it can be concluded that the phylogenetic structure observed is not merely an artefact of limited sampling.

3.3.3 Restriction profile of the FIV LTRs.

A restriction analysis of the LTR sequences was carried out using the Map and Mapplot functions of the GCG programmes on the University of Glasgow VAX computer. A comparison between the restriction profile of FIV LTR G-8 and FIV LTR Petaluma is shown in (Figure 3.7). This information was utilised in the subsequent production and characterisation of mutant sub-clones of the FIV LTRs.

Figure 3.7 Restriction Map of the FIV LTR.



Restriction digest profiles of the Glasgow-8 (G-8) and Petaluma (PET) (41) LTR sequences were established using the GCG Sequence Analysis programme on the VAX computer (University of Glasgow). The differences in the resulting restriction maps (especially the *Hind* III and *Taq* I sites) were utilised in the subsequent cloning and characterisation of the various deleted LTR constructs. See text (3.3.3) for further discussion.

3.4 Discussion.

3.4.1 Conserved sequences in the FIV LTR.

3.4.1.1 TATA box.

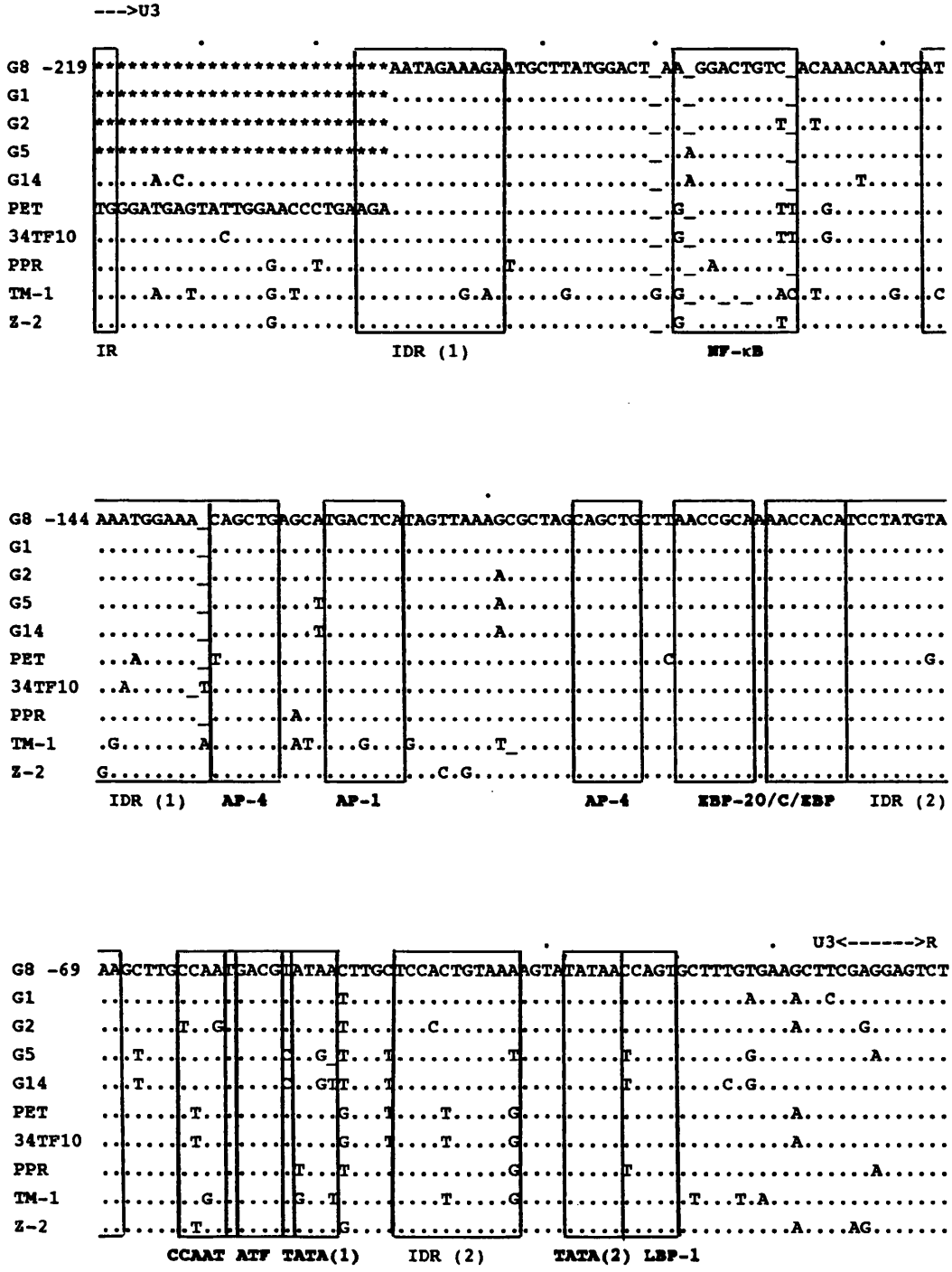
The TATA box mediates transcriptional initiation by binding a complex of transcription factors which includes TFIID (88, 246, 247). This complex of proteins can bind the enzyme RNA polymerase II which in turn initiates transcription of the DNA template (88, 246-252). It is not surprising therefore that the TATA element is a key component of viral and cellular promoters transcribed by RNA polymerase II (248-252).

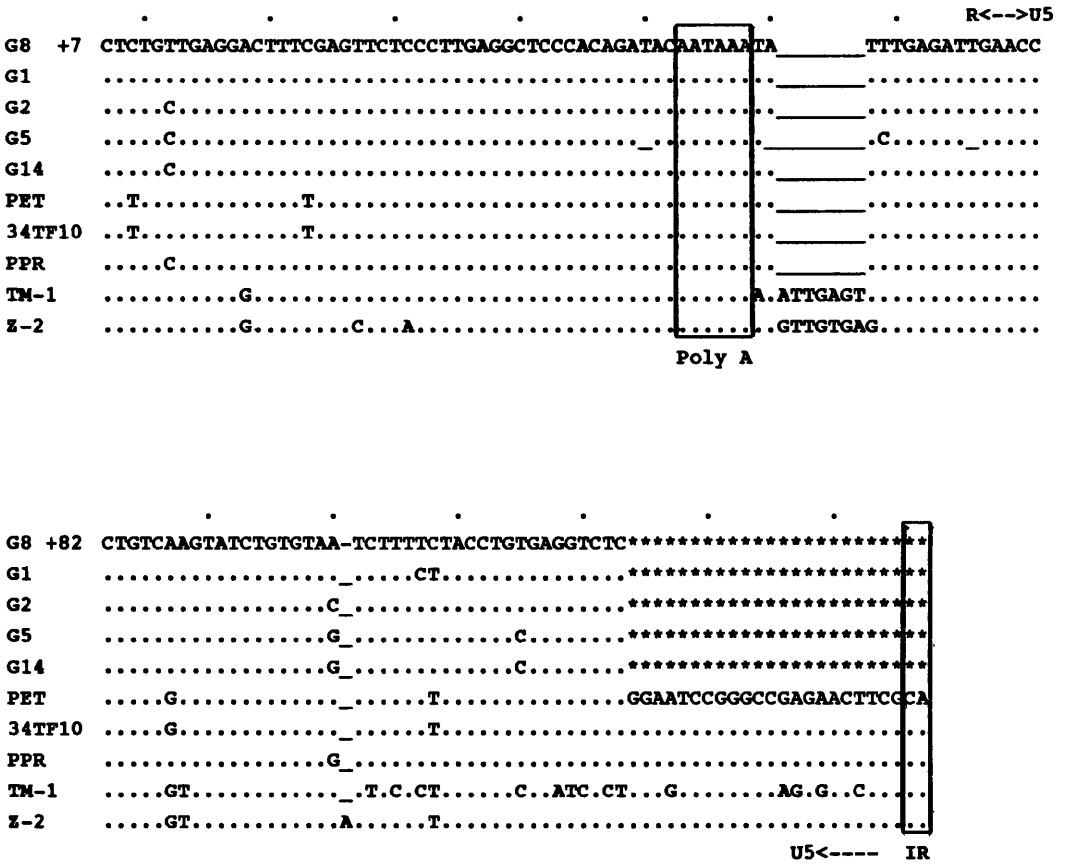
Lentivirus LTR promoters also belong to this class, for example the HIV-1 LTR contains a consensus TATA sequence which has been shown to bind several cellular factors including TFIID and LBP (253, 270). Furthermore it has been shown that due to the overlapping binding pattern of these proteins the rate of transcription of HIV-1 can be affected in a positive or negative manner (253). Following mutagenesis of the HIV-1 TATA element a marked decrease in both basal and *tat*-induced HIV-1 gene expression was observed (253, 254).

In the LTR sequence of several isolates of FIV (G-8, G-1, G-2, Pet, 34TF10 and Z-2, Figure 3.8) 2 TATA motifs at -25 and -50 were identified, however it was apparent that only the -25 box was conserved in all the isolates, (Figure 3.8). Given the role of TATA boxes in determining the transcriptional start site for many cellular genes (13), and assuming a similar role in the control of FIV gene expression the well conserved site would seem more likely to be the functional TATA box. Primer extension analysis, using poly A^+ RNA from FIV infected cells, determined the position of the cap site and allowed determination of the functional TATA box, which in cellular genes is usually located 20-30 base pairs upstream of the cap site (13). Therefore the primer extension studies confirmed the conclusions drawn from the sequence data in that the highly conserved

FIGURE 3.8

Potential Protein Binding Sites and other Conserved Motifs in FIV LTRs.





IDR: Imperfect direct repeat, IR: Inverted repeat.

Alignment is detailed as in Figure 3.3. Potential protein binding sites (**bold face**) and other conserved motifs (normal face) identified by sequence analysis (41, 198-200, 215) are boxed, see text (3.4.1 and 3.4.2) for description of their possible function.

sequence at -25 appeared to be the functional TATA box (42, 43).

3.4.1.2 Direct repeats.

Several retroviral long terminal repeats have been shown to contain large direct repeat sequences, which in turn contain multiple protein binding sites (22, 221-223). For example it has been shown that the LTR of FeLV from naturally occurring lymphomas frequently have sequence duplications (268). These duplications are analogous to those found in murine leukaemia viruses with accelerated disease potential (334-337). Although the FeLV duplications are unique to each independent isolate they each encompass a series of binding sites for feline nuclear factors and these duplications may lead to the outgrowth and heightened disease potential of some viruses (268).

The visna virus LTR contains two 43 base pair repeats that contain enhancer sequences and these regions are known to be important for the regulation of visna virus gene expression (22). Similarly both EIAV and CAEV contain direct repeats within the U3 regions of the LTR (222) and it has been suggested that variation in these repeat sequences may be important in EIAV host cell tropism and pathogenesis. (237).

An isolate of SIV (PBj14) which always causes fatal disease, within 8 days of inoculation is the most acutely pathogenic primate lentivirus so far described. Experiments to identify viral genes and gene products that influence pathogenicity revealed a high degree of conservation with other SIV isolates, except for a 22 base pair duplication in the enhancer region of the viral LTR. This duplication includes the site for the transcription factor NF-κB and it is conceivable that this duplication contributes to the acute course of disease induced by this strain of SIV (338).

The FIV LTR has 2 imperfect direct repeats (IDR, Figure 3.8), but neither is particularly well conserved throughout the different viral strains. No large repeats analogous to those discussed above have been found in the FIV strains

which have been studied to date.

3.4.1.3 Polyadenylation site.

The site of polyadenylation is predicted to be around base +74 (beginning of U5, Figure 3.8) from the location of the polyadenylation signal AATAAA which occurs within a highly conserved region in all the FIV LTRs (Figure 3.8) (43, 210).

3.4.1.4 Primer binding site.

The 5' LTR sequence is immediately followed by the 18 base sequence corresponding to the tRNA-lys primer binding site (41, 43, 211) which is similar to other lentiviruses thus far examined (41, 212, 213).

3.4.1.5 CCAAT box.

Mutational analysis of a number of eukaryotic promoters which contain a TATA box and are known to be transcribed by RNA polymerase II revealed the existence of a second upstream promoter element containing the sequence CCAAT (261). This sequence has now been shown to be required for transcription of a number of cellular and viral genes. (263-265). *In vitro* transcription studies revealed the existence in HeLa extracts of a protein (CTF) that recognises the CCAAT domain (261). Further analysis of the binding pattern of this protein revealed similarities between the consensus recognition sequences for CTF and nuclear factor-1 (NF-1) (263).

NF-1 was first discovered as a cellular factor required for the initiation of adenovirus replication (266), but is now known to represent a family of transcriptional regulatory proteins with binding sites in many cellular and viral promoters (7). Therefore it is now considered that CTF/NF-1 consists of a

family of proteins that can function both as a transcription selectivity factor and as an initiator for DNA replication (263).

NF-1 binding sites have been found in the MoMuLV, MMTV and FeLV LTRs (267, 268). A natural mutation in a highly conserved NF-1 binding site in the FeLV LTR, which abolished factor binding *in vitro*, is associated with significantly decreased promoter activity (267). Therefore these results show that NF-1 can act as a positive regulator of FeLV transcription and this compares closely with functional studies on the analogous site in murine retroviral LTRs (267-270).

In the LTR of several FIV strains a CCAAT consensus sequence can be found at -60.

3.4.2 Potential protein binding sites in the FIV LTR.

In addition to the well conserved regions outlined already a number of motifs corresponding to important protein binding sites in other viral promoters and enhancers (7, 13, 93, 214) were identified by sequence analysis of the FIV LTR (198, 215). When these putative sites were outlined on the sequence comparison (Figure 3.8), it was clear that not all of these sites were conserved in all the FIV strains.

3.4.2.1 AP-1 site.

AP-1 is a cellular transcription factor which regulates the expression of a variety of cellular genes (83, 255, 256). AP-1 mediates cellular responses to external stimuli, including serum, growth factors and phorbol esters, via its interaction with the consensus DNA recognition motif T(T/G)A(G/C)TCA, which has been found in a variety of cellular and viral promoter elements (83, 255, 256).

AP-1 is a heterodimeric complex containing products of both the c-*fos* and c-*jun* gene families (83). In addition Fos and Jun can interact with other cellular

proteins to modulate gene expression in either a positive or negative manner (257).

Two potential AP-1 binding sites have been noted in the HIV-1 LTR (258) however, the role that these motifs play on the regulation of HIV-1 gene expression remains to be established (21).

The visna virus LTR contains six separate motifs that are homologous to the recognition site for the cellular transcription factor AP-1 and a single sequence homologous to the recognition site for transcriptional factor AP-4 (24).

AP-4 was first identified as a cellular protein that binds to the SV40 enhancer (240). See Appendix B for further discussion. In several cellular promoters as well as some viral enhancers, AP-4 binding sites overlap with the recognition sequences of other transcription factors such as AP-1, suggesting possible factor-factor interactions (271). Results have shown that in visna virus both these classes of binding sites appear to be important for regulating basal levels of transcription and the AP-1 site most closely proximal to the TATA box was found to be essential for efficient viral *trans*-activation (22, 24).

Adjacent AP-1 and AP-4 sites have also been found in the SV40 promoter and *in vitro* studies using this system suggest that AP-1 and AP-4 proteins can act in concert to enhance late transcription of SV40 (240, 259).

Around position -120 in the FIV LTR an AP-1 and 2 AP-4 sites can be found by sequence analysis and all 3 sites are well conserved in each of the FIV strains.

3.4.2.2 Enhancer binding site.

Recognition motifs for the enhancer binding protein-20 (EBP-20) have been identified in several animal virus enhancers such as Murine Sarcoma Virus (MSV), SV40 and polyomavirus (260). Enhanced gene expression mediated by binding occurs via a common DNA sequence known as the enhancer core motif, which has the following consensus: 5'-TGTGG(A/T)(A/T)(A/T)G-3'. Further studies revealed that this same protein was capable of selective binding

to the CCAAT motif of several viral promoters such as the MSV LTR and the HSV *tk* gene (239, 261). This protein (EBP-20) was renamed, CCAAT enhancer binding protein (C/EBP) (262) and it is considered that this one protein can interact with 2 *cis*-acting regulatory elements which have no overt similarity at the DNA sequence level (263).

A fairly well conserved sequence, similar to the consensus enhancer binding site, is imperfectly repeated in the FIV LTR around nucleotide -86, and a CCAAT consensus sequence is found in several isolates at position -60.

3.4.2.3 SEF-1 site.

Preferential enhancement of transcription of SL3-3 murine retrovirus in T-cells was localised to a repeated sequence in the LTR enhancer region (130). A protein binding to this site, denoted SL3-3 enhancer factor 1 (SEF-1), is expressed specifically in T-lymphocytes and binds to the enhancer of leukaemogenic type C retroviruses and to the control regions of several cellular genes with high expression in T-lymphocytes (216, 217). SEF-1 is related or identical to PEA-2 (217) which is a differentiation-specific transcription factor known to activate the polyomavirus enhancer (218, 219). Furthermore, mutation of the SEF-1 binding sites disrupts the disease potential of SL3-3 murine leukaemia virus without significant impairment of virus multiplication, implying that the SEF-1 transcriptional activators are specifically required for the neoplastic transformation by SL3-3 (220). The core SEF-1 site closely resembles the EBP-20 binding site consensus and a similar site is tandemly repeated at -80 in the FIV LTR. See Appendix C for further discussion.

3.4.2.4 LBP-1 site.

The cellular DNA binding protein, LBP-1, interacts with two sites that flank the transcriptional start site of the HIV-1 LTR promoter. Binding to a sequence 3' to

the TATA box was shown to enhance HIV-1 LTR driven transcription, however binding to a second site, which overlaps the TATA box repressed transcription. It has been proposed that LBP-1 regulates transcription by blocking TFIID binding to the TATA element (207), however the mechanism of enhancement by LBP-1 binding at downstream sites is not yet clear (208). LBP-1 may regulate gene expression in a concentration dependent manner which in turn eliminates the binding of factors required for efficient transcription. Therefore LBP-1 may be important for controlling HIV-1 latency (209). A similar LBP-1 binding motif is found just downstream of the TATA box and is well conserved in the majority of FIV LTRs (Figure 3.8). The significance of this site in FIV has not yet been investigated.

3.4.2.5 ATF site.

Also evident in the FIV LTRs is a well conserved ATF motif at -50. ATF binding to the E4 promoter of adenovirus facilitates establishment of a preinitiation complex involving TFIID (224, 225) and ATF has been shown to bind regulatory proteins involved in the cyclic-AMP and E1A inducible transcription of adenoviral genes (226). Furthermore deletion and mutational analysis of this site in the HTLV-I and BLV LTRs has shown that proteins of the ATF family are involved in gene regulation via viral *trans*-activators and the cyclic-AMP pathway (227, 232).

