Characterization of Variant Equine Infectious Anemia Virus Long Terminal Repeat Sequences.

Charles Richard Madden
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CHARACTERIZATION OF VARIANT EQUINE INFECTIOUS ANEMIA VIRUS LONG TERMINAL REPEAT SEQUENCES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Biochemistry

by

Charles R. Madden
B.S., Kansas State University, 1989
December 1996
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................. ii

LIST OF TABLES ................................................................. iv

LIST OF FIGURES ................................................................. v

ABSTRACT ................................................................................... vi

CHAPTER
   1 LITERATURE REVIEW ................................................. 1
   2 MATERIALS AND METHODS ...................................... 36
   3 RESULTS AND DISCUSSION ....................................... 53
   4 SUMMARY AND CONCLUSIONS .................................. 95

REFERENCES ............................................................................. 102

APPENDIX: Permission Letter ....................................................... 110

VITA ............................................................................................ 112
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Retrovirus groups</td>
<td>6</td>
</tr>
<tr>
<td>2. Primer pairs and their deletion products</td>
<td>41</td>
</tr>
<tr>
<td>3. Variant LTR activity in FDD cell cultures</td>
<td>61</td>
</tr>
<tr>
<td>4. Variant LTR activity in FEA cell cultures</td>
<td>62</td>
</tr>
<tr>
<td>5. Variant LTR activity in Cf2Th cell cultures</td>
<td>63</td>
</tr>
<tr>
<td>6. Prtt deletion mutant activity in FDD cell culture</td>
<td>66</td>
</tr>
<tr>
<td>7. Prtt deletion mutant activity in FEA cell cultures</td>
<td>68</td>
</tr>
<tr>
<td>8. Prtt deletion mutant activity in Cf2Th cell cultures</td>
<td>68</td>
</tr>
<tr>
<td>9. ALTR3 deletion mutant activity in FDD cell cultures</td>
<td>72</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Retrovirus virion and genomic structure</td>
<td>2</td>
</tr>
<tr>
<td>2. Schematic diagram of the EIAV provirus and transcripts</td>
<td>13</td>
</tr>
<tr>
<td>3. Block diagram of the EIAV LTR</td>
<td>38</td>
</tr>
<tr>
<td>4. Sequence comparison of variant LTR isolates</td>
<td>55</td>
</tr>
<tr>
<td>5. Alignment of Prtt LTR deletion mutants</td>
<td>65</td>
</tr>
<tr>
<td>6. Alignment of ALTR3 deletion mutants</td>
<td>71</td>
</tr>
<tr>
<td>7. Quantitation of LTR variants in infected horse tissue DNA</td>
<td>77</td>
</tr>
<tr>
<td>8. Cell culture viability for infected and mock-infected FDD cell cultures</td>
<td>82</td>
</tr>
<tr>
<td>9. Visual appearance of FDD cell cultures</td>
<td>83</td>
</tr>
<tr>
<td>10. Reverse transcriptase production by infected FDD cell cultures</td>
<td>85</td>
</tr>
<tr>
<td>11. Analysis of viral mRNA by Northern blot hybridization</td>
<td>88</td>
</tr>
<tr>
<td>12. Analysis of cellular DNA by Southern blot hybridization</td>
<td>90</td>
</tr>
</tbody>
</table>
Equine infectious anemia virus (EIAV) is the causative agent of a persistent disease in horses. The long terminal repeat (LTR) region of EIAV contains the enhancer and promoter elements necessary for transcription of the proviral genome. The LTR is also prone to a high degree of sequence variability between different virus isolates. Sequence alignments of LTR variants from both tissue culture-adapted strains of the virus as well as sequential horse isolates showed a high degree of sequence variability between variants. Sequence variability was further localized to a hypervariable region approximately 60 bp upstream of the TATA box. This region contained insertions resulting in the duplication of flanking sequences and the putative cis-elements they contain. Transient gene expression analysis of the LTR variants in three different cell lines showed that the promoter activities of the LTR variants were different and that these differences were cell line dependent. Further analysis using deletion mutants indicated that the hypervariable and flanking regions contained several cis-elements important for promoter activity and that duplication of these elements increased activity under basal conditions. Analysis of infected horse tissue DNA using competitive PCR analysis did not indicate a high degree of sequence divergence localized to the LTR. Therefore, LTR heterogeneity may not be required for the onset of acute disease. To assess the impact of LTR variability on viral replication kinetics and cytopathogenicity, an infectious EIAV molecular clone was created in which the cognate LTR region was replaced with the corresponding region of a virus stock which demonstrates accelerated cytopathogenic effects. The study showed that the LTR increased replication capacity but did not influence cytopathogenicity.
CHAPTER 1
LITERATURE REVIEW

Retroviruses

The retrovirus family of viruses is arguably the most studied group of pathogens in human history. Initially identified in the early 1900s as filterable agents associated with diseases in animals, retroviruses were not linked to any human diseases until almost eighty years later with the discovery of the Human T-cell leukemia virus (HTLV), and Human immunodeficiency virus (HIV) (Wong-Staal, 1991).