3.4.2.6 NF-κB site.

When the first sequence of the FIV LTR was published (41) a NF-κB site was noted at -160 which had the sequence 5'-GGGACTGTTT-3'. This is close to the NF-κB consensus sequence 5'-GGGR(C/A/T)TYYCC-3' (228). Transcription of the HIV-1 genome is regulated in part by cellular factors and is stimulated by activation of latently infected T-cells. T-cell activation also correlates with the

induction of the factor NF-κB which binds to 2 adjacent sites in the HIV-1 LTR (229). This factor was initially found in high abundance in nuclear extracts from mature B-cells, but is present in a range of cell types as an inducible factor which is normally sequestered in a cytoplasmic complex (230, 231). NF-κB sites are important in the regulation of a number of other viral and cellular genes, including SV40, CMV, IL-2 receptor, Immunoglobulin κ gene and β interferon (228, 233). Furthermore the LTR of HIV-2 has a single NF-κB site as does the LTR of SIV_{mac}. Both these sites have been shown to bind proteins which increase levels of gene expression after T-cell activation (234, 235). However, as can be seen in Figure 3.8, the sequence around this site is quite variable in the FIV LTR and most isolates do not show a close match to consensus binding sequences.

3.4.3 Identification of enhancer motifs shared with other animal lentiviruses.

While the sequence of the FIV promoter/enhancer is clearly a unique structure, in that it is not closely related to any other viral LTR, it was noted that some of the potential protein binding motifs are present in the promoters/enhancers of other animal lentiviruses, notably the consensus AP-4/AP-1 motif at -120 in the FIV LTR is homologous to a site found at -60 in the visna virus LTR (Figure 3.9). In visna this site is known to be important for basal, TPA and *tat* mediated *trans*-activation (22, 24).

Similarly in EIAV a C/EBP sequence can be found which is very similar to the sequence in the FIV LTR (Figure 3.9) (236). In EIAV this sequence has proved to be a very heterogeneous site. Furthermore in some viral strains the C/EBP site is duplicated and it is thought that these sequence changes can alter host cell tropism and pathogenesis of the virus (237).

Figure 3.9

Comparison of Enhancer Protein Binding Motifs in Animal Lentiviruses.

AP-4/AP-1

```
G-8 U3              -139  GGAAACAGCTGA_GCATGACTCATA
                          * * ******* ** *** *****
Visna U3 (24)       -70   TGTAGCAGCTGATGCTTGAGTCATA

Consensus sequence        CC[A/G]CAGCTG[A/G]    T[T/G]A[G/C]TCA
(13, 238, 240)

                          <---AP-4        AP-1--->
```

C/EBP

```
G-8 U3              -85   TTAACCGCAA_AACCACAT
                          * ******** **** ***
EIAV U5 (236)       -77   TAAACCGCAATAACCGCAT

Consensus sequence (239)  C[TTT/AAA]CCACAC[TTT/AAA]CCACA

                                    <---C/EBP--->
                          <---C/EBP--->
```

(*) indicates similarities between the enhancer binding sequences of different animal lentiviruses. Consensus binding site sequences for each protein are shown below the U3 sequences. (-) indicate gaps introduced to give optimal alignment. Sequences are numbered with respect to their own cap sites.

This figure shows the similarity between potential protein binding sites in the FIV LTR and known protein binding sites found in the enhancer region of visna virus and EIAV. See text (3.4.3) for further discussion.

To discover whether the sequence motifs identified are genuine binding sites a direct analysis of protein interactions with the FIV LTR was required. Footprint analysis and functional studies with deletion mutants were undertaken to establish the functional roles of the protein binding sites and to locate other important regulatory sites within the FIV LTR.

CHAPTER 4

Chapter 4 Analysis of Protein Binding Sites in the FIV LTR.

4.1 Introduction.

The initiation of transcription of eukaryotic genes by RNA polymerases is controlled by complex interactions between non histone proteins and specific regulatory DNA sequences known as promoters, enhancers and silencers (7, 15, 92-94, 103). Purification and characterisation of such *trans*-acting protein factors has been aided greatly by the DNase I footprinting technique, which is a sensitive and accurate assay for sequence specific DNA binding proteins (183, 302, 313-315).

In order to ask whether the binding motifs identified by sequence analysis of the FIV LTR represented bona fide binding sites, and to examine their significance for the transcriptional regulation of FIV gene expression, the binding patterns of protein from crude nuclear extracts prepared from feline cells was examined.

4.2 Methods.

4.2.1 DNase I footprinting.

In the footprinting technique a purified DNA restriction fragment that contains the sequence(s) of interest is end labelled with [^{32}P] at a single 3' or 5' end. Protein is bound to the DNA and the nucleoprotein complex is then partially digested with deoxyribonuclease I (DNase I). DNase I gives random single stranded DNA nicks, but cleavage is inhibited where protein is bound. The cleaved DNA is then purified and denatured and the single stranded end-labelled DNA fragments are resolved on a denaturing polyacrylamide gel and autoradiographed. Digestion in the absence of added protein yields a ladder representing cleavage at most bases while the samples preincubated

with protein show the same cleavage pattern, except where protein was bound. The absence of cleavage products (a gap in the base ladder) defines a footprint and if sequencing reactions are electrophoresed in parallel, the precise location of the footprint can be determined (2.2.4.1) (175).

4.2.2 Generation of end labelled FIV LTR fragments.

A critical element in the execution of a footprinting experiment is the isolation of the end labelled DNA restriction fragment. The DNA of interest must be radiolabelled to a high specific activity (> 1 X 10^{7-8} cpm/μg), since in a typical reaction less than 20ng (> 10^4 cpm) of a < 1kb DNA restriction fragment will be cleaved, denatured and electrophoresed. This will result in each band in the base ladder having > 10 cpm which will give a visible image after autoradiography overnight.

The pIC19R-FIVLTR-G8 construct was digested with *Bam* HI (top strand) or *Bgl* II (bottom strand) leaving a 5 base extension at the 5' end. The digested DNA was dephosphorylated with calf intestinal phosphatase and the resulting 5' hydroxy group phosphorylated with (γ^{32}P) ATP and T4 polynucleotide kinase (2.2.5.2).

Since both ends of the DNA restriction fragment site were labelled in these reactions a second restriction digest with *Bgl* II (top strand) or Bam HI (bottom strand) was necessary. This generated 2 fragments each labelled at only one end. Due to the large size difference between these two fragments the (~350bpr) LTR insert could be separated and purified from the plasmid backbone (~2.7kb), on a 4% acrylamide gel. End labelled LTR fragments were recovered and used in footprinting experiments as outlined in (2.2.8).

4.2.3 Nuclear protein preparation.

The protein samples that were used in the following footprint reactions were

crude nuclear extracts prepared in low salt (0.14-0.30M NaCl), (2.2.7). Ammonium sulphate precipitation of the extracts removed non-specific DNA binding, high mobility group (HMG) proteins, which remained soluble. The precipitated material was collected by centrifugation and redissolved to yield the crude nuclear extract. This protein extract was extensively dialysed in order to remove the excess salt, which would otherwise inhibit DNase I enzymatic activity (183, 302).

4.2.4 Footprinting reactions.

1μg of DNA was end labelled and the specific activity of the fragment was routinely found to be > 10^7 cpm. Assuming that 50% of each labelled fragment was recovered it was estimated that 20ng of fragment contained > 10^4 cpm which provided sufficient signal for one footprint reaction.
Initial experiments were carried out to optimise the conditions for protein binding and successful cleavage of the DNA.

4.2.5 DNase I digestion of protein bound LTR fragments.

Since it was difficult to predict the conditions for optimum DNase I digestion it was necessary to solve this problem empirically.
A range of nuclear extract concentrations (0-80μl=0-600μg/protein) were incubated with the target DNA. The protein-DNA complexes were then digested briefly with DNase I. DNase I digestion was seen to vary with the amounts of protein and non specific competitor DNA in the sample. It was therefore necessary to titrate the reaction components under different conditions in a number of pilot experiments. In general, 40μl of protein (300μg) and 1μg of poly(dI)-poly(dC) gave the best results. Poly(dI)-poly(dC) is non specific competitor DNA which competes for the non-specific DNA-binding and/or nuclease activity which may be present in the protein extract

and these problems can be reduced further if all reactions are carried out on ice. This procedure is based on the assumption that the specific DNA binding proteins of interest will have a higher affinity for the end-labelled DNA sequences than the non-specific competitor DNA which is present in a 10-100 fold excess, by weight of DNA (183).

Using 1-10µl of serial dilutions of a stock DNase I solution (2mg/ml) optimum conditions were established. Routinely 1µl of a 1/10 dilution of stock DNase I was required to digest the DNA-protein complexes and a more dilute enzyme concentration of 1/100 was sufficient for digestion in the 'no protein' control samples.

4.3 Results.

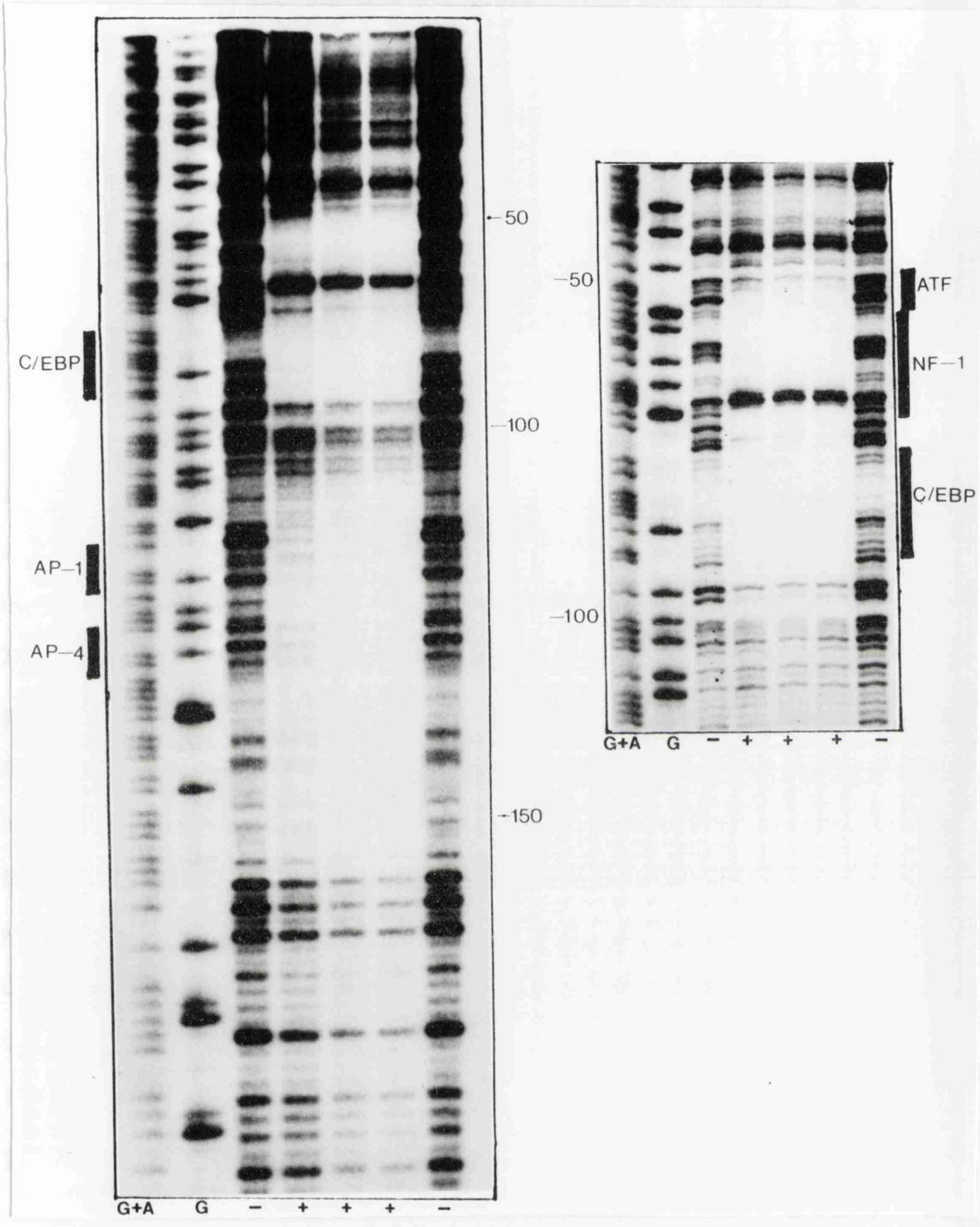
4.3.1 Protein binding sites identified in the U3 region of the FIV LTR.

In vitro footprinting protection assays using 5' end labelled LTR from the FIV G-8 strain bound to crude nuclear extracts made from feline fibroblast cells (CRFK subclone, ID10) identified three major protein binding sites within the U3 region (Figure 4.1).

By alignment with a Maxam and Gilbert sequencing ladder (2.2.4.2) (175), one footprint was located between -155 and -105 (Site 1) another around -50 (Site 3) and another broad and complex footprint from -80 to -60 (Site 2). In each of these cases the presence of several sequences representing known protein binding sites, previously identified in other viral systems, were apparent.

Protein binding sites within the U3 region of the LTR were most readily defined using the top strand of the FIV LTR (Figure 4.1). However in order to identify any protein binding sites within R and U5 the bottom strand of the LTR was labelled (Figure 4.7 and 4.8). This identified a fourth protein binding site (Site 4) at position +90 in U5 (Figure 4.7). Binding to this site was

Figure 4.1 Footprint Analysis of the U3 region of the G-8-FIV LTR.



This figure shows the footprint pattern (2.2.8) observed over the U3 region of the G-8 LTR (labelled top strand, 4.2.2) when incubated with increasing amounts (lane 4, 150µg, lane 5, 300µg, lane 6 600µg) of nuclear protein (2.2.7) extracted from CRFK cells (sub-clone ID10). Control lanes (3 and 7) indicate the DNA cleavage pattern in the absence of protein. The position within the 3 major footprints, of known protein binding sites previously identified in other viral systems, are named and indicated by black bars. Positioning of the footprints was achieved by comparison to a sequence ladder generated by Maxam and Gilbert reactions (2.2.4.2) (G+A, lane 1 and G lane 2). The sequence is numbered with respect to the cap site. The left hand gel shows a 3 hour run and the right hand gel shows a longer run of the same reactions. See text (4.3.1, 4.4) for a more detailed description.

very strong in nuclear extracts made from F422 cells whereas very little binding activity was seen in the nuclear extracts from CRFK cells. It remains to be established which protein(s) are binding to this site and consequently the significance if any of the alternate binding patterns found in the different cell lines to the expression of FIV.

Footprint analysis of the R region of the FIV LTR did not identify any protein binding sites. However as can be seen in Figure 4.8 the area around the initiation site (0) was hypersensitive to cleavage by DNase I digestion. This may be a consequence of an 'open' DNA configuration around this region induced by protein binding which *in vivo* would make this region more accessible to binding by transcription factors needed to initiate expression from this site (69). In the 'no protein' control lanes this site was not hypersensitive. Therefore this phenomenon is only seen in the lanes where protein was bound to the DNA and this may explain the hypersensitivity, in that when protein is bound to upstream U3 sequences it can in some way 'open' downstream sequences in readiness for protein binding (69). Protein binding around the initiation (CAP) site and TATA box is not seen in these assays but this may be due to instability or low concentrations of the relevant factor in the nuclear extracts tested (Dr. M. Plumb, Beatson Institute, Glasgow personal communication).

However further analysis was required in order to identify which specific protein binding sites were functional in the FIV LTR.

4.3.2 Specificity of binding sites; analysed by competition assays.

The specificity of binding within the identified U3 footprints was investigated further by competition assays using oligonucleotides based on consensus binding sites for well characterised transcription factors (Figure 4.2), previously identified in a number of viral systems. The oligonucleotides used in these competition studies are outlined in Figure 4.3. Footprints

Figure 4.2 Comparison of Protein Binding Sites.

Site	Source	Sequence	Reference
AP-4/AP-1	Consensus	TCAGCTGTGG TGACTCA C C T T	(240)
	SV40	CCAGCTGTGGAATGTGTGTCA	(240)
	FIV G-8	ACAGCTGAGC ATGACTCA	
	visna	GCAGCTGATG CTTGAGTCA	(22, 24)
	CAEV	TCAGCTGATG CTTGCTCA	(24)
C/EBP	Consensus	TGTGGAAAG TTT	(13, 197)
	FIV G-8(1)	TGTGGTTTTG	
	(2)	TGCGGTTAAG	
	EIAV	TGCGGTTATT TGCGGTTTAG	(236)
	MoMuLV	TGTGGTATG	(223)
	FeLV	TGTGGTTAA	(268)
NF-1	Consensus	TGG (N_{6-7})GCCA	(295)
	FIV G-8	TGTAAAGCTTGCCA	
	FeLV	CGGCTTGAG GCCA	(268)
	Mo-MuLV	CGGCTCAGG GCCA	(223)
	Adenovirus	TGGATTGAA GCCA	(266)
ATF	Consensus	GTGACGT A A	(226)
	FIV G-8	ATGACGT	
	BLV	GTGACGG	(318)
	Adenovirus E4	GTGACGT	(226)

This figure compares consensus sequences for DNA binding proteins with the sequences of protein binding sites previously identified in other viral enhancers as well as with the putative FIV LTR binding sites. These sequence comparisons were used to help design oligonucleotides for the competition studies (Figure 4.3 and 4.4).

Figure 4.3 Sequence of Oligonucleotides used in the Footprint Competition Analysis.

Oligonucleotide	Sequence	Source	Reference
AP-4	TGATAAATGGAAACAGCTGAGCA	MRC Retrovirus Research Laboratory (2.2.2.2)	FIV LTR
AP-1	CTAGTGATGACTCAGCCGGATC	Stratagene	Stratagene 'Hotfoot Kit'
SV40 core	GATCCATCTGTGGTTAAGCACCTGGA	Oswell, Edinburgh	(267, 268)
NF-1	GATCTTATTTTGGCTTGAAGCCAATATG	Oswell, Edinburgh	(268)
LV-b	GATCCGCAAAAACAGGATATCTGTA	Oswell, Edinburgh	(268)
ATF	GGGGGAAGTGACGTAACGT	MRC Retrovirus Research Laboratory (2.2.2.2)	(226)

This details the sequence of the double stranded oligonucleotides that were used in the footprint competition analysis, as shown in Figure 4.4. Sequences were based on known protein binding site consensus sequences (Figure 4.2) and were supplied, from various sources, as outlined above. LV-b is a general transcription factor which was identified in partially purified nuclear extracts and shown to bind to a sight in the MoMuLV LTR (223). Although an identical binding site was identified in the FeLV LTR no footprint was observed with nuclear extracts from feline cells (286). No LV-b binding site was identified in the FIV LTR by sequence analysis however the oligonucleotide for this site was included in these experiments as a negative control in order to identify any loss in binding activity due to non-specific interactions.

resulting from a preincubation of protein with 100ng of double stranded competitor oligonucleotide (2.2.8), before addition of labelled DNA and DNase I digestion (4.2.4 and 4.2.5) are shown in Figure 4.4.

These assays confirmed that the binding at Site 1 (-120) was specific for the AP-1 motif and the binding at Site 3 (-50) was to the ATF motif.

Although the Site 1 footprint extends into the AP-4 motif it was shown that a competitor oligonucleotide based on the AP-4 site (Figure 4.3) had no discernible effect on the footprint (Figure 4.4, Lane 5). It is possible that the footprint at this site is due exclusively to AP-1 binding or that AP-4 binding activity is insufficiently abundant or stable to extraction to register in the footprint assay. Resolution of this issue will require further characterisation of the protein complexes interacting with Site 1 by electrophoretic mobility shift assays (302).

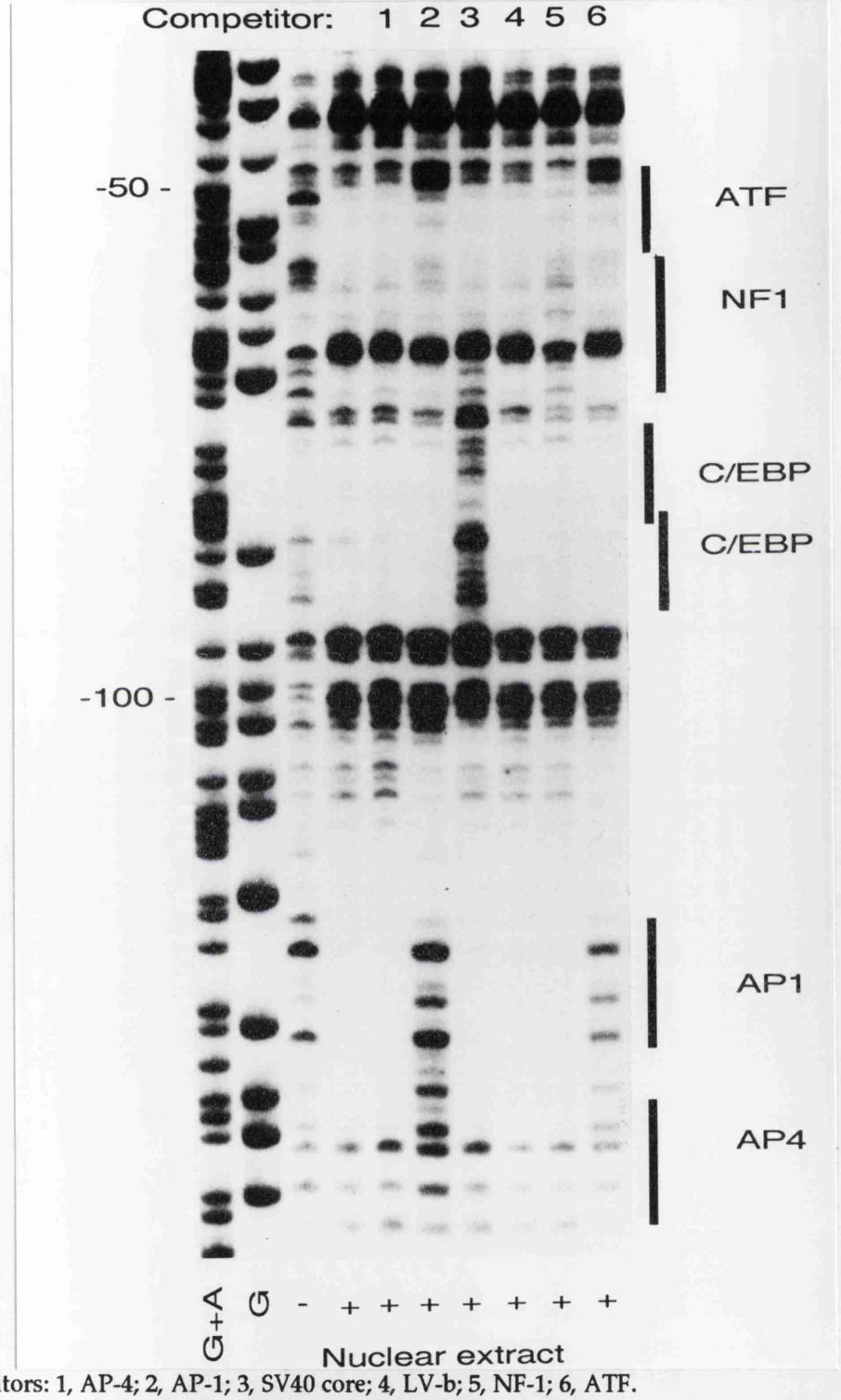
The competition analysis also allowed the dissection of the Site 2 (-80 to -60) footprint which could clearly be ascribed to an imperfectly repeated C/EBP site and an NF-1/CCAAT site at -70. These binding sites have been previously identified in several other viral enhancers where they have been shown to bind multiple cellular factors, some of which are known to be expressed in a tissue specific manner (216, 262, 267, 268, 290, 291).

4.3.3 Comparison of footprint patterns between different cell lines.

In order to identify any tissue specific binding factors the footprint patterns of nuclear extracts made from feline T-lymphoma cell lines (T17, T3, FL74, F422 and 3201) and feline fibroblasts (CRFK and AH927) were compared (Figure 4.5). F422 are permissive and AH927 are non permissive for FIV replication whereas the cell lines CRFK and 3201 can only be infected with selected strains of FIV, and only after adaptation of virus by repeated passage (198, 292, 293).

These assays revealed some clear differences in the abundance of binding

Figure 4.4 Competition Analysis of DNase I Footprints.



Competitors: 1, AP-4; 2, AP-1; 3, SV40 core; 4, LV-b; 5, NF-1; 6, ATF.

The sequence of the double stranded oligonucleotides that were used in these competition analysis are given in Figure 4.3.

Lanes 1 and 2 show Maxam and Gilbert sequencing reactions, Lane 3 shows the DNA cleavage pattern in the absence of protein, Lane 4 shows the cleavage pattern following incubation with 300μg of nuclear extract made from CRFK cells (ID10), lanes 5-10, shows the cleavage pattern after incubation with both nuclear protein (300μg) and a double stranded oligoucleotide. The sequence is numbered with respect to the cap site. See section (4.3.2) for further discussion.

factors in the cell lines tested (Figure 4.6).

In particular the levels of binding to the AP-1 and ATF sites were seen to vary markedly across cell lines. This finding is consistent with observations on human T-cell lines where AP-1 binding activity is inducible and low in unstimulated cells (294).

The results were also consistent with the observation that NF-1 binding activity is low as a result of post-transcriptional down regulation in the feline T-lymphoma cell lines T3, T17 and FL74 (267, 268). This was also a feature of the CRFK fibroblast cell line. However no obvious correlation between the expression of any specific binding activity and permissiveness for FIV replication was observed.

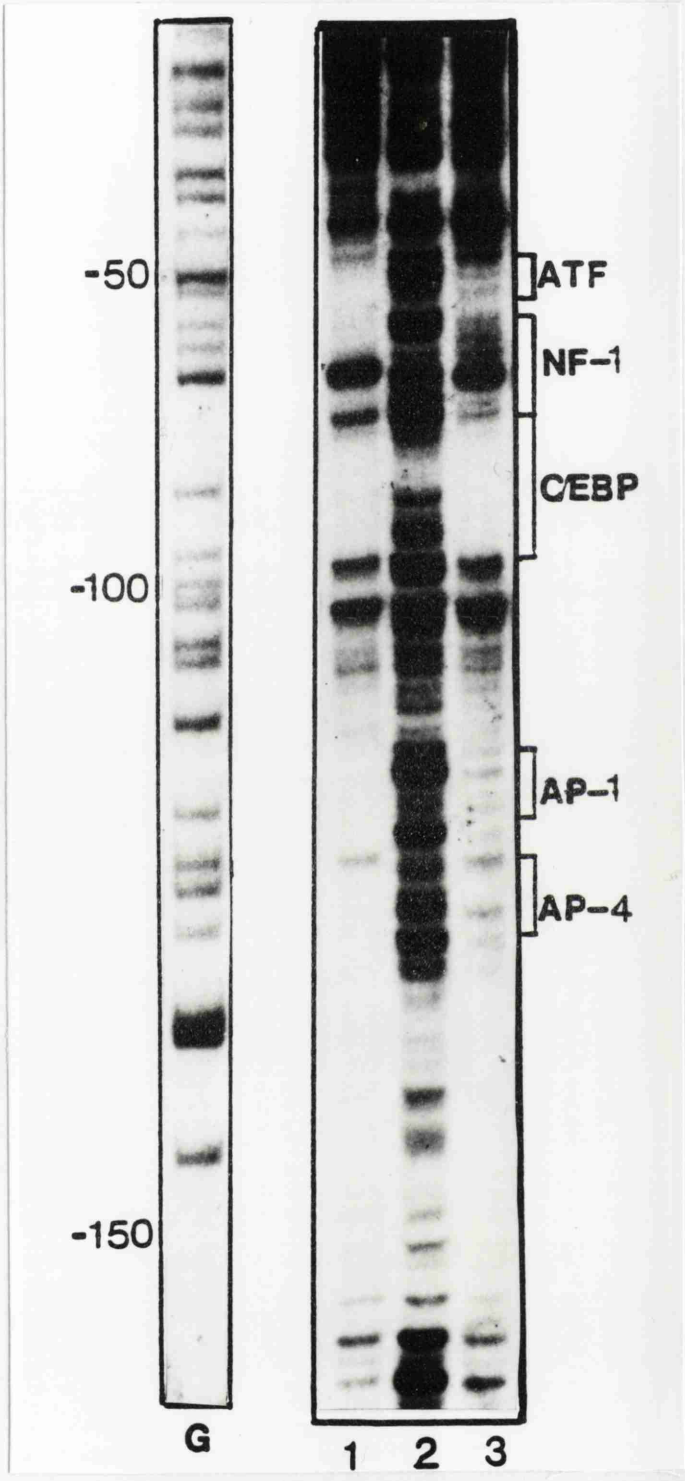
4.4 Discussion.

Footprinting analysis of the FIV LTR revealed a distinctive and complex array of at least three major binding sites within U3, which may play important roles in the transcriptional regulation of FIV. The recognition motifs for at least three distinct families of transcription factors were identified using competitive double-stranded oligonucleotides, AP-1 (-124/-118) (240), C/EBP (-94/-79) (262), NF-1 (-73/-59) (295) and ATF (-58/-53) (226).

Competition analysis also showed that adjacent binding sites were recognised independently by the respective protein(s), although there was evidence of cross competition between the AP-1 and ATF sites (Figure 4.4).

ATF is a common transcription factor which has been implicated in the transcription of adenoviral E1-A and cellular cyclic-AMP inducible genes (296, 297). Purification of ATF yielded a series of polypeptides that are related by DNA binding specificity and by immunological cross reactivity. This family of proteins included the transcription factor AP-1 whose recognition sequence is similar to the ATF binding site (287). This may

Figure 4.5 Comparison of the Footprints Generated by Protein from Different Cell Lines.



The above gel shows the differences in DNA cleavage patterns of the FIV LTR following incubation with equal amounts (300μg) of nuclear protein from fibroblastic cells (AH927) (lane 1), T-cells (T17) (lane 3) and in the absence of protein (lane 2). The sequence is numbered with respect to the cap site and was orientated by comparison to a Maxam and Gilbert sequence track (G). Putative DNA binding sites are outlined. See text (4.3.3) and Figure 4.6 for further discussion.

Figure 4.6 Abundance of Nuclear Factors Binding to the FIV-G8 LTR in Different Cell Lines.

		Factor Binding Site				
Cell Line	**Cell Type**	**AP-1**	**C/EBP**	**NF-1**	**ATF**	**FIV Replication**
CRFK(ID10)	Fibroblast	++	++	+	++	+
AH927	Fibroblast	++	++	++	++	-
F422	T-lymphoma	+	++	++	++	+/-
FL74	T-lymphoma	+	++	+	+	+
T3	T-lymphoma	+	++	+/-	+	+
T17	T-lymphoma	+	++	+/-	+/-	ND
3201	T-lymphoma	++	++	++	+	+/-

The above table shows the relative abundance of binding activity at the specified motifs in nuclear extracts from different cell lines, which bind to the LTR of the FIV-G8 strain. Cell name and type is as indicated. The abundance of the different factors is indicated by; +/-, very little binding; +, intermediate binding and ++, high binding levels. The ability of each cell to replicate FIV is indicated in the final column; +, FIV replication occurs; -, FIV replication does not occur; +/-, replication of some FIV strains after adaptation; ND, these cells have not been tested. See text 4.3.3 for discussion.

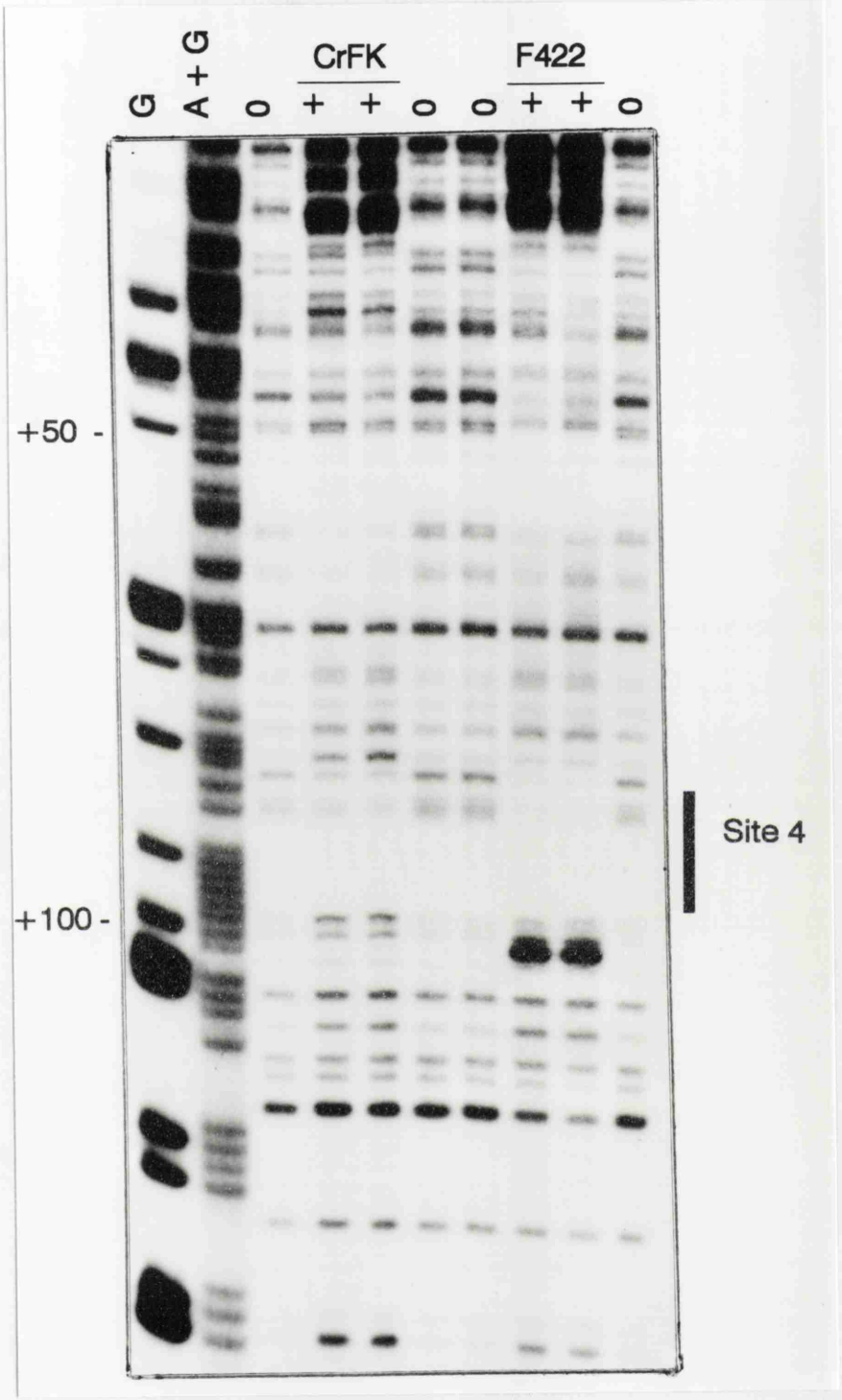
explain the cross competition that was observed in these assays (Figure 4.4). Furthermore heterodimeric proteins which include members of both the AP-1 and ATF families have been shown to bind to both the AP-1 and ATF DNA binding motifs (287) therefore this may be further evidence for the cross competition that was observed in the competition analysis.

For example, it has been shown that Jun A protein can dimerise with Fos to produce a heterodimeric protein that binds to AP-1 sites with greater efficiency than the Jun-Jun homodimer. Alternatively Jun A can efficiently dimerise with a member of the ATF family of proteins and in this case the heterodimer preferentially binds to the ATF recognition element (103, 288, 289). The AP-1 family of transcription factors was first defined as a class of proteins that bound to a specific DNA sequence in the control regions of SV40 genes (298). AP-1 has also been shown to be involved in mediating transcriptional activation in response to the phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-acetate). AP-1 is a complex mixture of proteins composed of members of both the *fos* and *jun* families and possibly other proteins (299, 300). See Appendix D for further discussion.

There are two tandem but imperfectly repeated recognition motifs for the core enhancer binding protein (C/EBP) between -94 and -79 in the FIV LTR. Multiple proteins which bind to this site in the SV40 core enhancer have been described (216, 262, 290, 291) but it is not yet clear which of these bind to the FIV LTR. See Appendix E for further discussion.

NF-1 is a DNA binding protein which was first discovered as a cellular factor required *in vitro* for the initiation of adenoviral DNA replication (266). NF-1 is now known to represent a family of transcriptional regulatory proteins with binding sites in many cellular and viral promoters where it is considered to be a transcription selectivity factor for RNA Pol II. (7). Furthermore NF-1 sites are found in the U3 regions of the LTR of a number of mammalian C-type retroviruses such as MoMuLV, MMTV and FeLV (268). Mutations within the NF-1 site have been shown to decrease *in vitro*

Figure 4.7 Footprint Analysis of the U5 region of the G-8-FIV LTR.



This figure shows the footprint pattern (2.2.8) observed over the U5 region of the G-8 LTR (labelled bottom strand, 4.2.2) when incubated with 300µg of nuclear protein (2.2.7) extracted from CRFK cells (sub-clone ID10) (+) or F422 cells (+). Control lanes (0) indicate the DNA cleavage pattern in the absence of protein. The position of the footprint is indicated (Site 4). Positioning of the footprint was achieved by comparison to a sequence ladder generated by Maxam and Gilbert reactions (2.2.4.2) (G, lane 1 and G+A, lane 2). The sequence is numbered with respect to the cap site. See text (4.3.1, 4.4) for a more detailed description.

gene expression driven by the FeLV LTR. However this effect was cell type dependent due to low NF-1 binding activity in certain cell types (267, 268).

Footprint analysis using the bottom strand of the FIV LTR showed a region in U5 around +90 (Figure 4.7) that could bind protein in nuclear extracts from F422 cells however in nuclear extracts from CRFK cells there was a much weaker binding pattern around this site. This may indicate that the abundance of the factor(s) which binds to this site is much higher or is more stable to extraction in T-cells than in fibroblasts or that the protein present in T-cells has a much higher affinity for the LTR binding site. This area of the LTR corresponds to a highly conserved region but the exact nature and specificity of protein binding at this site has not yet been studied.

Also from the bottom strand footprint analysis a hypersensitive site was seen around the region which corresponds to the start site (0) for viral gene transcription. This altered DNA configuration in the test lanes (Figure 4.8) may result from changes conferred on the DNA by protein binding to upstream U3 sequences (69) and this may reflect important *in vivo* functions which aid the initiation of gene expression (69).