Structure

Retroviruses are enveloped RNA viruses with a virion size of approximately 100 nm and a conical nucleocapsid (Fig. 1A). The envelope is derived from the plasma membrane of the host. Imbedded within it are viral glycoproteins, the products of the env gene, that form knob-like structures on the exterior of the virion and are essential for the recognition, attachment, and penetration of the virus into the host cell. The interior of the virion is dominated by the viral capsid. This structure is formed from the multiple protein products of the gag gene. In addition to the capsid, the interior of the virion contains several enzymatic activities required for retrovirus replication. Such as the reverse transcriptase, ribonuclease H (RNase H), and integrase enzymes encoded by the pol gene. Virion cores also contain host tRNA which functions as a primer during the reverse transcription of the viral genomic RNA and, in some cases, auxiliary proteins such as the viral infectivity factor (Vif) of HIV. Associated with the nucleocapsid is the positive strand, diploid RNA genome. These identical strands of RNA, from 7 to 10 kb in length, possess a 7mG cap at their 5' ends and are poly-adenylated like host mRNA.
Figure 1. Retrovirus virion and genomic structure. (Panel A). Structure of a general retrovirus virion. (Panel B) Sequence features of a retroviral genomic RNA (adapted from Coffin, 1991).
The retroviral genome contains three main open reading frames which form the \textit{gag, pol,} and \textit{env} genes (Fig. 1B). These genes produce the structural proteins and enzymes necessary for viral replication. Smaller open reading frames may also be present which can produce auxiliary gene products such as the transactivation protein (Tat) of HIV. The ends of retroviral genomic RNA are terminally redundant. A non-coding region, R, at the capped 5' end is repeated again at the 3' end of the genome just prior to the poly-adenine tail. R is followed at the 5' end by U5 and non-coding leader regions, while it is preceded at the 3' end by the U3 region. U5 contains the poly-adenylation signal sequence while U3 contains the viral promoter and enhancer elements necessary for transcription of the viral genome after infection (Coffin, 1991).

**Reverse Transcription and Integration**

The unique mechanism by which retroviruses replicate is one of the most defining characteristics of this viral family. Upon penetration of the host cell, the viral genomic RNA undergoes reverse transcription to form a double-stranded DNA copy or provirus. This process originates at a host derived tRNA molecule bound to a complementary primer binding site (PBS) which lies adjacent to the 5' U5 region. This bound tRNA serves as the primer for the viral reverse transcriptase enzyme which begins to create a DNA strand complementary (negative-sense) to the viral RNA. As the U5 and R regions are copied, they are degraded by the viral RNase H activity. Because the template is degraded as it is copied, the nascent DNA strand dissociates from the template when the 5' end is reached. The nascent DNA strand is then free to re-associate with the complementary R region at the 3' end of the template and reverse transcriptase continues to elongate the complementary DNA strand. This is referred to as strand jumping. During the
synthesis of negative-strand DNA, a positive-strand is being produced as well. The RNase H activity cleaves the template RNA at a precise point adjacent to the U3 region. This recognition sequence is referred to as the poly-purine tract due to its tendency to contain strings of adenine and guanine residues. The cut template RNA and negative-stranded DNA form the primer complex for reverse transcriptase. After the U3, R, U5, and PBS regions have been successfully copied, the positive-strand DNA jumps to complementary sequences at the opposite end of the negative-strand DNA. Both positive and negative DNA strands are then completed by reverse transcriptase to form the provirus. Not only is this mechanism unique due to its reversal of the conventional flow of genomic information, but it also results in a rearrangement of the viral genome at the same time. Unlike the RNA genome, the U3, R and U5 regions are clustered together and repeated at both ends of the provirus. Collectively, these three regions make up the long terminal repeat or LTR.

After being copied into a proviral form, the retrovirus integrates itself into the genome of the host. The exact mechanism by which this occurs is not completely understood but it is known that the reaction requires both the viral capsid as well as the integrase enzyme. Staggered cuts are made at both ends of the provirus resulting in 5' overhangs. In a linked reaction, staggered cuts are made within the host genomic DNA and these are linked to the cut provirus. Subsequently, any gaps or nicks in the DNA are repaired by a host repair enzyme. This results in an intact provirus, minus one to two base pairs from each end, being inserted into the host’s genome. During this reaction, linear provirus can become linked to itself forming a closed, circular piece of extra-chromosomal DNA.
Within an infected cell, both these forms of provirus are readily detectable (Coffin, 1991).

**Gene Expression**

Once integrated into the host genome, the provirus uses the host’s own transcriptional and translational machinery to create progeny virions. The U3 region of the LTR contains the single promoter for the entire retroviral genome. It contains the TATA box as well as most, if not all, enhancer elements. As these elements bind cellular transcription factors, they influence both the type and state of the host cell in which the virus may be transcribed as well as the rate at which transcription occurs. The actual transcription start site lies downstream of the TATA box and forms the demarcation between the U3 and R regions. Transcription then proceeds through the proviral genome to the U5 region of the 3' LTR which contains the poly-adenylation signal sequence. The point on the viral mRNA where poly-adenylation begins forms the boundary between the R and U5 regions. This full-length transcript may be transported to the cytoplasm where it forms the genomic RNA in progeny virions or be translated to produce the gag and gag-pol polyproteins. In a minority of cases, a translational error may occur resulting in the production of the pol gene products. Alternatively, the full-length transcript may stay within the nucleus and be spliced to form a variety of smaller viral mRNA species. These singly or multiply-spliced mRNAs are responsible for the production of the env polyprotein as well as auxiliary proteins. Virion assembly and subsequent budding occurs at the plasma membrane. Although the polyproteins produced by gag, pol, and env undergo some proteolytic cleavage prior to virion assembly and release, complete cleavage of the polyproteins and maturation of the virion occurs after budding (Coffin, 1991).
Taxonomy

The family *Retroviridae* is subdivided into three subfamilies; the *Oncovirinae, Lentivirinae, and Spumavirinae* based on a variety of factors including genomic complexity and pathology (Table 1).