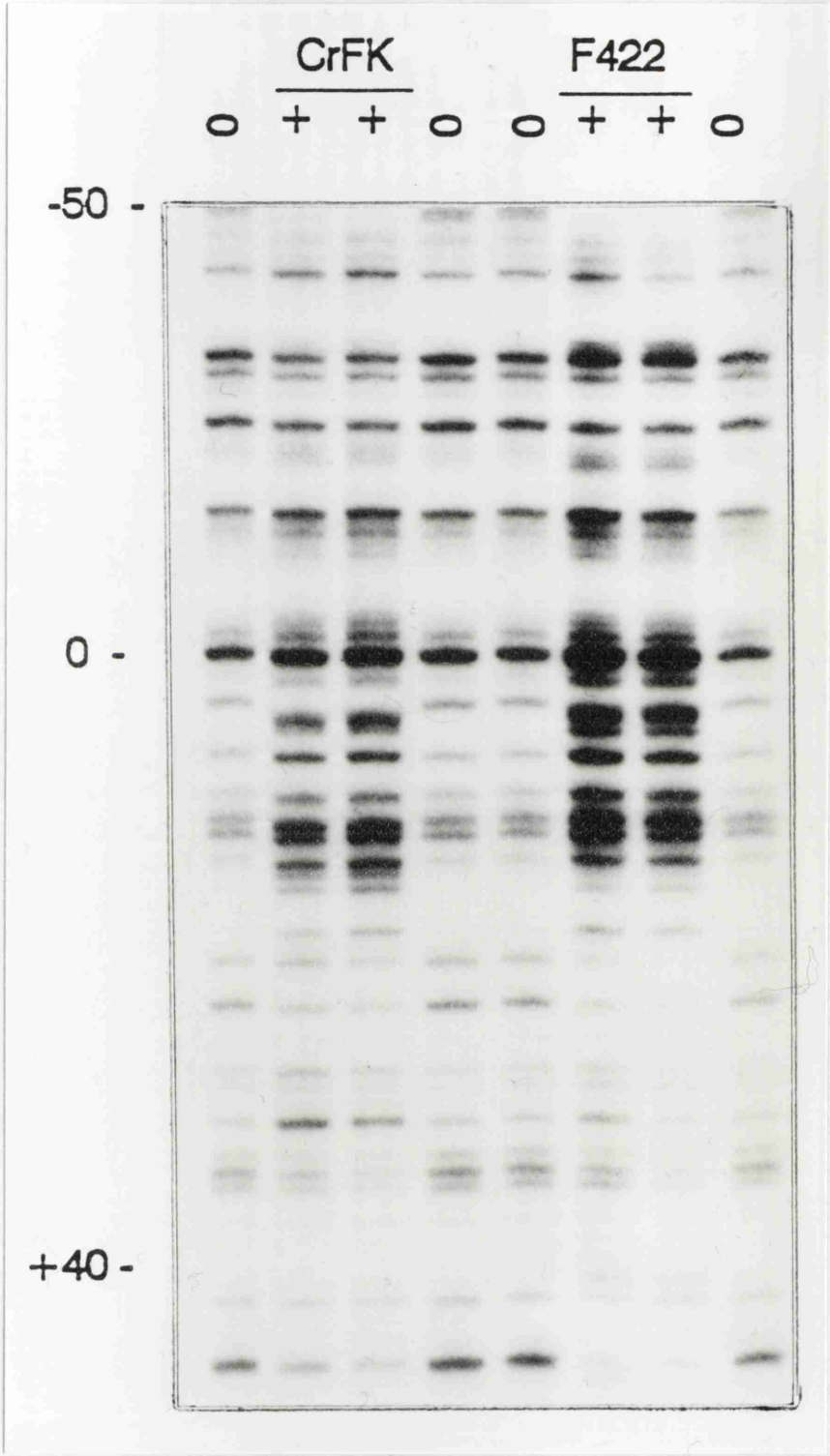
It is unlikely that all the protein binding sites in the FIV LTR have been found by these footprint analyses, and since some broad footprints were seen it is possible that more than one protein could bind at each site. One method which could be used to clarify the number of distinct proteins interacting with each site is electrophoretic mobility shift assays (302).

It would also be interesting to footprint the FIV LTR with nuclear extracts made from FIV infected cells since this may identify any virus specific or virus induced DNA binding proteins that were present.

In vivo footprinting in a range of cells would be of interest and results from these experiments may help to define how the different DNA binding proteins interact with the FIV LTR in a more physiologically relevant environment (369-371).

Finally if there were high levels of some proteins in the nuclear extracts that

Figure 4.8 DNase I digestion of the R region of the G-8-FIV LTR.



This figure shows the DNase I digestion pattern (2.2.8) observed over the R region of the G-8 LTR (labelled bottom strand, 4.2.2) when incubated with 300μg of nuclear protein (2.2.7) extracted from CRFK cells (sub-clone ID10) (+) or F422 cells (+). Control lanes (0) indicate the DNA cleavage pattern in the absence of protein. A hypersensitive site (dark bands) can be seen around the CAP site (0). Positioning of the footprint was achieved by comparison to a sequence ladder generated by Maxam and Gilbert reactions (2.2.4.2) (not shown). The sequence is numbered with respect to the cap site. See text (4.3.1, 4.4) for a more detailed description.

were tested it is possible that these proteins may 'dominate' in the footprint assays and occlude binding by other important regulators. If this is true it may be possible to miss the binding profiles of some of the less abundant nuclear proteins. Furthermore it is possible that some important proteins may be unstable to extraction however *in vivo* footprinting could be used to try to detect where these low level or unstable proteins bind (369-371).

Comparison of binding activity in nuclear extracts from a range of cell types revealed no correlation between susceptibility to FIV infection and the presence of any specific binding activity (Figure 4.5 and 4.6). AP-1 binding activity was lower in some of the T-cell lines and there were subtle cell type specific differences in the NF-1 and ATF footprints, presumably reflecting the recognition of these motifs by different tissue specific factors. It seems more likely therefore, that susceptibility to FIV infection, at the cellular level, is controlled primarily by other events during the viral life cycle, such as binding and entry to the host cell (9, 316, 317).

CHAPTER 5

Chapter 5 Dissection of FIV LTR Promoter Function **Page**

Chapter 5 Dissection of FIV LTR Promoter Function.

5.1 Introduction.

Structural and functional analyses of viral LTRs are necessary to determine which sequence elements and binding sites are important for the control of viral RNA synthesis. Following deletion or site directed mutagenesis, *cis*-acting sequences in the LTRs of several lentiviruses (20-22) have been shown to be involved in regulating gene expression. These mutagenised LTRs were then linked to reporter genes such as chloramphenicol acetyl transferase (CAT) of luciferase (114, 121). To determine the functional consequences of these mutations the constructs were transfected into eukaryotic cell lines. Transfections performed in this manner have identified regulatory elements critical for basal and/or inducible expression in a number of viral systems (22, 286, 301). Therefore in a similar fashion, the roles played by the protein binding sequences identified in the FIV LTR by DNase I footprinting, were analysed following deletion mutagenesis and transfection.

5.2 Methods.

5.2.1 Generation of FIV LTR deletion panel.

A series of oligonucleotides based on the U3 sequence of the FIV LTR-G8 was synthesised (Figure 5.1) (2.2.2.2). These oligonucleotides were used together with the same 3' oligonucleotide (Primer 6, Figure 5.1) in PCR reactions (2.2.2.3) to create a panel of deleted LTR fragments. The oligonucleotides were designed to progressively remove the protein binding sites which were identified in the DNase I footprinting analyses (4.3.2).

Figure 5.1 Sequence of Oligonucleotides used in PCR and Sequencing Reactions.

Oligonucleotides for PCR:

Oligonucleotide	Start position in FIV LTR	Sequence 5'-3'
Primer 5	-219	TGGGATGAGTATTGGAACCCTGAAGA
Primer 20	-176	GCTTATGGACTAAGGACTGTC
Primer 19	-147	ATGATAAATGGAAACAGCTGA
Primer 12	-126	GCATGACTCATAG
Primer 9	-113	TTAAAGCGCTAGCAGCT
Primer 10	-68	AAGCTTGCCAATGACG
Primer 11	-47	TTTGCTCCACTGTAA
Primer 6	+147	TGCGAAGTTCTCGGCCCGGATTCC

Oligonucleotides for Sequencing Reactions:

Universal primer	5'-GTTTTCCCAGTCACGAC-3'
Reverse primer	5'-CAGGAAACAGCTATGAC-3'

This figure details the sequence of the oligonucleotides that were used in PCR reactions to generate full length (Primer 5 and Primer 6) and deletion mutants of the FIV LTR (Primer 9,10,11,12,19 or 20 with Primer 6). See text (5.2.1) for details. Universal and reverse primers were used in sequencing reactions (2.2.4.1) to check the sequence and orientation of the recombinants. See text (5.2.2) for details.

5.2.2 Construction of reporter plasmids.

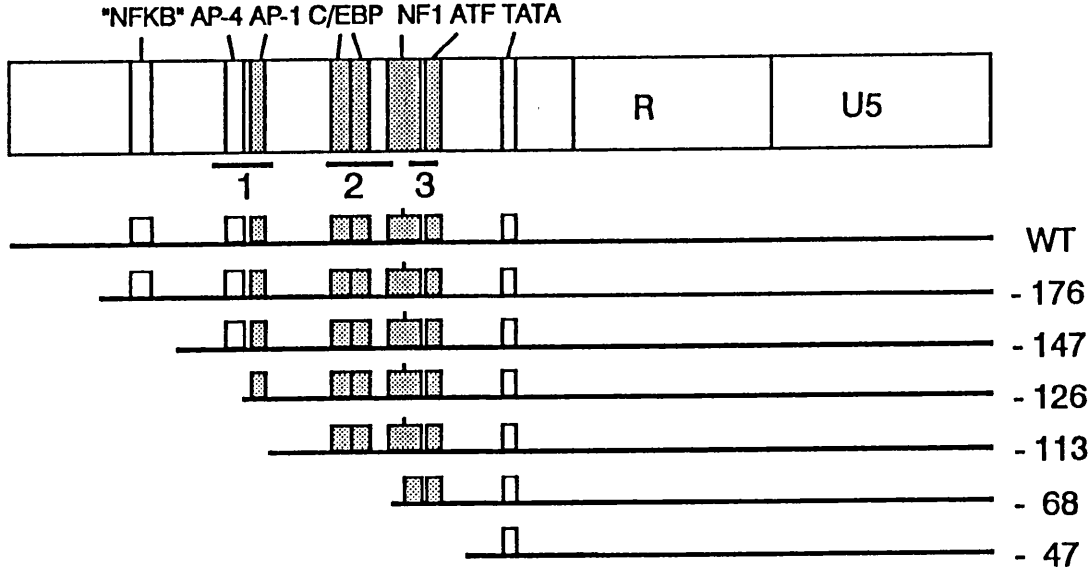
The FIV LTR deletion fragments were cloned into the *Hinc* II site of the plasmid, pIC-19R (146) (2.1.4 and 2.2.3). The resulting recombinants were analysed by DNA sequencing using universal and reverse primers (Figure 5.1) (2.2.4.1). The mutated LTRs were excised from pIC-19R using a *Bam* HI-*Bgl* II restriction digest and cloned into the *Bam* HI site of the plasmid pCAT12 (149, 150) (2.1.4 and 2.2.3). Again these constructs were sequenced (2.2.4.1) to check that the LTR insert was in the correct orientation, to drive the expression of the downstream CAT gene. A schematic diagram of the deletion panel that was generated is shown in Figure 5.2.

5.2.3 Eukaryotic cell transfections.

The wild-type and deletion mutant LTR CAT plasmids were transfected into a range of cell lines using the calcium phosphate precipitation procedure (2.2.9.1). To allow comparison between different transfection experiments it was important that the amount of DNA added to the cells remained constant throughout each experiment. Therefore the concentration of each plasmid DNA was checked by $OD_{A260/280}$ and agarose gel electrophoresis (2.2.2.4) prior to transfection (Figure 5.3).

Further checks were performed to ensure that cells had taken up similar amounts of DNA during transfection. After 48 hours, cytoplasmic or DNA extracts were prepared from the cells (2.2.9.1 and 2.2.10.3). The protein concentration of each extract was estimated (2.2.10.2) and CAT activity measured (2.2.10.4) after any necessary adjustment of the protein concentration. Transfection efficiency between different samples was checked by re-extracting DNA from the transfected cells and quantitating the specific amounts of DNA by Southern blot analysis using the CAT gene as a probe (2.2.10.3). Also in some cases co-transfection was performed with a

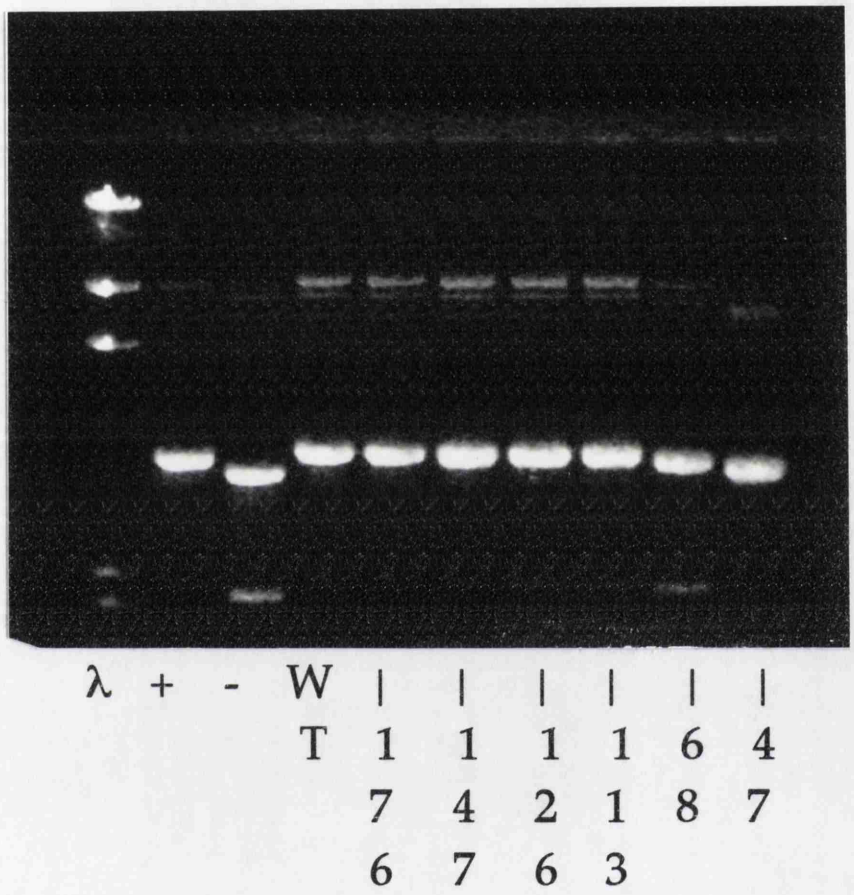
Figure 5.2 Schematic of the FIV LTR Deletion Panel.



Binding sites in the U3 region of the FIV LTR identified by DNase I footprinting analysis are indicated by hatched boxes, putative NF-κB, AP-4 and TATA binding sites are indicated as clear boxes. WT indicates the full length LTR fragment. The size and position of the 5' ends of the deletion mutants which were generated by PCR (5.2.1) are shown, the boxes above each line indicate which protein binding sites were retained in each mutant, numbers refer to the position in U3 where the deletions begin.

Figure 5.3

Agarose Gel of Plasmid Constructs used in Transfection Studies.



The above picture shows a comparison between 0.5μg of each undigested plasmid which was used in the various transfections. See text (5.3.1) for details.

Lane 1, λ *Hind* III digested markers; Lane 2 positive (+) control plasmid; Lane 3, negative (-) control plasmid; Lane 4, full length FIV-LTR-CAT plasmid (WT); Lanes 5-10 deletion mutant FIV-LTR-CAT plasmids, numbers refer to the position in U3 where the deletion begins.

plasmid carrying a functional β-galactosidase (β-gal) gene, pHFBG2 (151) (2.1.4). When cytoplasmic extracts of these transfections were made, β-gal assays were performed (2.2.10.1). Assuming that the uptake of both the β-gal plasmid and the LTR-CAT constructs was equal the resulting value for β-gal activity served as an internal control for transfection efficiency between different samples (302).

5.3 Results.

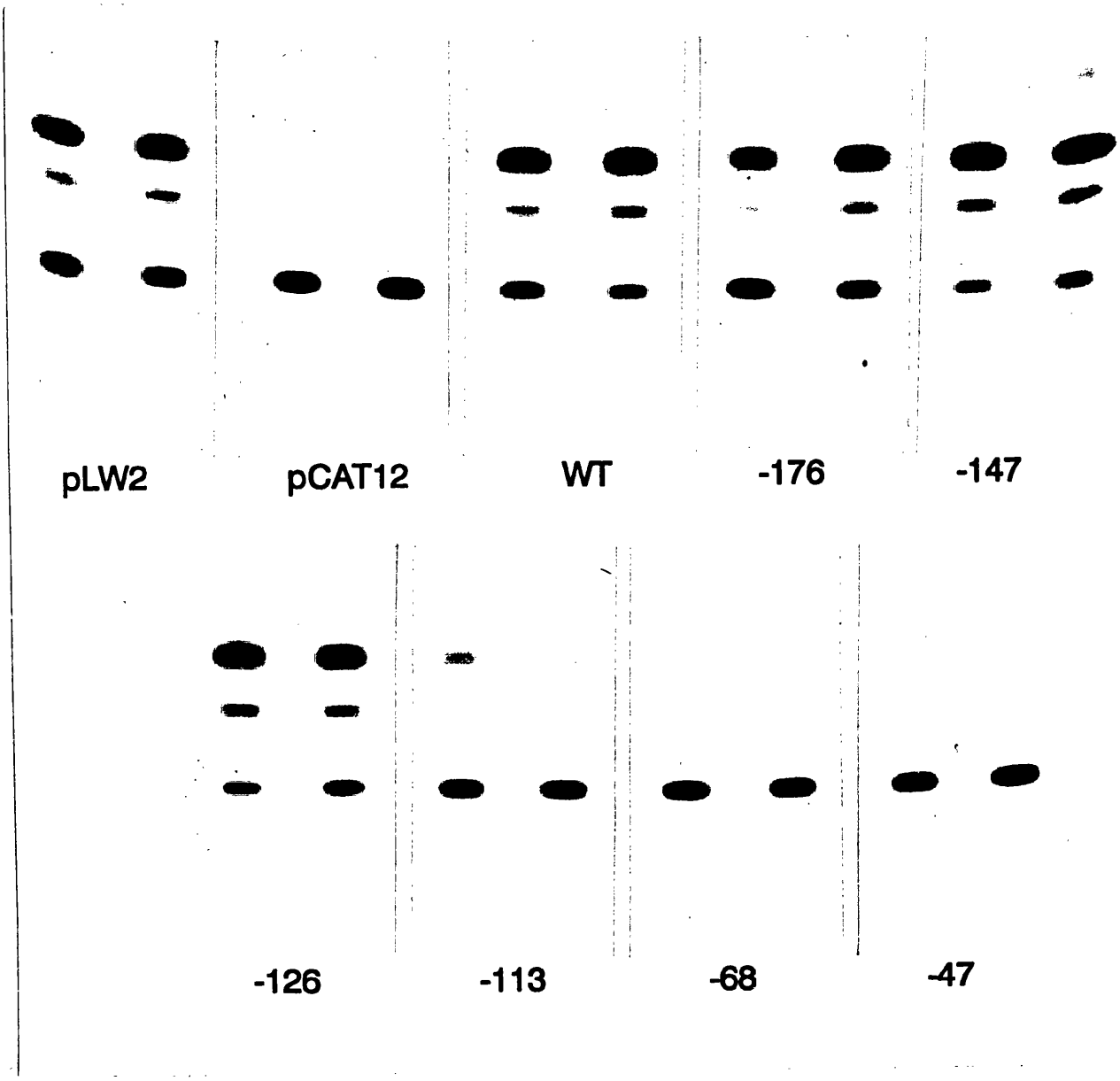
5.3.1 Eukaryotic transfections.

Following satisfactory and comparable readings of external DNA load, internal DNA uptake and protein concentration within the cell extracts, CAT activity between the different constructs were compared. Figure 5.4 shows the CAT activity of the mutant panel transfected into feline fibroblast cells (CRFK sub-clone ID10) and Figure 5.5 is a list of the relative CAT activities of the mutant panel in various cell lines normalised to the full length LTR control. Each figure represents the average of five experiments where each sample was transfected in duplicate. The cell lines compared here are feline fibroblasts either permissive (CRFK) or resistant (AH927) to FIV infection and also heterologous cells refractory to FIV infection (HeLa).

The deletion mutants showed some consistent properties regardless of cell type (Figure 5.6). The effect of deleting the AP-4/AP-1 motif was very marked, particularly when the core of the AP-1 binding site was removed (-113) (Figure 5.6). This deletion resulted in a 10-25 fold loss of activity relative to the wild-type LTR. Also notable was the virtual disappearance of promoter activity when the ATF and 3' half of the NF-1 site were deleted (-47). From these results it appears that the AP-1 site and the ATF site are of prime importance for basal promoter activity in the cell lines examined. See Appendix F for further discussion.

Figure 5.4

Representative CAT Assay of an FIV LTR Transfection



Pictured above is an autoradiograph of a representative CAT assay on cell extracts made from transfected CRFK (sub-clone ID10) cells, where each sample was analysed in duplicate.
pLW2, positive control plasmid, pCAT12, negative control plasmid, WT, full length FIV-LTR-CAT construct, (-176 to -47) numbers refer to the start position, in U3, of the deletion mutant FIV-LTR-CAT constructs as outlined in Figure 5.2. See text (5.3.1) for discussion.

Figure 5.5

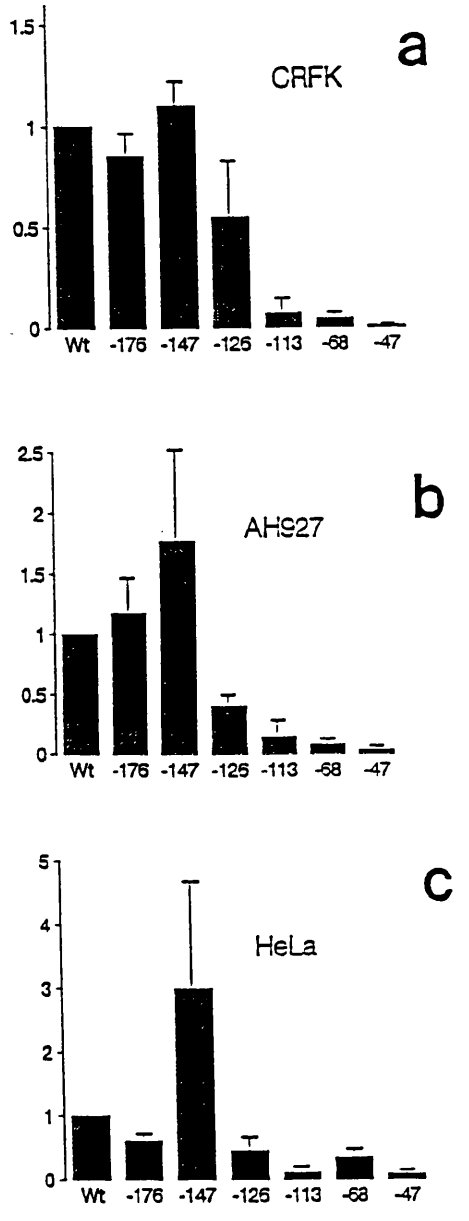
Relative Activities of Wild-type and Deletion Mutant FIV-LTR-CAT Constructs in Different Cell Lines.

		Cell Line		
LTR	**Site Deleted**	**CRFK**	**AH927**	**HeLa**
Wild-type	-	1.00	1.00	1.00
-176	-	0.85	1.09	0.60
-147	"NF-κB"	1.11	1.18	2.99
-126	AP-4	0.55	0.31	0.44
-113	AP-1	0.07	0.06	0.10
-68	C/EBP (NF-1)	0.04	0.04	0.34
-47	(NF-1) ATF	<0.01	<0.01	0.08

This table lists the relative activities of wild-type and deletion mutant FIV-LTR-CAT constructs after transfection into three different cell lines. The numbers on the left refer to the position in U3 where the deletion begins and the motif that was deleted in each case is indicated. The figures show the relative CAT activities of the mutant panel normalised to the full length LTR (1.00). Each figure represents the average of 5 experiments where each sample was transfected in duplicate. See Figure 5.6 for plots of the above figures and text (5.3.1) for further discussion.

Figure 5.6

Plots of the Relative Activities of Wild-type and Deletion Mutant FIV-LTR-CAT Constructs in Different Cell Lines.



These graphs show the relative activities of wild-type and deletion mutant FIV-LTR-CAT constructs after transfection into three different cell lines. The numbers on the x-axis refer to the position in U3 where the deletion begins (Figure 5.2). The figures on the y-axis correspond to the relative CAT activities of the mutant panel normalised to the full length LTR (1.00) (See Figure 5.5 for data). Results were an average of 5 experiments where each sample was transfected in duplicate and standard deviations are represented by extension bars. See text (5.3.1) for further discussion.

5.3.2 Identification of a *cis*-acting negative regulatory element in the FIV LTR.

The activity of the LTR mutant panel in HeLa cell transfections suggested the presence of a *cis*-acting negative regulatory element between -176 and -147, however this was much less marked in the feline fibroblast cells. This area of sequence in the FIV LTR contains purine rich stretches and includes the putative NF-κB site first identified by Olmsted *et al* (41), however this region is not well conserved in all of the viral LTR sequenced to date (Figure 3.8). Following identification of this negative regulatory effect a closer inspection of this area in footprinting analyses showed that with high levels of nuclear extracts (80μl = 600μg) from feline fibroblasts a weak footprint begins to appear (Figure 4.1 lane 6).

5.4 Discussion.

This study has identified binding sites for an array of transcription factors in the promoter proximal region of the FIV LTR. Deletion mutagenesis showed that these sites play a vital role in basal promoter activity in a variety of cell lines. In particular, AP-1 and ATF sites at -120 and -55 respectively are required for full basal promoter activity. Deletion of the AP-1 site led to a 10-25 fold drop in activity while deletion past the ATF site virtually abolished basal transcription.

ATF binding to the E3 and E4 promoters of adenovirus facilitates the establishment of a preinitiation complex involving TFIID (226) and it is conceivable that a similar role is played by the ATF site in FIV.

However this may not be the sole function of ATF since this cellular transcription factor has also been implicated in the regulation of cellular cyclic-AMP inducible promoters and it remains to be determined whether similar controls are important in FIV infected cells (224, 287, 306).

As mentioned earlier (Chapter 3) the FIV LTR is not closely related to any other viral promoter element but there are similarities to some binding motifs which have been found in the promoter/enhancers of other viruses. Therefore FIV may share some features of transcriptional regulation with the maedi visna and EIAV families. A notable resemblance was seen in the consensus AP-4/AP-1 motifs in the visna and FIV LTRs (Figure 3.9). Cellular factors that interact with the AP-1 sites in the visna virus LTR have been shown to be critical for basal and *trans*-activation (22). Lately it has been shown that the proteins Fos and Jun are directly involved in the differential regulation of visna virus gene expression in macrophages through an AP-1 site in the viral LTR (307) and again this may reflect as yet unidentified mechanisms which control gene expression in FIV infected cells.

Transfection studies with the deletion mutants did not help to delineate the functions of the C/EBP sites and the NF-1 site. The removal of these sites did not greatly affect basal levels of activity. It is possible that these sites respond to signals which are present only in certain cell types or that they only function during specific stages of the FIV life cycle and are therefore not detectable during transient transfections assays. For example alternate binding patterns have been seen with the Moloney murine leukaemia virus (MoMuLV) LTR where binding to the core enhancer and NF-1 sequences is observed only in differentiated mouse cell lines but not in undifferentiated embryonal carcinoma cells or erythroleukaemic cell lines indicating that there can be subtle differences in the transcription factors available in different cell lines and it has been considered that this may influence the

tissue specificity and general pathology of retroviral diseases (45, 223, 308, 309).

A negative regulatory effect was also noted at the 5' end of the FIV U3 domain, although this effect was cell-type dependent and was not precisely mapped. It is interesting to note that the relevant area of the LTR includes a purine rich sequence (-135/-155) and that negative regulatory activity has been mapped to similarly purine rich protein binding sites in the HIV and IL-2 gene promoters (114, 310, 311).

Negative control of gene expression has also been observed in the HIV-1 LTR. A negative regulatory element (NRE) has been identified between positions -340 and -185 in the HIV-1 LTR (Figure 1.6) (114). Mutations in this region have been shown to alter the levels of HIV-1 gene expression. Mutation of the COUP-TF binding site in the HIV-1 LTR results in a 2-3 fold increase in HIV-1 gene expression (303, 304). Similarly HIV-1 constructs containing a deletion in the LTR which includes the USF-binding site show increases in both gene expression and growth kinetics. Thus the USF-binding site may also serve as a negative regulatory element within the HIV LTR (305).

A number of both positive and negative cellular factors such as NF-AT (311), interleukin binding factor (ILF) (312), COUP-TF (303, 304), and USF (305) have been shown to interact with the HIV-1 LTR and it has been postulated that transcriptional repression maybe relieved by the release of positive effectors (eg NF-AT NF-κB and ILF) from inhibitory proteins which can in turn displace the negative regulators (eg COUP-TF and USF) by binding to similar overlapping binding sites in the LTR (312).

Therefore a more detailed analysis of this area in the FIV LTR will be required in order to try to identify any protein binding sites around this region and/or positive or negative factors which maybe operating at this site.

So far results seem to indicate that any factor(s) which can bind to this region is present at low levels in the nuclear extracts tested resulting in the very weak footprints observed. The results of footprint reactions with HeLa extract would be interesting as a more dramatic effect on FIV gene expression was observed in HeLa cell transfections therefore there may be more of the relevant factor in these cells. Alternatively the protein(s) of interest may not be stable to extraction and therefore a footprint would not be observed.

It is clear that more experiments are required before the exact nature of this effect on the control of FIV-LTR-driven gene expression can be established. T-cell transfection would be of particular interest as this would show the effect of the negative regulatory domain in a more physiologically relevant environment. However to date none of the FIV susceptible T-cell lines yielded measurable CAT activity by any of the available transfection procedures (2.2.9.2, 2.2.9.3) and therefore could not be assessed for FIV transcriptional activity. It may however be possible to study the activity of the FIV LTR in T-cells if a more sensitive reporter gene is used. Studies on the FeLV LTR have shown that the activity of this promoter in T-cells could be monitored more successfully when it was linked to the human growth hormone gene (pOGH) (Dr. R. Fulton, Glasgow University personal communication, 267). Therefore a new panel of FIV deletion constructs cloned upstream of the a similarly sensitive reporter gene may provide a more detailed analysis of the activity of the FIV LTR in T-cells.

CHAPTER 6

Chapter 6 Viral *Trans*-activation.

6.1 Introduction.

In addition to cellular factors, virus-encoded proteins are also involved in the regulation of lentiviral gene expression. All lentiviral genomes sequenced to date contain small ORFs between the *pol* and *env* genes. These ORFs are expressed as small multiply spliced transcripts which encode *trans*-acting proteins known to regulate viral gene expression by diverse mechanisms (Reviewed in 6).

The S open reading frame of visna virus encodes a ~10kDa nuclear protein that is able to *trans*-activate visna virus LTR-dependent transcription (161, 319).

Sequences in the visna virus LTR that respond to viral *trans*-activation are located upstream of the transcription initiation site in the viral promoter/enhancer region of U3. Studies have implicated the region homologous to the cellular binding site for AP-1 as the *cis*-acting element in virus mediated *trans*-activation (24). Similarly *trans*-activation of CAEV is mediated through DNA sequences in the U3 region of the viral LTR (222, 279).

Trans-activation by visna virus and CAEV via U3 sequences appears to contrast with *trans*-activation of other lentiviruses, HIV, SIV and EIAV which occurs through orientation dependent *cis*-acting elements in the R region of the LTR (Reviewed in 6).

A recent study (321) has shown that bovine immunodeficiency virus (BIV) LTR driven gene expression is upregulated in BIV infected cells and it is postulated that this is due to a BIV *tat*-like function (321). However the exact mode of action of BIV Tat is as yet unknown (320, 321).

The major response element of the HIV Tat is the *trans*-activation response element (TAR), which is located downstream from the site of transcriptional

initiation in the R region of the viral LTR (Figure 1.6) (21). It has been shown that both the functional expression of the viral *tat* gene product and an intact copy of the cis-acting target sequence TAR are essential for HIV-1 replication in culture (30, 113, 114).

Similarly *trans*-activation of HIV-2, SIV and EIAV is mediated by LTR sequences that extend through and downstream from the site of transcription initiation (322-327).

The mechanisms by which these proteins activate viral gene expression are not fully understood, however it seems likely that they act at multiple levels during viral gene expression. Studies on the mechanism of how HIV-1 Tat regulates HIV gene expression have proven to be complex and both transcriptional and post transcriptional mechanisms have been proposed (as reviewed by 6, 21).

Therefore a study was initiated to examine the role of transcriptional *trans*-activation in the regulation of FIV gene expression.

6.2 Methods.

6.2.1 Generation of FIV ORF-2 clones.

A number of small ORFs have been identified in the 3' half of the FIV genome (Figure 6.6) and some of these (ORFs 1, 2, D, F, I and H) are conserved in at least 2 clones of FIV (198). It was suggested that ORF-2 might encode a putative *tat*-like function due to its size and location in the FIV genome (Dr. J. Elder Scripps Clinic, La Jolla, California, personal communication, 198). However no obvious nucleotide or protein sequence homology was evident between ORF-2 and the primate lentivirus *tat* genes (198).

To look for the presence and the sequence content of the ORF-2 of the FIV-G8 strain, PCR primers (see below) were designed on the published FIV-

Petaluma sequence (2.2.2.2, 41).

5991 6012

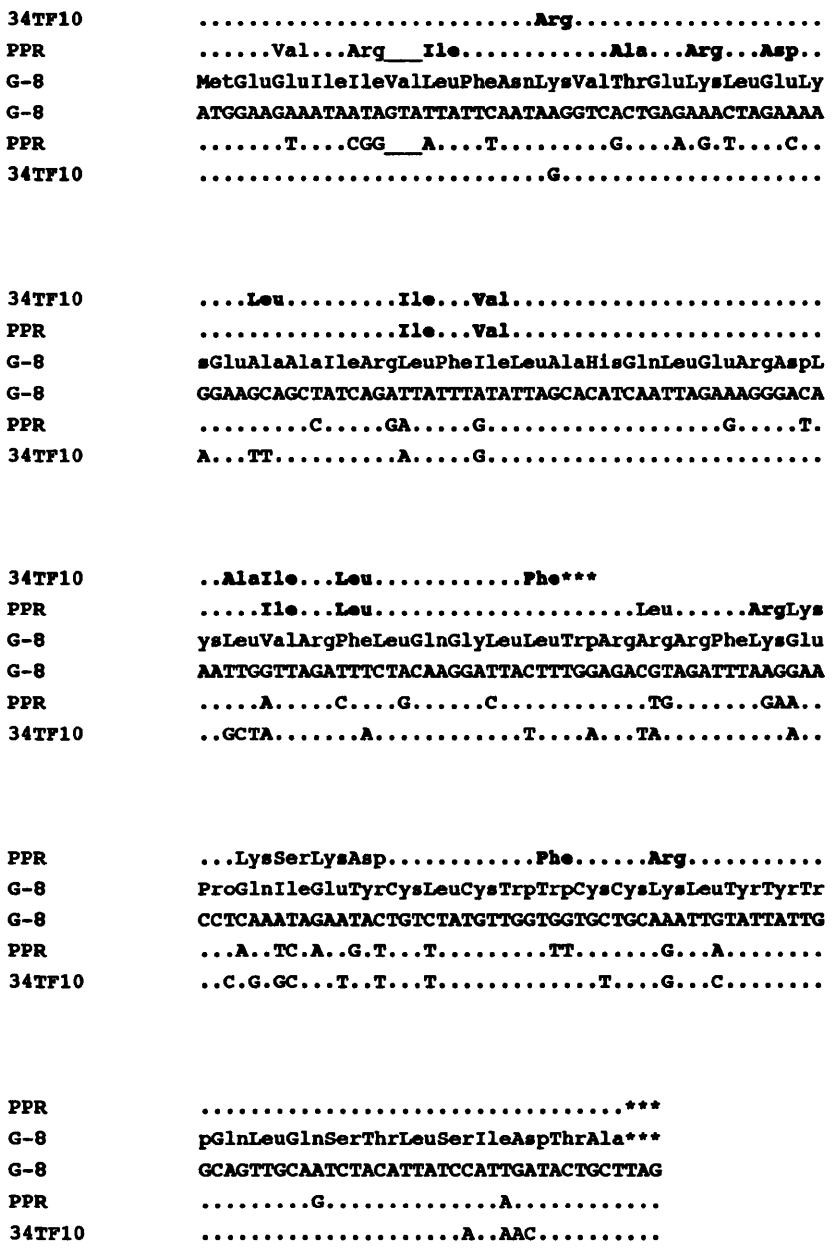
T1: 5'-GCCGGGATCCATGGAAGAAATAATAGTATTAT-3'

6345 6322

T2: 5'-GCCGGAATTCGTGTTGCTATATCAAAATCTAATA-3'

The numbers above the sequence refer to the position of the oligonucleotide in the FIV-Petaluma genome (41) and the underlined bases show the restriction enzyme sites and the 'GC' clamps which were incorporated to aid subsequent digestion and cloning. These oligonucleotides were used as primers in PCR reactions with FIV-G8 infected cell DNA (2.2.2.3). This reaction generated a 356 base pair product flanked by unique restriction sites. The product was gel purified (2.2.2.4, 2.2.2.5) and digested with *Bam*-H1 and *Eco*-R1 restriction enzymes (2.2.2.6). This cohesive-ended fragment was ligated to *Bam*-H1/*Eco*-R1 digested pIC-20H (2.1.4, 2.2.3.1) and positive recombinants were screened and amplified (2.2.3.2-2.2.3.4.2). This region of FIV G-8 was sequenced (2.2.4.1) (Figure 6.1) using universal and reverse primers (Figure 5.1) and a protein of ~8kDa was predicted. From the sequence analysis and the comparison of the predicted amino acid sequence it was evident that this region of the genome is highly conserved between different FIV isolates (> 80% in amino acid identity) and that most of the actual amino acid changes are conservative giving an amino acid similarity of > 90%. Figures 6.1 and 6.2 show the comparison between the DNA sequence and the deduced amino acid sequence of ORF-2 from the G-8, PPR and 34TF10 (198) FIV strains.