Table 1. Retrovirus subfamilies

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Isolates</th>
<th>Pathology</th>
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<tr>
<td><strong>Oncovirinae</strong></td>
<td>Rous sarcoma virus</td>
<td>acute, exogenous</td>
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<td></td>
<td>Rous-associated virus 1-50</td>
<td>non-acute, exogenous</td>
</tr>
<tr>
<td></td>
<td>Feline leukemia virus</td>
<td>non-acute, exogenous</td>
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<tr>
<td></td>
<td>Mouse Mammary tumor virus</td>
<td>non-acute, exogenous</td>
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<tr>
<td></td>
<td>Bovine leukemia virus</td>
<td>non-acute, exogenous</td>
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<tr>
<td></td>
<td>Human T-cell leukemia virus</td>
<td>non-acute, exogenous</td>
</tr>
<tr>
<td></td>
<td>Rous-associated virus type 0</td>
<td>benign, endogenous</td>
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<tr>
<td><strong>Lentivirinae</strong></td>
<td>Human immunodeficiency virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simian Immunodeficiency virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feline immunodeficiency virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Visna/maedi virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Equine infectious anemia virus</td>
<td></td>
</tr>
<tr>
<td><strong>Spumavirinae</strong></td>
<td>Human foamy virus</td>
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<tr>
<td></td>
<td>Simian foamy virus</td>
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Oncoviruses have been identified in a variety of animal species including humans and although usually exogenous, certain strains such as Rous-associated virus type 0 are endogenous. Many oncoviruses, but not all, cause transformation...
of *in vitro* cell cultures and induce tumors within their natural hosts. While some oncoviruses actually contain oncogenes and rapidly cause disease, there are many members that do not encode oncogenes but are still involved in tumorigenesis and still others that cause no disease at all. Therefore they are classified as acute, non-acute, and benign. Most oncoviruses are genetically quite simple. Few produce any auxiliary proteins other than an oncogene. HTLV, the causative agent of adult T-cell lymphoma, is one of the most complex oncoviruses in that it produces two auxiliary proteins Tax and Rex. Both of these proteins perform regulatory functions. Tax interacts with cellular transcription factors, bound to specific *cis*-elements within the U3 region, and stimulates transcription. The presence of Rex results in the production of more full-length or singly-spliced viral mRNA transcripts relative to the multiply-spliced transcripts that produce itself and Tax. These proteins are similar to those produced by lentiviruses (Coffin, 1991).

Lentiviruses are probably the most significant subfamily of retroviruses from a pathology standpoint as this group includes the HIV virus, the causative agent of the acquired immunodeficiency syndrome (AIDS). Since its discovery in the early 1980s, HIV is believed to have infected more than 13 million people world-wide. By the year 2000, that number may reach 40 million. Despite intense research efforts this virus continues to exhibit a fatality rate of nearly 100% (Cowley *et al.*, 1995). Lentiviruses differ significantly from the oncoviruses in pathology and genomic complexity. These viruses usually cause slow, progressive infections that can lead to auto-immune or immunodeficiency diseases within the host. They are also more genetically complex than the oncoviruses because they produce a number of auxiliary proteins. The most universal of these proteins are Tat and Rev. The HIV Tat protein, in conjunction with cellular proteins, interacts
with a stem-loop structure, the transactivation response element (TAR), contained in nascent viral mRNA transcripts and may either stimulate initiation of transcription or may prevent premature termination by the RNA polymerase II complex (Laspi
ta et al., 1989; Selby et al., 1989). This mechanism is not universal among lentiviruses. In the case of Visna/Maedi virus for example, there is no TAR element present and the analogous Tat protein acts indirectly at the viral LTR in a mechanism similar to that of HTLV Tax. Despite these differences, the lentivirus Tat protein dramatically increases viral mRNA production. Rev also acts upon viral mRNA at a region of extensive secondary structure, the rev-responsive element (RRE), and facilitates the transport of viral mRNA into the cytoplasm and the subsequent loading of polysomes (Tiley et al., 1992). This results in an increase in the ratio of full-length or singly-spliced viral mRNA to multiply-spliced viral mRNA thereby increasing production of the structural proteins while decreasing the production of auxiliary proteins such as Tat and Rev (Perkine et al., 1989). Most lentiviruses are also believed to produce a viral infectivity factor, Vif. This small protein is packaged within the virion and appears essential for viral infectivity in vivo as mutations in Vif can prevent spread of the virus (Von Schwedler et al., 1993). In addition to Tat, Rev, and Vif, the primate lentiviruses SIV (Simian immunodeficiency virus) and HIV produce the auxiliary proteins Nef, Vpu, and Vpr/Vpx. The genes which produce these proteins are often dispensable in vitro thus they have not been as extensively studied as Tat and Rev but they appear to dramatically affect virulence in vivo. Nef was originally identified as a negative regulatory protein that acted upon the LTR to decrease transcription initiation. A theory that remains somewhat controversial. Nef is also believed to play a role in the endocytosis and lysosomal degradation of CD4, the primary
cellular receptor for HIV and SIV (Nebreda et al., 1991). Vpu is a protein unique to HIV-1 and appears to perform two different functions. It is involved in both virion egress as well as degradation of intra-cellular CD4 (Vincent et al., 1993). Virtually nothing is known about the in vivo function of Vpr (HIV-1 and HIV-2) and Vpx (HIV-2 and SIV), although some studies have shown that Vpr may act upon the HIV LTR to enhance transcription (Clements and Payne, 1994).

The least information is known about the subfamily Spumavirinae, or foamy viruses. Viral isolates belonging to this family have been isolated from a wide variety of species including humans. Although they have not been shown to cause disease, although a study of patients suffering from nasopharyngeal carcinoma indicated a high prevalence Human foamy virus (HFV) positive individuals. This may indicate an involvement in tumorigenesis. They are dubbed foamy viruses due to their cytopathogenic effects when propagated in tissue culture. Similar to lentiviruses on a genomic level, the foamy viruses are believed to produce at least three auxiliary proteins including one that functions as a transactivator similar to Tat (Aquuzzi, 1993).