Figure 6.1 Comparison of Nucleotide Sequence and Deduced Amino Acid Sequence of ORF-2 from Three FIV Isolates.



Comparison of nucleotide sequence and deduced amino acid sequence of ORF-2 from the 34TF10 and PPR clones of FIV (198) and the PCR generated fragment of FIV-G8. Nucleotide and amino acid changes are indicated and **bold** 3 letter codes indicate conservative amino acid changes. Stop codons are shown as (***) and gaps (_) are introduced to give optimal alignment. See Figure 6.2 for percentage relatedness between the sequences and text (6.2.1, 6.4) for further discussion.

Figure 6.2

Percentage Identities of Nucleotide and Deduced Amino Acid Sequence of ORF-2 from Three FIV Isolates.

	Nucleic Acid	Amino Acid	
	Identity	Identity	Similarity
PPR:34TF10	78%	73%	91%
PPR:G-8	82%	75%	89%
34TF10:G-8	87%	80%	98%

This figure shows the percentage identities between the nucleic acid sequence and the deduced amino acid sequence of the PPR and 34TF10 molecular clones (198) of FIV and the PCR fragment of ORF-2 from FIV G-8. The amino acid similarity calculation ignores conservative amino acid changes. See Figure 6.1 for sequence details and (6.2.1, 6.4) for further discussion.

6.2.2 Production of ORF-2 expression vectors.

The ORF-2 gene fragment was sub-cloned into several expression vectors. The GST-fusion-gene vector system, pGex (2.1.4) was digested with *Bam*-H1/*Eco*-R1 and ligated to the digested PCR product (2.2.12.1). Putative recombinant clones were screened (2.2.3.4.1) and positive recombinants were amplified (2.2.3.4.2) and checked by sequence analysis (2.2.4.1) prior to induction with IPTG and analysed for protein expression on SDS-Page gels (2.2.12.2). Several such attempts were made to generate a functional protein-expressing clone but unfortunately none were found.
The eukaryotic expression vectors pCMV-IE (2.1.4) and pBabe (2.1.4) were digested with *Bam*-H1 or *Bam*-H1/*Eco*-R1 respectively. ORF-2 fragments were excised from pIC-20H-ORF-2 with *Bam*-H1/*Bgl*-II or *Bam*-H1/*Eco*-R1. These fragments were ligated to the expression vectors and positive recombinants were selected, purified and checked by sequence analysis using the T1 and T2 oligonucleotides, as detailed above (6.2.1)

6.2.3 Eukaryotic cell transfections.

Eukaryotic cell transfections were performed as outlined in (2.2.9.1) and controls for DNA concentration, DNA uptake and protein concentration in the cell extract, as outlined in (5.2.3), were maintained throughout these experiments.

6.2.4 FIV infection.

CRFK cells were incubated with Glasgow-8 FIV-infected cell supernatant (kindly provided by Dr. M. Hosie). After 12 hours fresh medium was added and the cells were cultured for a further 24 hours before transfection. At all stages in the procedure FIV infection was monitored using the antibody

based enzyme linked immunosorbent assay (ELISA) kit (Idexx). All of these tests were performed by Dr. M. Hosie.

6.3 Results.

6.3.1 Transfection of FIV infected cells.

CRFK cells were infected with the Glasgow-8 strain of FIV as outlined above (6.2.4). Cells were plated and transfected (2.2.9.1) with the wild-type FIV-LTR-CAT construct, the deletion panel of LTR-CAT constructs (Figure 5.2) and the control plasmids pLW2 (positive) and pCAT12 (negative). CAT assays (2.2.10.4) were performed on the cell extracts (2.2.9.1) and Figure 6.3 shows the average counts (2.2.10.4) from three experiments, where each test was performed in duplicate (also Figure 6.5 panel a and c). The figures are expressed relative to wild-type activation (1.00). These results showed no significant increase in the activity of the full length FIV-G8 LTR in FIV infected cells. However a *trans*-activation effect of up to 10 fold was observed when the AP-1/AP-4 site was deleted and this increase in gene expression driven by the FIV LTR remained even after deletion of the C/EBP and ATF sites (3 and 7 fold respectively) (see Figure 6.5 panel a and c).

6.3.2 Co-transfection with infectious molecular clones of FIV.

In a second approach FIV *trans*-activation was studied following co-transfection with molecular clones of FIV. The molecular clones of FIV were obtained from Dr. J. Elder (Scripps Clinic, La Jolla, California, 2.1.4, 43, 198). These molecular clones, FIV-PPR and FIV-34TF10, were co-transfected with either wild-type FIV-LTR-CAT plasmid, FIV-LTR-CAT deletion mutants or the positive and negative control plasmids into CRFK cells (sub-clone ID10) (2.2.9.1). Cells were harvested 48 hours after infection and tested for CAT

Figure 6.3

Relative Activities of Wild-type and Deletion Mutant FIV-LTR-CAT Constructs in Infected and Uninfected CRFK Cells.

		Cell Line		
LTR	Site Deleted	CRFK	CRFK + FIV	Fold Activation
Wild-type	-	1.00	1.00	1.0
-176	-	0.85	1.00	1.2
-147	"NF-κB"	1.11	1.03	0.9
-126	AP-4	0.55	0.87	1.5
-113	AP-1	0.07	0.70	10.0
-68	C/EBP (NF-1)	0.04	0.12	3.0
-47	(NF-1) ATF	<0.01	0.07	7.0

This table details the activity of wild-type and deletion mutant FIV-LTR-CAT constructs after transfection into uninfected and infected (+ FIV) CRFK cells (sub-clone ID10). The numbers on the left refer to the position in U3 where the deletion begins and the proposed site that was deleted in each case is indicated. The figures show the relative CAT activities of the mutant panel normalised to the full length LTR (1.00) in uninfected cells. Each figure represents the average of 3 experiments where each sample was transfected in duplicate. The fold activation between the levels of expression in uninfected cells compared to FIV infected cells is given in the final column. See Figure 6.5 panels a and c for plots of the above figures and text (6.3.1, 6.4) for further discussion.

activity (2.2.10.4). The recorded levels of CAT activity for this experiment are given in Figure 6.4. The figures are expressed relative to wild-type activation (1.00) and the fold activation in the presence of the molecular clones is given in brackets. From these results it can be seen that again there is no discernible variation in the expression of the full length LTR and very little effect on deletion mutants (-176 to -126). However, consistent with the previous results, a more dramatic effect was seen when the AP-1 site was deleted (-113) (Figure 6.5 panel a, b and d). In the co-transfection with the FIV-PPR molecular clone re-activation was lost after deletion of the C/EBP site and then partially regained after deletion of the ATF site (Figure 6.5 panel a and b). In contrast co-transfection studies using the FIV-34TF10 clone (Figure 6.5 panel a and d) did not significantly alter the pattern for the LTR-CAT transfection alone, suggesting some functional difference in the 34TF10 clone. The activity of the positive and negative control plasmids showed little variation throughout the experiment. See Appendix F for discussion.

6.3.3 Co-transfection with the FIV ORF-2 gene.

It has been postulated that ORF-2 in the FIV genome may express a putative *trans*-activator protein Tat (198). Several attempts to express this open reading frame as a GST-fusion protein were unsuccessful, hence no antibodies to this protein have yet been produced. Screening for ORF-2 expression from eukaryotic expression vectors CMV-IE-ORF-2 and pBabe-ORF-2 (6.2.2), with pooled serum from FIV infected cats also proved unsuccessful. However it is not known whether this was due to a low level of antibodies to this gene product in FIV infected cat serum or due to low protein expression from the constructs. Furthermore co-transfection studies using these expression vectors together with wild-type FIV-LTR-CAT and control constructs were carried out in a number of cell lines (CRFK, AH927 and HeLa, 2.1.5). However no significant increases in the level of FIV-LTR

Figure 6.4

Relative Activities of Wild-type and Deletion Mutant FIV-LTR-CAT Constructs after Co-transfection with FIV Molecular Clones.

			Molecular Clone	
LTR	**Site Deleted**	**CRFK**	**CRFK + FIV-PPR**	**CRFK + FIV-34TF10**
Wild-type	-	1.00	1.00 (1.00)	1.00 (1.00)
-176	-	0.80	0.60 (0.75)	0.45 (0.56)
-147	"NF-κB"	1.30	0.90 (0.69)	1.60 (1.20)
-126	AP-4	0.70	0.75 (1.07)	0.67 (0.96)
-113	AP-1	0.05	0.76 (15.2)	0.06 (1.20)
-68	C/EBP (NF-1)	0.09	0.02 (0.22)	0.13 (1.40)
-47	(NF-1) ATF	<0.01	0.04 (4.00)	0.05 (5.00)

This table details the activity of wild-type and deletion mutant FIV-LTR-CAT constructs after co-transfection into CRFK cells (sub-clone ID10). The numbers on the left refer to the position in U3 where the deletion begins and the proposed site that was deleted in each case is indicated. The figures show the relative CAT activities of the mutant panel normalised to the full length LTR (1.00). The fold activation in the levels of expression after co-transfection with FIV molecular clones (PPR, 34TF10, 43, 198) is shown in brackets (). See Figure 6.5 panels a, b and d for plots of the above figures and text (6.3.2, 6.4) for further discussion.

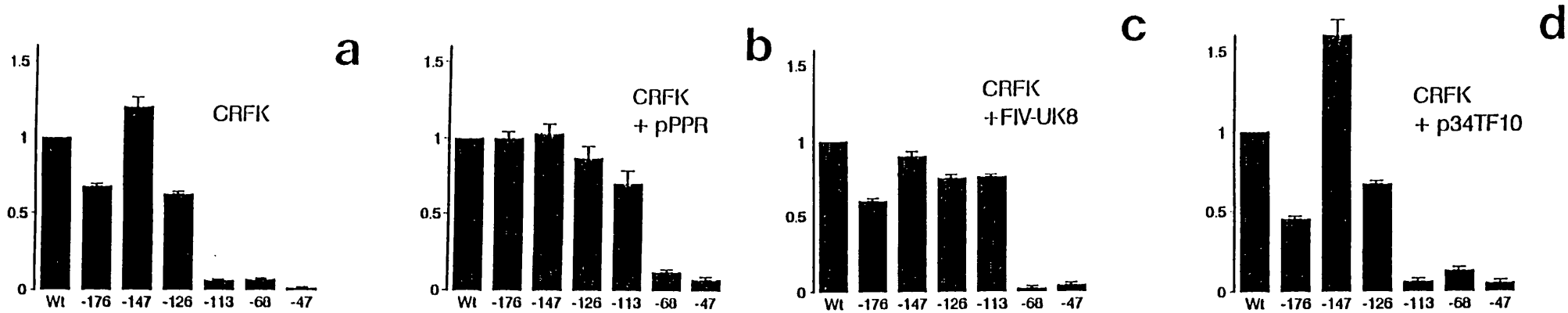
driven gene expression was recorded in these experiments (results not shown). Therefore the identity of the ORF-2 product as a Tat-like protein remains unproven.

6.3.4 Heterologous *trans*-activation.

As noted previously there are interesting parallels between FIV and visna virus which, unlike EIAV, lack predicted gene products and RNA structures resembling HIV Tat and TAR (24, 161, 327). Furthermore the AP-1 / AP-4 site in the visna virus LTR which is important for basal expression and is thought to be the Tat *trans*-activation target (24) is closely homologous to the FIV LTR site (Figure 3.9). It therefore seemed possible that FIV and visna virus share a common *trans*-activation mechanism involving direct interactions between viral and cellular transcription factors and that the better characterised visna virus Tat might *trans*-activate the FIV LTR. This was indeed suggested by preliminary studies in which the FIV-LTR-CAT constructs were found to be much more active (20X) in Vero cells (2.1.5) stably expressing visna virus Tat than in Tat minus control cells (162). However when these Vero cell experiments were repeated using the deletion panel of FIV LTR mutants it was not possible to localise the *trans*-activation effect to any particular binding site in the FIV LTR since all mutants were equally efficiently *trans*-activated as was the positive control plasmid (pLW2). Furthermore we found no measurable *trans*-activation when a plasmid encoding visna virus Tat (2.1.4) was co-transfected into CRFK cells (sub-clone ID10) with the deletion panel of FIV-LTR-CAT reporter constructs.

Figure 6.5

Plots of the Relative Activities of Wild-type and Deletion Mutant FIV-LTR-CAT Constructs Under Different Cellular Conditions.



These graphs show the relative activities of wild-type and deletion mutant FIV-LTR-CAT constructs in CRFK cells (sub-clone ID10) (Panel a), in CRFK cells infected with G-8 FIV (Panel c) or following co-transfection with full-length molecular clones of FIV-PPR (Panel b) and FIV-34TF10 (Panel d). The numbers on the x-axis refer to the position in U3 where the deletions begin (Figure 5.2). The figures on the y-axis correspond to the relative CAT activities of the mutant panel normalised to the full length LTR (1.00) (See Figures 6.3 and 6.4 for data). Results were an average of three experiments where each sample was transfected in duplicate and standard deviations are represented by extension bars. See text (6.3 and 6.4) for further discussion.

6.4 Discussion.

The existence of viral encoded proteins which regulate lentiviral gene expression have been well documented (Reviewed in 6, 21). Two distinct mechanisms of lentiviral *trans*-activation have been defined (6, 126). Experiments have shown that upstream U3 sequences in the viral LTRs are important for viral *trans*-activation during visna and CAEV infection (24, 222, 279) and that the downstream R region (TAR) of the LTR is of primary importance in HIV, SIV and EIAV (6). However sequence analysis of the FIV LTR and RNA secondary structure predictions showed no obvious TAR-like stem loop.

Therefore in an attempt to define any viral *trans*-activation effect on FIV gene expression a number of preliminary experiments were carried out. However, very little evidence of *trans*-activation was seen with the wild-type G-8-FIV-LTR-CAT construct in transfection experiments carried out in FIV infected CRFK cells. Furthermore co-transfection experiments with molecular clones of FIV together with full length FIV LTR constructs showed no significant *trans*-activation, in any of the cell lines tested.

These results compare with studies of the visna virus LTR which was found to have a high basal activity in most cell lines (24) and *trans*-activation of the visna virus LTR was also low in virus infected cells. However more significant levels of *trans*-activation were observed when visna virus Tat was over expressed in a stable environment (24, 161, 319).

Several small ORFs, evident in the FIV genome have been shown to be conserved in a number of FIV clones (198). Due to the size and location in the FIV genome, it was postulated that ORF-2 may encode a *tat*-like gene product (Figure 6.6A) (198). This area of the genome is highly conserved between different viral strains (Figure 6.1 and 6.2) and it therefore seems likely that this region encodes an important viral protein/function.

Furthermore there are splice donor and splice acceptor sites (198) that would

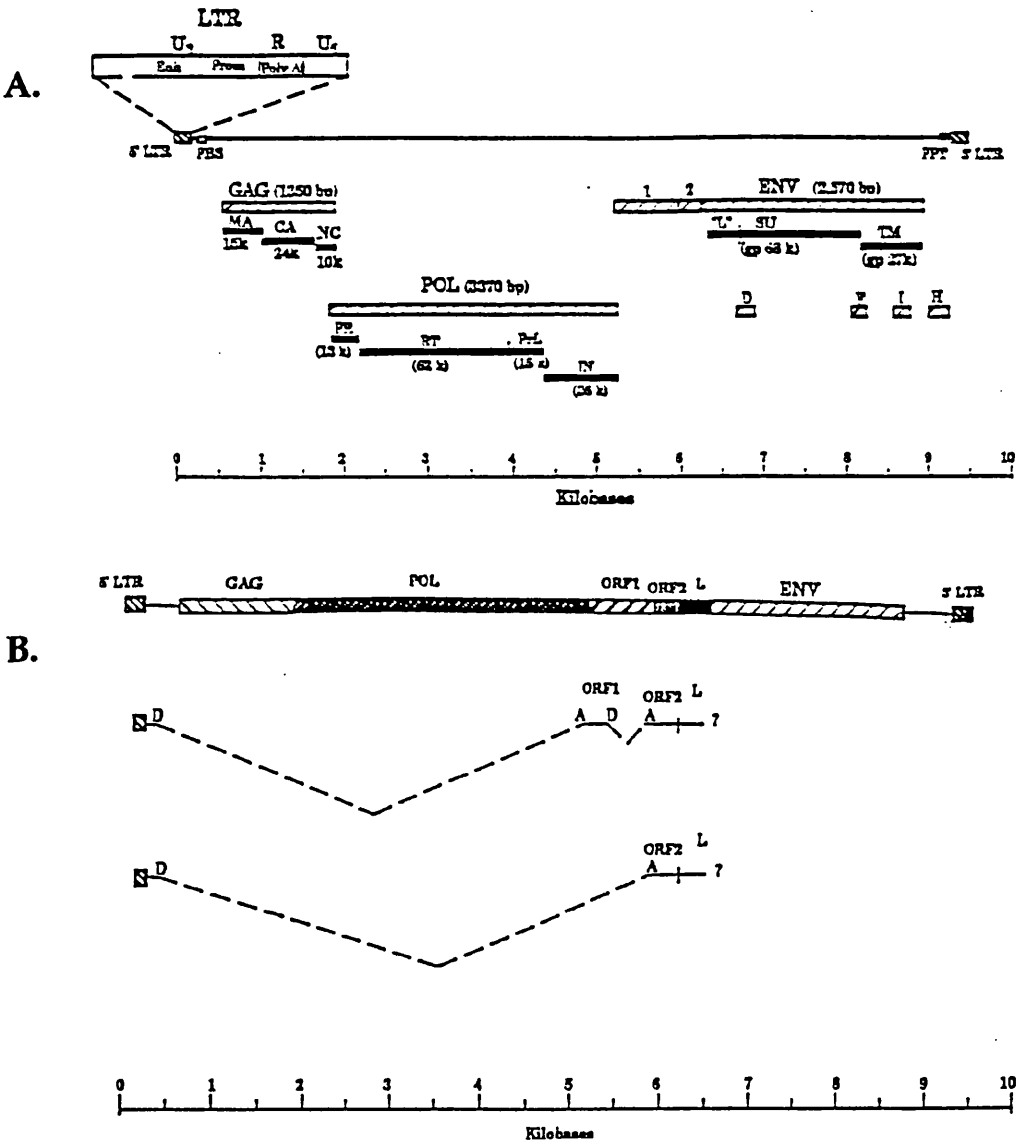
allow expression of this region as a putative product in FIV infected cells (Figure 6.6B). Several attempts to express this open reading frame in a prokaryotic expression vector failed, making the validation of eukaryotic vectors expressing the FIV ORF-2 protein product less satisfactory. Furthermore transient co-transfections with these ORF-2 expressing clones and wild-type FIV-LTR-CAT plasmids did not give significantly greater *trans*-activation levels than the experiments using infected cells or molecular clones. Therefore it remains to be confirmed whether or not a functional ORF-2 protein product is expressed by any of the constructs.