**Equine Infectious Anemia Virus**

Equine infectious anemia, first described in 1843, is a persistent disease affecting members of the genus *Equus*. In 1904, the disease was linked to a filterable agent, the Equine infectious anemia virus. The virus is transmitted to susceptible hosts by contaminated blood, the main vector being hematophagous insects, where it infects cells of the monocyte/macrophage lineage (Sellon et al., 1994). Diagnostic tests, the agar gel immunodiffusion and competitive ELISA tests, are required by federal quarantine laws governing the interstate transport of animals. Estimates place the percentage of infected horses, within the United
States, at between 0 and 2% depending upon geographic location (Loftine et al., 1990).

Horses with EIA may undergo three different phases of the disease. Within 30 days of the initial infection, the horse may develop high titer viremia resulting in fever, weight loss, thrombocytopenia, and severe anemia. This is the acute phase of the disease and in severe cases may be fatal. EIA is unique among lentiviral syndromes due to this rapid emergence of clinical disease. Horses that recover from the initial febrile episode may experience additional disease episodes at irregular intervals during the first year of infection. Horses that experience multiple and severe febrile episodes are described clinically as being in the chronic stage of EIA. Both the number of episodes experienced during this phase and the time interval between them varies. During the 1980s, researchers discovered that emergence of antigenic variants of the virus corresponds to sequential disease episodes (Montelaro et al., 1984). Thus EIA became the first disease of lentiviral etiology whose persistence was linked to the antigenic variation of the virus. Horses which harbor the virus yet show no visible signs of disease are described as being asymptomatic. Asymptomatic horses can have a recurrence of clinical symptoms should they become immuno-compromised at some point in their lifetime. In addition, asymptomatic horses are capable of transmitting the disease and therefore serve as a reservoir for the virus.

The clinical symptoms of EIA are believed to result from widespread immune system activation within the host in response to viremia. Complement coated erythrocytes are present in horses during the acute and chronic phases of the disease. It is believed that viral glycoproteins or viral glycoprotein/antibody complexes associate with the surface of erythrocytes eventually resulting in
complement mediated lysis or phagocytosis of the affected erythrocytes (Sentsui and Kono, 1987). In addition to hemolysis, anemia in horses with EIA is exacerbated by a decrease in erythrocyte precursors. As viral proteins do not appear to be associated with immature erythrocytes, the reason for their depletion is unknown but may be due to an activation of macrophages and subsequent release of factors, interleukin 1 and lactoferrin, that affect iron metabolism (McGuire et al., 1969). A decrease in platelet count, thrombocytopenia, is caused by a similar immune-mediated mechanism. During disease episodes, host platelets are bound to excessive amounts of IgG and IgM which may cause their subsequent removal by phagocytosis (Clabough et al., 1991; Sellon et al., 1994).

**Structure**

EIAV is classified as a lentivirus based on pathology, morphology, serology, genetic organization, and enzymatic activities. It is an enveloped RNA virus, approximate diameter 140 nm, with a conical core. The exterior envelope is derived from the plasma membrane of the host cell and is studded with glycoprotein spikes. The viral glycoprotein gp90 forms the exterior domain of the surface spike while the transmembrane segment is formed by gp45. Both are cleaved from a precursor poly-protein encoded by the env gene. The conical core of EIAV is formed from four non-glycosylated proteins, p26, p15, p11, and p9, which are products of the gag gene. In addition, the core contains the pol gene products which include the reverse transcriptase, protease, deoxyuracil triphosphatase (dUTPase), RNase H, and integrase enzymes. EIAV also produces two regulatory proteins whose functions are analogous to HIV Tat and Rev, as well as possibly two auxiliary proteins of unknown function (Ttm and the ORF S2 product). The diploid RNA genome consists of capped and poly-adenylated RNA...
8.2 kb in length. The exact processes of reverse transcription and integration have not been elucidated for EIAV but it is believed to behave similarly to that of other lentiviruses (Sellon et al., 1994).

**Genomic Organization and Expression**

The EIAV proviral genome (Fig. 2), like all lentiviruses, is subject to a high degree of sequence variability both within a single host and throughout a host population. Therefore the nucleotide lengths discussed hereafter must be considered approximations. The genome begins with a 5' LTR region which varies in length, approximately 300-330 bp, between different virus isolates. This is followed by a leader sequence, 143 bp long, which contains a splice donor site used in subsequent viral mRNA splicing reactions and the first exon of the EIAV tat gene. The leader region ends at the ATG initiation codon for the gag gene. This gene, approximately 1500 bp in length, encodes a precursor polyprotein which yields the four core proteins upon proteolysis. Overlapping the gag gene by 251 bp is the pol gene. Approximately 3400 bp in length, this gene produces the protease, reverse transcriptase, RNase H, dUTPase, and integrase enzymatic activities. The dUTPase enzyme is only present in a sub-set of the lentivirus group of viruses. Those viruses that infect macrophages such as EIAV and Feline immunodeficiency virus (FIV) may require this enzyme in order to replicate in this terminally differentiated and non-replicating cell type. Immediately following the pol gene is a small open reading frame designated as ORF S1 which is only about 150 bp long and lacks its own initiation codon. This region forms the second exon of the EIAV tat gene. Following the stop codon of ORF S1 by about 7 bp is another short open reading frame ORF S2. This 200 bp region could potentially produce a 66 amino acid protein which bears no sequence homology to other known lentiviral proteins.
Figure 2. Schematic diagram of the EIAV provirus and transcripts. Open reading frames and their products are illustrated for the proviral genome followed by diagrams of possible transcripts produced as well as their respective protein products.
and has no known function although it has been hypothesized to be the EIAV equivalent of Vif. ORF S2 almost completely overlaps the env gene. The env gene of EIAV is approximately 2600 bp long and produces the polyprotein gp135 which is subsequently cleaved to form the surface protein gp90 and the transmembrane protein gp45. Within the env gene region lies another overlapping open reading frame, ORF S3, approximately 400 bp long. This region, like ORF S1, contains no initiation codon but with subsequent splicing of the mRNA forms a large portion of the putative EIAV rev gene. After the stop codon of env lies a short region 17 bp long, containing the poly-purine tract, which is followed in turn by the 3' LTR (Sellon et al., 1994).

In addition to the main structural and enzymatic proteins, the genome encodes a number of auxiliary proteins similar to other lentiviruses. In order to accomplish this, EIAV utilizes several splice donor and acceptor sites to create singly and multiply-spliced mRNAs (Fig. 2). To produce a functional tat gene, the splice donor site within the leader sequence is linked to a splice acceptor site at the end of pol. This creates a singly-spliced mRNA 3.2 kb in length that produces tat and env gene products. To produce the putative Rev protein, the splicing machinery uses the splice donor site within the leader sequence as well as the splice acceptor site at the end of pol to remove the gag and pol regions as previously discussed for the 3.2 kb mRNA. In addition, a splice donor site at the end of ORF S1 is linked to a splice acceptor site just downstream of ORF S2. Finally, a donor site in env is linked to an acceptor site at the beginning of ORF S3. This creates a mRNA species containing the leader sequence linked to ORF S1, then to approximately 100 bp of env, and ORF S3. This multiply-spliced, 1.5 kb mRNA species is believed to produce both an intact Tat protein in addition to Rev. Two
additional species of viral mRNA have been identified in infected cells. One is multiply-spliced and similar to the mRNA that produces Tat and Rev but it lacks the first exon of \textit{rev}. Therefore it is believed to produce only Tat. The other is a singly-spliced mRNA produced by the splicing of the leader sequence directly to the C-terminal portion of \textit{env}. This mRNA produces a chimeric protein of unknown function, dubbed Ttm, encoded by the first exon of \textit{tat} and the C-terminal portion of \textit{env} (Derse et al., 1993).

\textbf{Tissue Tropism}

EIAV infects peripheral tissue macrophages \textit{in vivo}. Within chronically infected horses, viral antigen can be detected in lymph nodes, kidney, liver, lung, thymus, bone marrow, adrenal, stomach, pancreas, cerebrum, cerebellum, pituitary gland, and spleen. The highest concentrations are within the spleen, liver, lymph nodes, and bone marrow. All these tissues are enriched with peripheral macrophages. Hybridization studies indicate that viral antigen is localized to macrophage-like cells within these tissues. Analysis of viral DNA and \textit{in situ} hybridization studies agree with this tissue distribution and localized viral mRNA to mature tissue macrophages exclusively (Sellon et al., 1994).

\textbf{Sequence Variation}

Like all retroviruses, EIAV isolates demonstrate a great degree of sequence divergence. In some cases, sequence divergence results in an altered phenotype and the creation of a new quasi-species or strain of the virus. Pair-wise sequence comparison between sequential EIAV isolates from an infected horse indicated a sequence variation as high as 1.43\% overall. Much of this variation was localized to the \textit{env} and LTR regions which, individually, showed sequence divergence rates as high as 5.6\% (Payne et al., 1987). The high degree of sequence variation is
believed to result from the error prone nature of the reverse transcriptase enzyme coupled to selective pressure by the immune system of the host. The retrovirus reverse transcriptase enzyme lacks any exonucleolytic proofreading activity and can have \textit{in vitro} misincorporation rates as high as 1 in 1000 nucleotides. In addition, the unique nature of retrovirus replication, involving the transfer of nascent DNA strands to other locations in the genome, leads to a high degree of recombination and rearrangement. Although no exact measurement has been made of the frequency of these events, one study found that approximately 50% of HIV-1 proviruses isolated from an individual contained some sort of sequence rearrangement such as a deletion, addition, or duplication. In many cases, these aberrations create unique viral phenotypes by changing the cytopathogenicity, tissue tropism, or replication capacity (Coffin, 1991).

\textbf{In Vitro Propagation}

Propagation of virulent EIAV field isolates \textit{in vitro} is extremely difficult. Leukocyte cultures have a very short life-span, approximately 10 to 12 days, and, when infected with EIAV, can demonstrate rapid, within 72 hours post-infection, cytopathogenic effects. Due to this difficulty, Malmquist \textit{et al.} (1973) adapted the Wyoming strain, a highly virulent field isolate, for growth in an equine dermis (ED) cell line. The ability to replicate in equine fibroblasts corresponded to a severe decrease in virulence. Subsequently, it was found that this Prototype strain was able to establish persistent, non-cytopathogenic infections in feline embryonic (FEA), fetal canine thymic (Cf2Th), and fetal equine kidney (FEK) cell cultures. The Prototype strain is also able to replicate in fetal donkey dermal (FDD) cells cultures but induces cytopathogenic effects within 22 days post-infection. Multiple passage of Prototype in FDD cell cultures led to a new virus strain, the FDD-
adapted strain, which demonstrates cytopathogenic effects within 12 days post-infection. Most recently, a number of EIAV molecular clones have been developed. Although infectious, none of these clones appear to cause disease (Sellon et al., 1994).