The constructs generated in this study contain a single exon encoding the putative *tat* gene (198). However Tat products are often encoded by multiply spliced transcripts in other viral systems (6, 8, 46, 47, 57, 75, 161, 339, 340). Recent analyses of the splicing pattern of FIV transcripts in infected cells did not reveal messages below 1.4kb (328). Therefore since ORF-2 only encodes a 356 base pair product it may be that the putative FIV Tat product is also encoded by more than one exon (67, 328, 368). To date cDNA cloning of FIV infected cells has identified several multiply spliced transcripts some of which contain an in-frame ORF-2 (Figure 6.7) but no Tat-like function has been observed from any of these clones (67, 368).

Therefore it is apparent that more work is required in order to identify the FIV *trans*-activator. However since the full-length FIV LTR has a high basal activity (67, 346) and may only respond to *trans*-activation under certain cellular conditions (See Chapter 7 for further discussion) it may be necessary to use deletion or site-directed LTR mutants in further *trans*-activation studies. Furthermore it would be beneficial to construct stable cell lines containing wild-type and mutated molecular clones of FIV as these are more representative of the *in vivo* environment usually encountered by the virus than those generated by transient transfection.

The sequences in the visna virus LTR that respond to viral *trans*-activation are located upstream of the transcriptional initiation site in the viral

Figure 6.6 Genomic Organisation of FIV.



A: This figure shows the consensus genomic organisation of FIV. Features of both LTRs are shown in the 5' LTR. The *gag* region encodes the matrix (MA), capsid (CA) and nucleocapsid (NC) proteins. The *pol* region encodes protease (PR), reverse transcriptase (RT), protease-like protein (PrL) and integrase (IN). The *env* region encodes the putative L protein (43, 161) as well as the major (SU) and minor (TM) glycoproteins of the viral envelope. In addition to the above genes six short ORFs (1 (putative *vif* gene (73)), 2, D, F, I and H) were evident and conserved in both the US strains of FIV. Polypurine tract is indicated as (PPT) and the primer binding site (PBS) is shown. Adapted form (198).

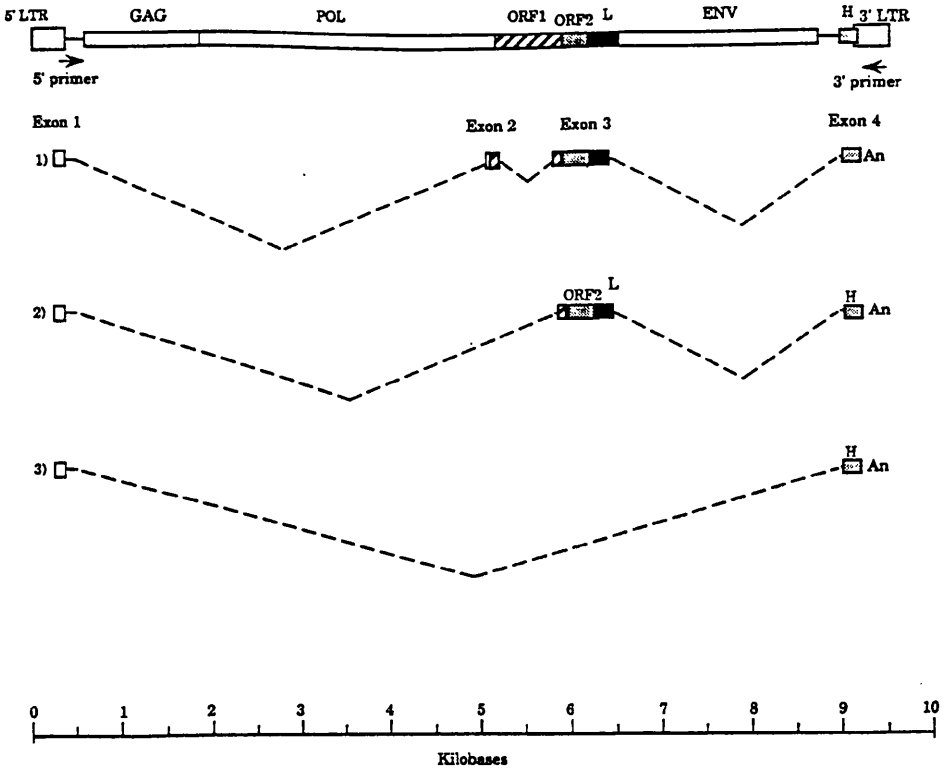
B: This figure shows the potential splice donor (D) and splice acceptor (A) sites in the 34TF10 isolate of FIV. The major ORFs of FIV are shown along the top. Two potential sub-genomic RNA species of the 34TF10 clone of FIV (43) are shown below. Both of these potential transcripts include ORF-2 which may code for the FIV Tat function (198). See text (6.4) for further discussion. Adapted from (198).

promoter/enhancer region of U3 (24). Preliminary studies have implicated AP-1 binding sites as possible *cis*-acting elements in virus mediated *trans*-activation (24). Experiments have shown that in a transfected Vero cell line which stably expresses visna virus Tat, *trans*-activation of the visna LTR can be as high as 45 fold and deletion mutants of the LTR implicated the AP-1/AP-4 site as the major recognition element for this increase in expression (24). In similar co-transfection experiments using visna-virus-Tat constructs and CAEV-LTR-CAT or SV40 promoter-CAT construct an increase in CAT activity driven by these promoters was also seen (162). Furthermore a 20 fold increase in the activity of the FIV-LTR was observed. However in contrast, the expression driven by the HIV-1 LTR was not affected by visna virus Tat expression (162). Experiments using the panel of FIV-LTR deletion constructs showed that levels of gene expression from all the constructs were similarly elevated in Vero-Tat cells so that the effect could not be attributed to any specific enhancer element. However since all the constructs contain the sequences from -47 onwards it cannot be excluded that visna Tat functions at a specific site in this minimal promoter region.

These studies do not rule out the possibility that the reported and observed effects of visna Tat are due to clonal variation of the Vero-*tat* cells since even the expression from the positive control plasmid was increased significantly in the Vero-*tat* cell line, alternatively this may indicate a lack of promoter specificity in the activity of the visna Tat product (See Chapter 7 for further discussion). Further mutational analysis of the FIV LTR and the development of an inducible visna Tat expression system would help to resolve these questions.

So far results from experiments on the FIV LTR deletion mutants in FIV infected cells or in co-transfections with molecular clones seem to indicate that *trans*-activation of FIV occurs through U3 sequences which is similar to the mechanism outlined for visna virus gene regulation.

Figure 6.7 Map of FIV cDNA Clones.



This figure shows a map of the cDNA clones isolated from FIV infected cells that have been studied to date. At the top is a diagrammatic representation of the ORFs of FIV. The splicing pattern and ORFs of all three cDNA clones are indicated. The dotted lines represent introns and the rectangles represent exons. 'An' indicates the poly (A) tail. See text (6.4) for further discussion. Adapted from (67).

In contrast the HIV-1 LTR contains binding sites for several constitutive and inducible transcription factors however the basal level of transcriptional activity of this promoter is quite low in most established cell lines (286, 331). Expression of the HIV-1 *trans*-activator Tat results in a large (up to 100 fold) increase in HIV-1 LTR dependent gene expression (31, 115). *Trans*-activation of HIV-2 and SIV is achieved by a similar mechanism and HIV-2 and SIV TAR regions have been shown to provide fully functional targets for *trans*-activation by HIV-1 Tat (322-325, 332).

In co-transfection experiments, in a number of cell lines, we did not observe any *trans*-activation by HIV-1 Tat on the FIV LTR driven gene expression. In all the cell lines tested the HIV-1-LTR-CAT plasmid (2.1.4) showed a very low basal level of activity compared to the FIV-LTR-CAT plasmid. However when the HIV-1 LTR was co-transfected with a plasmid which expresses HIV-1 Tat, RC-P1, (2.1.4) (154), a significant increase in the level of *trans*-activation was observed, but a similar effect on the FIV-LTR was not observed (results not shown). This may indicate further that the FIV *trans*-activator does not function via a Tat-TAR mechanism but is similar to visna virus and CAEV in that *trans*-activation occurs through upstream U3 sequences. However it may be due to the fact that HIV-1 Tat cannot function in the FIV system in the same way that HIV-1 and HIV-2 Tat-TAR mechanisms are not reciprocal (6, 322-325, 332). Therefore further experiments are required before the exact mode of action of a FIV *trans*-activator is fully understood.

CHAPTER 7

Discussion

In the course of evolution retroviruses have found many ways to regulate the expression of their genes and thus their replication. They have borrowed heavily from the very cells they infect, acquiring elements that respond to or interfere with cellular differentiation, hormonal and/or physiological activation or even components of endogenous viruses. In these cases high expression also results in cytopathology, ie proliferation leading to unchecked growth and neoplastic transformation or cell death. Our challenge is to understand the physiological processes and proviral elements that lead to increased viral gene expression and replication, in the hope that this may lead to the ultimate goal of rational interference with the viral life cycle and with the development of retroviral diseases.

Several well characterised retroviral systems have highlighted the interaction between cellular processes and replication. Examples are the developmental regulation of Moloney murine leukaemia virus; the hormonal regulation of MMTV; the regulation of HIV by lymphoid activation signals; the regulation of HIV and human T-cell leukaemia virus (HTLV) by homologous viral *trans*-activators and finally regulation of HIV by heterologous viral *trans*-activators. In all of these cases long terminal repeats (LTRs) and virus encoded *trans*-activators help determine viral host range, tissue tropism and pathogenesis of disease (Reviewed in 386).

Therefore in an attempt to define the mechanisms involved in the regulation of FIV gene expression this study was initiated.

FIV, which belongs to the lentivirus group of retroviruses is closely related in cell tropism and genome organisation to HIV, SIV and the ungulate lentiviruses. (32, 33). It is known that FIV is cytopathic in tissue culture and has a tropism for the $CD4^+$ subset of T-cells, macrophages and other cells (44, 292, 387, 388, 397).

Two infectious molecular clones of FIV (34TF10 and PPR) have been fully sequenced and studied in some detail (198). Infectious clones have constraints on their sequence variability as critical viral functions must be maintained for the virus to remain viable. Thus essential functional domains of FIV should be conserved between infectious molecular clones. However the US strains of FIV that were studied differed in an important biological property, their *in vitro* host cell range. In other retroviruses host cell alterations have resulted from changes in the LTR and/or the *env* regions of the virus (18, 389-396). It is likely that one or both of these regions are also responsible for the host cell range of FIV. However the tissue restriction shown by the molecular clones of FIV did not correlate with the ability of the LTR to function as a promoter in various cell types. This study (Chapter 4) has also shown that there is no obvious correlation between any cell-type specific binding activity and permissiveness for FIV replication. Consequently tissue restriction of FIV replication may involve other factors such as early stages of infection (e.g. attachment or entry and integration) or post-transcriptional events (e.g. translation of viral messages or assembly and release of infectious virions). Therefore further studies are required before the exact determination of the factors influencing host cell range can be identified.

FIV proviral DNA is approximately 9.4kb in length (43) and encodes both structural and regulatory gene products (Figure 6.6). In addition to the *gag* (virion core products), *pol* (reverse transcriptase, endonuclease and integrase) and *env* (envelope glycoproteins) genes FIV also encodes a post-transcriptional regulator of viral mRNA processing, *rev* (67, 367) and *vif* which is necessary for cell free virus infection in feline T-lymphoblastoid cells (73). There is also a conserved open reading frame (ORF-2) whose function is as yet unknown. The genetic organisation and transcription pattern of FIV is similar to other members of the virus group which includes

primate immunodeficiency viruses (HIV-1, HIV-2 and SIV) visna virus, CAEV, and the lentiviruses isolated form horses (EIAV) and cows (BIV) (6, 386). FIV appears to have fewer genes than the primate lentiviruses and consequently displays a simpler splicing pattern (67, 198, 328, 368) and it is likely that this will also be reflected in differences in the post-transcriptional regulation of FIV mRNA synthesis.

A comparison of the FIV LTR obtained from different viral strains revealed a high degree of similarity (Figure 3.3) with divergent base positions clustered at several sites. This finding is largely consistent with other lentiviral LTRs. However a significant heterogeneity has been found in the LTR of different EIAV isolates (236, 237, 386). A number of studies have shown that variants of EIAV contain duplications and insertions of factor binding sites within a 30bpr region of the LTR. This diversity may also be manifested in varied patterns of expression in certain tissues or in response to specific activation signals. It is not yet known whether the distribution of LTR variants changes during the course of infection and whether these differences in the EIAV LTR sequence correlate with tissue tropism, pathology or disease course. However this large sequence variation has not been found in the FIV disease system. Therefore it will be interesting to study more FIV LTR sequences from a wider range of different countries and also from highly pathogenic strains of FIV to see if there are any changes in the LTR sequences which may have produced an altered viral phenotype.

It will also be of interest to look for alterations in FIV LTR sequences which are recovered from any FIV positive tumours that are found. Since tissue specific enhancer duplications have been identified in the U3 regions of the LTR in FeLV proviruses cloned from naturally occurring T-cell tumours (268) or from splenic lymphomas (Dr. R. Fulton, Glasgow University, personal communication) and in the MuLV system (216, 290) and it is considered that cell type differences in binding activities to the motifs

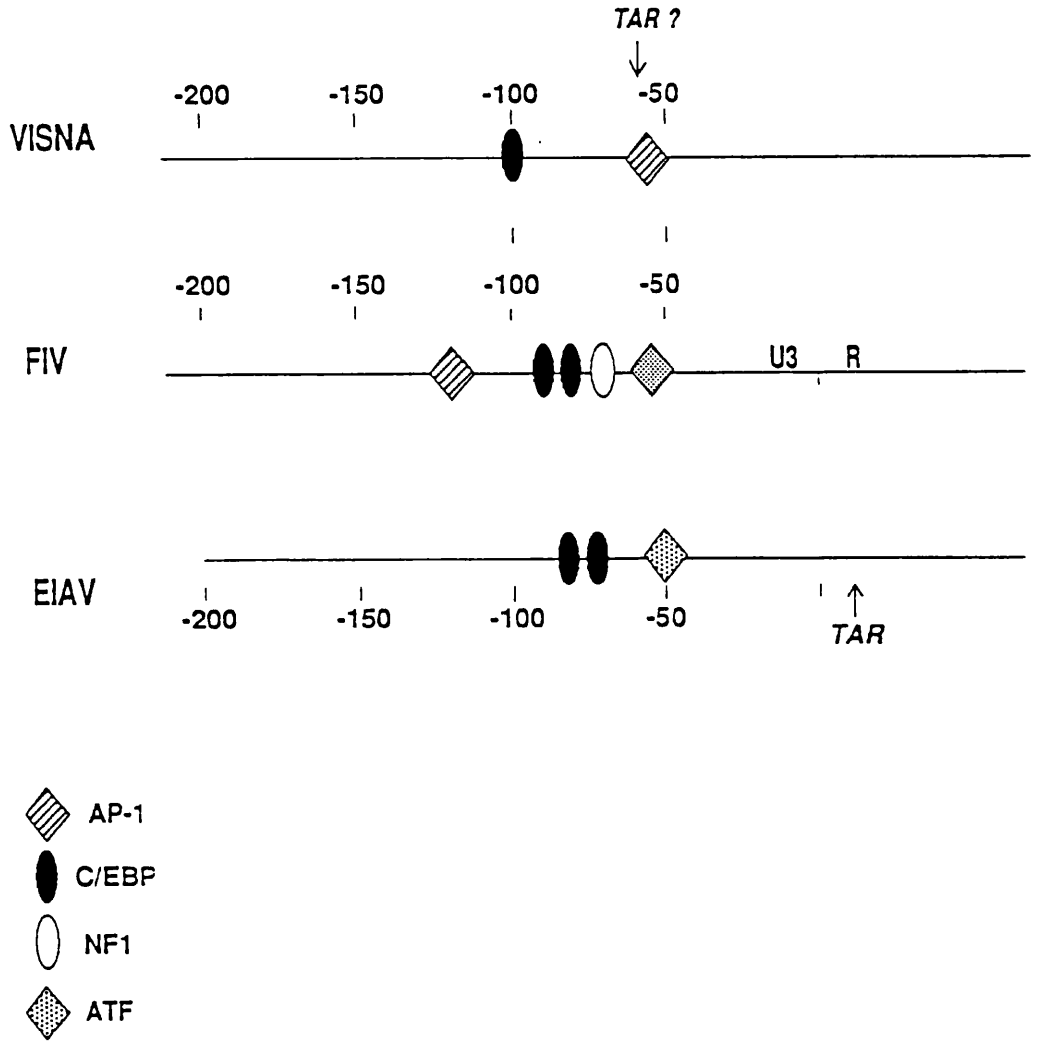
present may underlie the selective process which leads to outgrowth of viruses with specific sequence duplications (268). Furthermore monoclonally integrated HIV-1 sequences have been found in a number of human lymphomas isolated from AIDS patients (54, 330). However it is not yet clear whether the HIV-1 infection had a central role in the lymphocyte transformation process. To date several tumours from FIV positive cats have been studied by Southern blot analysis but no FIV proviral DNA sequences have been identified in the tumour genome (J. Callanan and A. Terry, personal communication).

The organisation of the FIV LTR is unique but shares common features with the LTR elements of other animal lentiviruses, notably visna and EIAV (Figure 7.1).

Binding site 1 in the FIV LTR (-130/-120) centres on a consensus AP-4/AP-1 motif which is closely homologous to regulatory sites in the SV40 late promoter (240) and the visna virus LTR. In visna this site is known to be important for basal activity and serum induction (22, 24) and has been shown to bind the heterodimeric complex of Fos and Jun proteins (307). In the FIV LTR the AP-4/AP-1 site also appears to be necessary for *trans*-activation by feline herpes virus type 1 (397). This suggests a related mechanism for regulation of FIV and visna virus by inducible and regulatable cellular transcription factors.

The most striking similarity in organisation of the FIV LTRs is seen in the WY012 strain of EIAV which has 2 C/EBP motifs and an ATF motif in similar spatial relationship (Figure 7.1) to the functional TATA box (386) which may indicate similarities in the regulation of gene expression by both EIAV and FIV and/or highlight shared mechanism of control.

Figure 7.1 Schematic Diagram of the Similarities between the Positions of the Protein Binding Sites Identified in Several Animal Lentivirus LTRs.



This schematic diagram shows the similarity between the types and spatial positioning of the protein binding sites that have been identified in the FIV, EIAV and visna virus LTRs. See discussion for further details. LTRs are numbered with respect to their CAP site (0).

DNase I footprinting analysis identified binding sites for multiple transcription factors in the U3 region of the FIV LTR (Figure 4.1). Deletion mutagenesis showed that these sites play a vital role in basal promoter activity in a variety of cell lines (Figure 5.5 and 5.6). In particular AP-4/AP-1 and ATF sites at -120 and -55 respectively are necessary for full basal activity. The full-length FIV LTR was not detectably *trans*-activated but the activity of a deleted promoter construct lacking the AP-4/AP-1 motif could be partially restored by *trans*-activation (Figure 6.3, 6.4 and 6.5).

These results shed further light on mutational analysis of the FIV LTR performed prior to direct identification of the protein binding sites. Deletion mutants of the FIV TM-1 LTR which were assayed in fcwf-4 feline macrophage-like cells showed a similar 10-fold loss of activity on deletion of the AP-4/AP-1 motif (397). However in that study deletion of the C/EBP motifs led to reduction of promoter activity to background levels while similar deletion mutants in this study retained measurable activity. This apparent inconsistency may be due to the different target cells used in the respective transfection studies or to functional differences between the FIV LTR elements studied. For example the TM-1 isolate differs from FIV-G8 in the NF-1 motif (Figure 3.8) which was identified as a protein binding site in the footprinting analysis. NF-1 is a positive regulator of the feline leukaemia virus LTR (267) and it is conceivable that it plays a similar role in FIV-G8 but not FIV-TM-1. It will be interesting to study the difference between TM-1 and G8 LTR directed gene expression in different cell lines following deletion or mutation of this site.