Cytopathogenicity

As little is known of retrovirus cytopathogenicity, the mechanism by which it occurs and the viral determinants that influence it are of considerable scientific interest. There are probably several different mechanisms by which retroviral infections can induce cell death and therefore the viral determinants that influence it may differ from one system to another. Experimental studies usually involve the exchange of gene segments between cytopathogenic and non-cytopathogenic strains. Numerous studies of this type have been performed on primate lentiviruses with very diverse results. Genetic regions that influence cytopathogenicity have been found throughout the lentivirus genome but are most often localized to the transmembrane, TM, and surface, SU, proteins produced by the env gene. The TM protein of retroviruses contains a membrane spanning region in its amino terminus that mutational analysis indicates is responsible for cell to cell fusion or syncitia formation in primate lentivirus infected cell cultures. In addition, the TM protein contains a region near its carboxyl terminus that may form an amphipathic helix. Should these helices aggregate within the plasma membrane and form channels, they would have a deleterious effect on the osmotic pressure across the plasma membrane. This theory explains why some cells infected with HIV demonstrate a large increase in cell volume prior to cell death. Studies performed with HIV strains containing deletions in the carboxyl terminus of the SU protein demonstrate syncitia formation but show an attenuation in the production of enlarged single
cells (Garry, 1989). Not all cytopathic infections demonstrate the physical changes within the host cell required by the two previous theories. It has also been proposed that the HIV SU protein may induce programmed cell death or apoptosis in both T-cells and neural cells when bound in excessive amounts to the HIV receptor molecule CD4. This effect can be mimicked by incubation of these cell types with anti-CD4 antibodies (Gougen, 1993). The viral determinants of cytopathogenicity are not exclusively limited to the structural genes however. Some cytopathogenic infections seem to be caused by an over-expression of the virus. There are examples of cytopathic HIV infections which produce an estimated $2.5 \times 10^6$ copies of viral mRNA per cell or levels of viral protein as high as 40% of the cellular total. In these cases, it is possible that the cell is simply being overwhelmed by viral products resulting in a shutoff of host protein production and inevitable cell death. This situation is believed to occur when the host cell loses its resistance to super-infection (Garry, 1989). Most retrovirus infections lead to a down-regulation of the host protein that acts as the virus receptor which prevents a cell from being infected multiple times or super-infected. In studies with cytopathogenic avian retroviruses, excessive amounts of unintegrated proviral DNA was found in the cytoplasm of infected cells indicating multiple rounds of viral infection. Subsequent experiments with UV irradiated virus capable of infection and reverse transcription but deficient in gene expression indicated that the presence of a large amount of viral proviral DNA was not, in itself, cytopathogenic. It is possible that super-infection of a host cell also leads to over-expression of viral mRNA or viral gene products causing cell death. The viral determinants which control the host cells resistance to super-infection are, once again, usually located within the $env$ gene. Mutations in $env$ may lead to a change
in receptor recognition allowing the virus to circumvent the previous down-regulated receptor (Coffin, 1991).

The accelerated cytopathogenic effects induced by the FDD-adapted virus strain provides an interesting system for studying factors that influence virus cytopathogenicity. Perry et al (1992) examined the sequence differences between Wyoming strain proviral DNA, propagated in horse macrophage cultures, and the infectious molecular clone CL22, which is capable of replicating in ED cells and is not virulent. The results indicated that both the gag and pol regions were relatively conserved, with only 0.26% and 0.67% nucleotide difference respectively. Most of the sequence divergence occurred within the env and LTR regions. The surface protein, gp90, region of env showed a nucleotide difference of 5.57%, an amino acid difference of 11%, while the LTR indicated a divergence of 5.14% to 5.30%. These results are in approximate agreement with those obtained from other studies examining sequence changes upon tissue culture adaptation or disease progression within the host. These studies all agree that the LTR and env, including the S3 ORF, are prone to a high degree of sequence variability. As gp90 is essential in the process of host cell recognition and attachment, it would seem logical that changes within this protein are necessary for any change in cell tropism such as tissue culture adaptation. Unfortunately, replacement of the CL22 gp90 coding region with the corresponding region from Wyoming strain proviral DNA resulted in a molecular clone still capable of infecting ED cells. Although this result was surprising, it seemed to agree with the data obtained on the FDD-adapted strain of EIAV.

In a previous study, the cytopathogenic infection of FDD cell cultures by FDD-adapted virus was compared to the persistent infection of FEK cell cultures.
by Prototype virus. Rasty et al. (1990) reported, as determined by immunofluorescence studies, that in the cytopathogenic infection approximately 30% of the cell population was EIAV antigen positive while in the persistently infected cell culture almost 100% of the cell population exhibited EIAV antigen. This result was supported by data obtained by Southern hybridization analysis of total cellular DNA isolated from infected cultures. These results indicated approximately one third the amount of proviral DNA in the cytopathogenic infection relative to the persistent infection. Evidence indicated that the viral determinants which affect the cytopathogenicity of the FDD-adapted strain are probably not involved in cell recognition, attachment, or penetration of the virus as the percentage of cells infected in the cytopathic infection was actually less than that of the persistent infection and there was no accumulation of viral DNA indicative of super-infection. In addition, FDD cell cultures infected with the FDD-adapted virus did not demonstrate the morphological changes such as cell swelling or syncitia formation indicative of some cytopathic HIV infections. All this evidence seemed to discount the env region as the likely candidate responsible for the behavior of the FDD-adapted virus.

The study by Rasty et al. (1990) did reveal some possible explanations for the behavior of the FDD-adapted virus strain. Using Northern hybridization analysis, it was shown that FDD cell cultures infected with the FDD-adapted virus produced approximately 30 times as much viral mRNA as FEK cell cultures persistently infected with the Prototype virus. In addition, the cytopathogenic infection produced detectable levels of viral mRNA much earlier in infection. This was a very interesting result in light of some theories about the mechanism of retrovirus cytopathogenicity which indicate that the over-production of viral mRNA
or viral gene products is cytotoxic. One region of the genome that directly influences the production of viral mRNA is the long terminal repeat.

**Long Terminal Repeat**

The most characterized lentivirus LTR is, of course, that of HIV. The HIV LTR contains a core enhancer/promoter region in its U3 region approximately 90 nucleotides in length that is centered 65 bp upstream of the RNA cap site. This core unit contains the TATA box, two NF-kB and three SP-1 binding motifs and is essential for virus replication.

NF-kB is constitutively expressed in all mammalian cells yet plays a major role in restricting the replication of HIV to cell types involved in immune system functions such as T-lymphocytes and monocytes. Its level of expression is highest in B- and T- lymphocytes where it induces expression of the immunoglobulin k, interleukin-2a receptor, and interleukin-2 genes. In most cell types, including unactivated T-cells, it is associated with an inhibitor protein, I-kB, which masks its nuclear localization signal causing it to be retained in an inactive state within the cytoplasm. T-cell activation leads to the phosphorylation and degradation of I-kB allowing NF-kB to migrate to the nucleus and stimulate transcription. A similar effect can be induced in other cell types by treatment with mitogens, cytokines, or other activating agents. In monocytes, NF-kB binding activity is induced by HIV infection itself. These elements are not necessary to maintain active viral replication of the virus *in vitro* but are required to activate transcription from the latent provirus.

Three tandem Sp-1 binding sites, which behave as proximal promoter elements, lie immediately downstream of the NF-kB sites. Although Sp-1 is a ubiquitous transcription factor and stimulates expression of a number of cellular
genes, its expression is usually the highest in cells undergoing terminal differentiation. This protein contains a zinc-finger structural motif which recognizes DNA regions rich in GC dimers. Of the three Sp-1 binding sites, only the two sites proximal to the HIV promoter are active. It is essential for viral replication that one of these two binding sites be maintained and in close proximity to the TATA box as Sp-1 interacts directly with TATA box binding protein associated factor 110. It has been suggested that Sp-1 acts to stabilize pre-initiation complexes. The complete absence of these sites eliminates promoter activity under both basal and transactivated conditions. Sp-1 is also capable of interacting in a synergistic manner with other transcription factors bound to more distal elements such as NF-kB, Ets-1, or additional Sp-1 monomers.

Approximately 24 bp upstream of the transcriptional start site is the HIV TATA box. Similar to other eukaryotic promoters, the HIV TATA box binds the TATA box binding protein (TBP) component of transcription factor IID (TFIID) and provides the location for the assembly of the RNA polymerase II complex. The actual role of the TATA element is somewhat more complex however as mutations within this region that do not effect basal transcription can dramatically reduce transactivation by Tat. In addition, mutations within this region that do reduce basal transcription can be corrected for by corresponding mutations within the Sp-1 sites. These experiments strongly suggest interactions between the RNA polymerase complex at the TATA box and both Sp-1 and Tat. The TATA box of HIV is flanked on both sides by palindromic E box motifs (CANNTG) which have also been shown to bind cellular proteins that stabilize the TFIID complex while displacing TBP. Mutations within these motifs effect transcription under both basal and transactivated conditions.
Overlapping the TATA region is the initiator element or SSR (start site region). Originally identified in the terminal deoxynucleotidyl transferase gene, this element allows the initiation of transcription in the absence of a TATA box. The RNA polymerase complex formed on this element differs from the complex formed at the TATA box in that it does not respond to transactivation by Tat. This complex has only been demonstrated in vitro.

Downstream of the transcription start site, nucleotides +1 to +59, is the TAR element of HIV-1. TAR forms a stem-loop structure in nascent viral RNA that is essential for viral replication and is both proximity and orientation dependent. The actual structure rather than the sequence is most important for activity although the nucleotides comprising the loop are important as is a uracil residue within a three nucleotide bulge on the stem region. It is this bulge that actually binds the viral protein Tat while the loop region is known to bind a variety of other cellular factors. Competition studies show that TAR DNA fails to inhibit Tat recognition of TAR RNA thereby proving that TAR functions primarily as an RNA element. The exact mechanisms by which the Tat/TAR complex acts to enhance viral expression could involve both transcript elongation and initiation. In the absence of Tat, the HIV-1 promoter produces a large amount of short transcripts comprising only the region between the transcription start site and TAR. This region has been named IST for inducer of short transcripts and mutations within this region interfere with short transcript accumulation. In the presence of Tat, the number of short transcripts decreases and longer viral RNA transcripts predominate. Experiments have also shown that Tat is able to modify transcription factor complexes artificially stalled at TAR allowing them to elongate. Both of these observations indicate that the Tat acts to elongate stalled HIV-1 transcripts.
probably through interactions with transcription factors TFIIF and TFIIS. Presumably, TAR simply acts to bind Tat and bring it into close proximity with the HIV promoter. Other studies indicate that Tat may stimulate transcription initiation as well. Mutations mapping to the TATA or Sp-1 binding regions can influence transactivation without influencing basal transcription. These synergistic interactions between upstream elements and Tat may implicate it in transcription complex assembly. Therefore, Tat may act to stimulate transcription initiation as well as elongation.

The remainder of the U3 region (upstream of the core region) contains an array of possible transcription factor binding sites such as those for, LEF, Ets-1 or Ets-2, and AP-1. Mutational analysis of these sites often yield variable results depending on cell type and, in some cases, these sites negatively regulate transcription. Therefore the relevancy of these sites is still controversial. It has been speculated that these sites confer responsiveness of the LTR to diverse cellular signals allowing for changes in cellular tropism (Antoni et al., 1994; Garcia and Gaynor, 1994; Gaynor, 1995; Jones and Peterlin, 1994).