In another previous study on the FIV Petaluma (34TF10, 346) strain mutations introduced into either of the AP-1 or ATF motifs led to 8-10-fold decreases in basal promoter activity in CRFK cells. These mutations appeared to have some selective phenotypic consequences since the AP-1

mutation had a greater effect on protein kinase C-mediated responses while the ATF mutation was more deleterious to c-AMP responses mediated by protein kinase A (346). In view of the cross-competition between AP-1 and ATF by an overlapping set of factors (287) it might be considered that one intact site could mediate both responses, albeit less efficiently. It would therefore be interesting to examine the effect of mutating both sites which might be expected to have much more drastic consequences for promoter function.

In a recent study (398) it was shown that the AP-1 binding site in the LTR of FIV-TM-2 is important for achieving maximal expression of the FIV genome but that the site is not required for the replication of FIV in feline T-lymphocytes *in vitro*. But it remains possible that this region is required for full biological activity *in vivo*. In transient transfection assays the AP-1 site of the FIV LTR has been reported to be important for the responsiveness to T-cell activation signals (346) and it is therefore likely that the AP-1 site in the FIV LTR plays a role in an initial increase of viral gene expression in response to T-cell activation signals. After initial augmentation of transcription, viral replication might occur irrespective of the presence of AP-1 and depend on other cellular factors in the cells, although it remains possible that the AP-1 in T-cells can bind to and therefore function via an alternative site such as ATF (398).

DNase I footprinting assays also defined a protein binding site in the U5 region of the G8 LTR (Figure 4.7). This protein appears to be most abundant in nuclear extracts from feline T-cells. However competition analysis across this site with a range of consensus oligonucleotides based on known protein binding site sequences did not give any clues as to the identity of proteins binding at this site. Site directed mutagenesis of this region should be undertaken to examine possible functions for this binding site. In studies on

the HIV LTR, no protein binding sites have been identified in U5 although some sites have been found in the R region of the LTR (386). It is not yet entirely clear what function these sites have but it has been proposed that they may have either a repressive function on the initiation of LTR driven gene expression (207-209) or that they may be involved in the attenuation of transcription (136).

These studies also identified a weak negative regulatory element which mapped to the 5' end of U3 in the FIV-G8 LTR (Figure 5.5). The effect was slight in AH927 and CRFK cells but much more marked in HeLa cells. This is similar to the results of a recent study on EIAV where a NRE was identified between -110/-90. However the NRE effect was cell type specific, being detected in FEA and HeLa cells but not in D17 cells (canine osteosarcoma). DNase I and gel mobility shift assays have shown that this region of the EIAV LTR can form specific complexes with cellular proteins but the precise binding determinants and the nature of the factors that interact with this site are not yet known (329). 5' deletion mutants of Japanese and US FIV isolates showed a similar slight increase in fcwf-4 (397) and CRFK cells but a much larger effect in G355-5 brain-derived cells (346). It is interesting to note that the relevant area of the LTR includes a purine rich sequence (-135/-155) and that negative regulatory activity has been mapped to similar purine rich binding sites in the HIV and IL-2 gene promoters (114, 310, 311). Cellular factors such as NFAT (310) and interleukin binding factor (311) have been shown to interact with such motifs and it has been postulated that transcriptional repression is relieved by degradation of inhibitory proteins or their displacement by positive regulators (311). It would therefore be interesting to examine the effect of deleting this domain on virus replication *in vitro* and *in vivo*, and to study the footprinting pattern around this motif with an extensive range of nuclear protein extracts, especially with those extracted from HeLa or G355-5 cells.

The virus-coded *trans*-activators of HIV and the primate lentiviruses are essential for virus replication but despite the presence in FIV of small open reading frames in similar genetic location it has hitherto been unclear whether FIV encodes a Tat-like function (Figure 6.6 and 6.7). The results of these experiments show that significant *trans*-activation can be detected if the LTR is truncated past the AP-1/AP-4 motif (Figure 6.3 and 6.4). To explain these observations a model can be suggested in which complete activation of the full-length LTR is achieved via protein-protein interactions which occlude a viral *trans*-activator. Only when one of the target binding sites is removed by DNA deletion or one of the necessary factors (e.g. AP-1) is absent would *trans*-activation be seen. It should be noted that established cell lines often have aberrant constitutive expression of AP-1 as a function of their transformed state (345) and that such cells may therefore be unrepresentative of FIV infected cells *in vivo*. FIV target cells *in vivo* may conceivably include AP-1 deficient environments where viral *trans*-activators could play a significant role. Therefore further efforts should be made to transfect cells which are known to have low AP-1 levels and then to study the effect on LTR driven gene expression upon induction of AP-1. See Appendix G for further discussion.

Further work will be required to identify the FIV product(s) responsible for activating the minimal promoter element. The ORF-2 gene is a candidate since it is fully open in the PPR and FIV-G8 isolates but prematurely terminates in the 34TF10 clone (Figure 6.1) (198). However so far results have been unable to show any activity with constructs containing the isolated ORF-2. It is possible that ORF-2 does not encode the complete gene product or that a second virus-encoded protein is required to form a functional *trans*-activating complex. Alternatively there may be other functional differences between the three FIV strains which are responsible for these observations.

Ravazzolo *et al* (399) have recently published a study which shows that there was no significant *trans*-activation of the CAEV LTR in their system, which is in agreement with the results of this study on FIV. They also noted that the CAEV LTR was as readily *trans*-activated as that of visna virus when the effector protein was visna Tat suggesting that the lack of *trans*-activation is an intrinsic property of the CAEV Tat protein (400). It is unclear why visna virus should encode a more potent *trans*-activator than CAEV (399) or FIV (162) but one explanation may be that the Icelandic visna virus strain (K1514) is a highly adapted strain which has been selected for high titre growth in sheep fibroblast cells. Therefore it is possible that some naturally occurring point mutations in the *tat* gene of visna virus have been selected for and they in turn provide a more potent *trans*-activator in this cell type. Nevertheless the *trans*-activating activity of the non-primate lentiviruses FIV, CAEV and visna Tat is very low when compared to those reported with the HIV-1 system (6, 386). Therefore the significance of the Tat mediated *trans*-activation in these systems could be questioned. Indeed the basal level of activity of these promoters in fibroblasts is so high that one may wonder why these viruses need a *trans*-activating factor. However fibroblasts are not believed to be the main target cells for these viruses *in vivo* and could be a poor system to study the *trans*-activation by Tat. Although the level of visna *tat trans*-activation in the human promonocytic cell line U937 (399) was not found to be significantly higher than that observed in the fibroblast system, it remains to be seen if it would be more effective in primary cultures of goat or sheep monocytes. Alternatively it has been shown that visna Tat is moderately active on heterologous promoters including FIV, SIV and SV40 (162). This is in sharp contrast to the specificity of HIV-1 Tat but reminiscent of the weak and promoter non-specific *trans*-activation by HIV-1 Vpr (379). Given the weak but persistent similarity between CAEV *tat* and HIV and SIV *vpr* (401) a reappraisal of the role of some non-primate lentivirus *tat* genes is clearly needed.

There are interesting parallels between FIV and visna which unlike EIAV lack predicted gene products and RNA structures resembling HIV Tat and TAR (327). Also the AP-4/AP-1 site of the visna virus LTR, which is important for basal expression and is the target for *trans*-activation by *tat* is closely homologous to the FIV LTR site characterised in this study. It therefore seemed possible that FIV and visna virus share a common *trans*-activation mechanism involving direct interaction with cellular transcription factors. Although these results support a common role for the AP-4/AP-1 site in the regulation of basal expression in response to cellular signals experiments with truncated LTR promoters failed to support a role for this site in FIV viral *trans*-activation. In contrast the responsive deleted promoters retain C/EBP and ATF binding sites. Further deletion mutants show that the C/EBP sites are necessary for this response but do not rule out the possibility that the ATF motif is also required. In support of a possible role for this site in *trans* regulation, ATF has been shown to bind regulatory proteins involved in cyclic-AMP and E1-A-inducible transcription (226) and ATF sites are important for regulation by $p38^{tax}$ in the bovine leukaemia virus LTR (318). Furthermore ATF sites are important in the control of HTLV-1 gene expression (386, 402, 403). It has been shown that cellular proteins of the CREB/ATF family interact with a 21 base pair repeat (TRE-1) in the HTLV-1 LTR and this same site is known to confer the *trans*-activation responsiveness, via Tax-1, on viral gene expression (386). Therefore it would be interesting to study the activity of different site specific FIV LTR mutants and their response to different cell signalling pathways (e.g. induction of the cyclic-AMP pathway with forskolin) or if possible to study the effect of heterologous *trans*-activators by co-transfection experiments with BIV $p38^{tax}$ or adenovirus E1-A.

With hindsight it is apparent that this study was limited by the assay systems that were available and it would definitely be advantageous to

pursue T-cell transfections. This would allow one to study LTR driven gene expression in a more realistic environment and also to have the added advantage of low levels of certain binding factors which can be upregulated if and when desired. Furthermore all the work described here has been carried out with transient transfection assays which involve high copy numbers of reporter plasmids. This is a poor reflection of the state of viral DNA in an *in vivo* infection. Therefore stable cell lines with integrated FIV provirus or LTR-reporter gene plasmids would provide a better and more authentic environment and might also provide a more controllable model system. Since discrepancies between the effect of mutations on LTR-driven reporter gene expression and replication of the infectious molecular clones of FIV have been noted (67, 198, 346, 368, 398) we must be cautious in drawing too many conclusions from the *in vitro* transient assay data. It is therefore apparent that this area of research should be continued using either stable cell lines with integrated molecular clones of FIV which contain LTR or open reading frame (ORF) deletions or by following the disease progression in animals infected with mutated molecular clones of FIV.

In acquired immunodeficiency syndrome caused by HIV it is suspected that co-factors might be involved in the acceleration of the clinical stages of AIDS. The majority of opportunistic infections observed in AIDS patients are herpesvirus infections and it is thought that these viruses may act as co-factors that stimulate HIV gene expression which often results in the early death of the AIDS patient (404, 405). It has been reported that *in vitro* the LTR of HIV could be activated by herpesvirus (406-408, 411) papovavirus (407) adenovirus (408) and HTLV-1 (310).

Feline herpesvirus type-1 (FHV-1) causes a severe upper respiratory tract disease in cats (409). This disease occurs world-wide and is thought to be responsible for nearly half of all feline respiratory illness (410).

A study which was initiated to determine the effect on FIV LTR driven gene

expression by FHV-1 infection (411) demonstrated that FHV-1 did indeed *trans*-activate the FIV LTR *in vitro* indicating that this FHV-1 may act as a co-factor which could contribute to the acceleration of clinical disease in FIV infected cats.

It has also been reported that FeLV infection greatly potentiates the severity of the transient primary and chronic secondary stages of FIV infection *in vivo* (11) suggesting that FeLV may also act as a co-factor which can accelerate FIV pathogenesis. In an attempt to study this phenomenon *in vitro* FeLV infected fibroblasts were transfected with the FIV-LTR-CAT reporter plasmids however no effect on the level of gene expression was detected (results not shown). This may indicated that the FeLV-FIV interaction occurs via a mechanism that does not involve the viral LTR. However it should be kept in mind that up-regulation of FIV may occur through the LTR in other cell lines some of which may be target cells for the virus(es) *in vivo*. Therefore a more detailed analysis of the FIV-FeLV interaction is needed before the mechanism is understood.

In conclusion this study shows that host transcription factors which are expressed in response to cellular activation bind to the FIV LTR and play a prominent role in regulating its activity. Moreover viral *trans*-activation may have an accessory and possibly cell type specific role. Further work with viruses mutated in specific factor binding sites and small open reading frames will help to elucidate the complexities of FIV regulation *in vivo* and may assist in the design of attenuated viruses for vaccine application.

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Appendix A

In order to check the fidelity of the PCR reactions, FIV-G8 was amplified, from infected cell DNA, on four separate occasions. On comparison of the complete sequence of these four clones no changes were observed. Therefore we were confident that the differences observed in the LTR sequences isolated from the different viral strains were real and had not been introduced during the amplification and cloning procedure.

Appendix B

Enhancer binding protein AP-4 is a transcription factor that activates both viral and cellular genes by binding to the palindromic DNA sequence, CAGCTG. The gene encoding the human AP-4 protein has been cloned and characterised (271). AP-4 is a helix-loop-helix regulatory protein and distinct structural motifs responsible for transcriptional activation, DNA binding and dimerisation have been identified. AP-4 can form very stable homodimers *in vivo* which may interfere with the ability of other helix-loop-helix or leucine repeat factors to function in some cell types. Mutational studies on the different domains of AP-4 have indicated that under certain conditions AP-4 can form heterodimers with other proteins. Therefore it has been suggested that post-translational modifications of AP-4 may allow distinct heterodimers to form *in vivo* indicating novel levels of control which need further investigation (271).

Appendix C

Analysis of the EIAV LTR (329) has identified several *cis*-acting elements that control viral gene expression. Gene regulation of EIAV is mediated by multiple discrete elements some of which display cell type and condition specificity. Four basic motifs were identified, a methylated DNA binding site (MDBP), 2 PEA2 binding motifs, a PEA1/AP-1 site and an *ets*/PEA3 motif. These sites were shown to bind protein and be important for LTR function (329). This structure showed some similarity to the component elements of the polyomavirus enhancer (431-434).

Sequences homologous to the PEA2 site are also present in the promoters of other lentiviruses such as visna virus, CAEV, FIV and the hepatitis B enhancer sequences (329). In the polyomavirus enhancer the PEA2 site (GACCGCA) overlaps with an AP-1/PEA1 site and it has been proposed that PEA1 binding factors could weakly interact with the PEA2 site (435) thereby generating further control mechanism for gene expression. It will therefore be interesting to investigate these sites and their interaction in the lentiviral LTRs that have similar overlapping binding sites.

Members of the enhancer binding protein (EBP) family can bind to a sequence similar to the PEA2 site (218, 219). It is known that the EBPs are expressed in HeLa cells (436), but no binding was seen with HeLa extracts and the EIAV PEA2 site. Therefore the proteins which bind to the PEA2 element have yet to be cloned and characterised and the significance of this site in the FIV LTR needs further investigation.

Appendix D

Cross competition between AP-1 and ATF binding may be the result of co-operative binding between the two factors. It may be possible that either AP-1 or ATF must bind to the FIV LTR before the correct conditions are achieved which will allow the second protein to bind, resulting in the double binding pattern that was observed in our competition analysis. In order to test this theory single and double mutants of these sites in the FIV LTR would have to be constructed and then binding patterns across these mutations would allow a more detailed analysis of this phenomenon.

Appendix E

C/EBP is a member of a class of proteins which utilises a characteristic motif for protein-protein interactions known as the leucine zipper. Several genes encoding members of this family of proteins have been cloned and have been shown to contain 4 or 5 leucine residues that are spaced exactly 7 residues apart (262, 412). This allows α-helices to from in the proteins which in turn allow the monomers to interdigitate to form heterodimers. However it is sequences outwith this zipper region that are involved in binding to DNA (83) and it has become increasingly clear that the leucine zipper acts as an independent dimerisation region for different kinds of DNA binding domains (12).

Appendix F

The raw data from the various experiments were analysed in a T-test where p=<0.05 indicated a significant difference between the results. A significant drop in activity was observed in all the cell lines tested when the AP-4/AP-1 site was deleted. Deletion of the ATF site gave a significant drop in activity in CRFK and HeLa cells indicating further that these sites seem to be of prime importance in the control of FIV gene expression. In HeLa cells the deletion between -176 and -147 produced a significant increase in the level of gene expression indicating that the presence of a putative negative regulatory element does affect the level of wild-type gene expression in this cell line. The other deletions did not show any statistically significant effect upon gene expression.

Results of further T-tests indicated that upon transfection of FIV-G8 infected cells deletion of the AP-4/AP-1 site no longer gave a significant decrease in the level of gene expression as compared to uninfected cells. Indicating that a virus specific product may have an effect on viral gene expression under certain conditions. In the presence of a viral infection deletion of the C/EBP, NF-1 and ATF sites did give a significant reduction in the levels of gene expression. However levels of gene expression driven by the mutants remained significantly higher in the infected cell line compared to the low levels of activity that were recorded in the uninfected line, again indicating that a virus specific factor may function to drive viral gene expression in infected cells.

In co-transfection studies with FIV molecular clones deletion of the ATF site generated a significant reduction in gene expression in both experiments. However there were differences in the significance of deleting the AP-1 site. This had little effect in the presence of FIV-PPR but a significant drop in activity was recorded in the presence of FIV-34TF10. Furthermore deletion of the putative NRE was significant in CRFK cells co-transfected with FIV-

34TF10 but had no effect in cells containing FIV-PPR. Therefore it appears that the different viral strains can have different effects upon the level of viral gene expression in transfected cell lines and further analysis of the LTR and viral gene products will be required before the exact nature of the different mechanisms can be fully explained.

Appendix G

The proto-oncogene *c-fos* encodes a 55-62kd nuclear phosphoprotein (Fos) which has a pivotal role in the transduction of extracellular and intracellular signals which result in changes in gene expression (413, 419).

Fos functions as a transcriptional regulator for a set of target genes which it can either activate (420-424) or repress (424, 426-428, 430). Fos has been shown to function in conjunction with a number of Fos-associated proteins (FAPs) (429) the best characterised being the sequence specific DNA binding protein Jun with which it forms a heterodimeric complex (256, 420, 425).

The *c-fos* gene is subject to tight transcriptional controls. In most cells *c-fos* is expressed at very low levels but can be rapidly and transiently induced by exposure to various external stimuli (414-416, 419). Induction of the human *c-fos* gene by serum growth factors is mediated through the serum response element (SRE) (417) which contains the dyad symmetry element (DSE) (418), which is the binding site for the serum response factor. Experiments have shown that Fos can repress its own expression via negative feedback regulation of its own promoter (426). It has been postulated that this occurs through the binding of Fos and Jun heterodimers to consensus AP-1 binding sites in the *c-fos* promoter (426, 427, 430).

A recent study to investigate the role of the cellular protein Fos and Jun on the activation of the visna virus gene expression has shown that Fos and Jun bind to an AP-1 site in the viral LTR in cells that have been stimulated with TPA (307). It was found that the formation of this DNA-protein complex could be substantially reduced in extracts which had been preincubated with anti-Jun and anti-Fos antibodies, indicating that Jun and Fos are an integral part of the complex which binds and activates the visna virus LTR. Further studies are now underway in order to determine if viral factors can upregulate Jun and Fos production in infected cells thereby creating a feedback mechanism for viral gene expression. This type of regulation may

be due to the action of the visna virus Tat protein although the mechanism of action is not yet clear. Due to the obvious importance of the AP-1 site in the FIV LTR it is now clear that the experiments detailed above should be mirrored in the FIV system in order to identify any similar mechanisms that may be involved in the control of FIV infection.