Of the transcriptional activators that bind to elements upstream of the core enhancer/promoter region, one of the most interesting may be the lymphoid enhancer factor 1, LEF-1. Identified in human T- and B- cells, this factor does not seem to enhance transcription itself but acts synergistically with other enhancer elements in close proximity such as the Ets-1 site. LEF binding induces a bend within the DNA of 110°. This is believed to facilitate the protein-protein interactions between upstream enhancer elements and proximal promoter elements such as Sp-1. In support of this mechanism, a minimum distance is required
between LEF and the promoter as deletion of the intervening DNA abrogates LEF function (Giese et al., 1992; Jones and Peterlin, 1994).

Ets-1 is a member of the proto-oncogenic ets family of transcription factors which includes Ets-1 and 2, PU.1, Spi-1, Elf-1, PEA3 and others. Members of this family have been linked to a variety of steps involved in hematopoiesis especially myeloid and lymphoid cell specific gene expression. PU.1, for example, up-regulates the production of the macrophage colony stimulating factor receptor and the macrophage scavenger receptor during macrophage differentiation. Another family member, Elk-1, binds to the serum response element upstream of c-fos. Little is known about the mechanisms by which these proteins are regulated. During B-cell activation, phosphorylation of Ets-1 by caseine kinase II results in its release from the DNA. In T-cells however, Elf-1 is sequestered by the under-phosphorylated form of the retinoblastoma protein, Rb. Upon T-cell activation, the phosphorylation of Rb leads to the release of Elf-1 allowing it to activate transcription. These proteins are believed to contain helix-turn-helix structural motifs which interact with the DNA. The minimal consensus core sequence recognized by these factors is C/A GGA A/T but nucleotides in proximity to the core motif may influence exactly which factor binds to a given site as multiple members of the ets family may be present within the same cell type. However, in the case of the immunoglobulin heavy chain enhancer, multiple ets proteins are capable of binding at the same recognition site. ets recognition sites are often found in multiples and in close proximity to other transcription factor binding sites, such as those for AP-1 and Sp-1, with which they often act in synergy. In the HTLV-1 LTR, Ets-1 or Ets-2 interact cooperatively with Sp-1 to activate transcription. Combinatorial interactions between ets proteins and AP-1 have been
demonstrated to activate transcription of polyoma virus, granulocyte-macrophage colony stimulating factor, and the macrophage scavenger receptor (Jones and Peterlin, 1994; Moreau-Gachelin, 1994; Wang et al., 1994; Wu et al., 1994).

AP-1, originally human activating protein 1, is a transcription factor formed primarily from the products of the c-fos and c-jun proto-oncogenes. AP-1 protein complexes are usually Fos/Jun heterodimers but may also consist of Jun/Jun homodimers. Both Fos and Jun contain a leucine zipper motif flanked by basic residues which form the region where protein dimerization and DNA binding occur. Similar motifs are found in other transcription factor families such as ATF, CREB, and the glucocorticoid receptor. Due to this similarity, Fos and Jun are capable of forming complexes with these proteins as well. Although the consensus recognition sequence for AP-1 is TGACTCA, the heterogeneous nature of the protein complex itself and the influence of surrounding nucleotides leads to a large degree of variability in AP-1 recognition sites and its effect on transcriptional activity. AP-1 complexes, in conjunction with the glucocorticoid receptor, can actually inhibit transcription of the proliferin gene. AP-1 activity in cell cultures may be temporarily induced by treatment with protein kinase C activators such as phorbol esters but its regulation on the cellular level is much more complicated than its state of phosphorylation as both fos and jun are auto-regulatory and affect the expression of several other transcription factors (Kerppola and Curran, 1991).

At the time this study was initiated, very little was known about the transcription factor complexes formed by the EIAV LTR. Compared to other lentiviruses, it is quite short with a U3 region of approximately 200 bp, an R region of approximately 80 bp, and a U5 region approximately 40 bp long. The average length of a lentivirus U3 region is 450 bp, the R region 100 bp, and the U5 region...
70 bp in length (Coffin, 1991). The short length of the U3 region, where most cis-acting regulatory signals are usually confined, indicated that the transcription factor-LTR complexes formed in the case of EIAV were probably fewer in number relative to complex lentiviruses such as HIV. Derse et al. (1987) performed a functional comparison of two different LTR variants isolated from proviral DNA. They identified the putative TATA box and poly-adenylation signals based on sequence analysis and identified the RNA cap site by S1 nuclease mapping. In addition, they replaced the SV40 promoter sequences of the CAT expression vector pSV2/CAT with the LTRs and quantitated their transient expression levels in a variety of cell lines. The results of this study indicated that the EIAV LTRs were very weak promoters relative to the SV40 virus promoter in all the cell lines tested but their activity was greatly enhanced, by over 200 fold, when the cell cultures were infected with EIAV. This was the first evidence that EIAV produces a product which transactivates its LTR. In a following study, Sherman et al. (1988) used deletion mutants of the LTR coupled to the CAT gene to demonstrate that deletion of the U5 and R regions abrogated transactivation in a Cf2Th cells. The transactivation response element was further localized by deletion studies in FEA cells to the R region (Dorn and Derse, 1988). More recent studies have since identified the TAR element of EIAV as a stem-loop structure formed by nucleotides +1 to +25. Similar to HIV-1, EIAV Tat interacts with an RNA element and requires additional cellular factors such as Tat associated protein kinase TAK (Herrmann, 1995; Derse et al., 1993). Although much work was performed identifying the TAR and tat regions of the genome, very little attention was given to identifying the basal transcriptional complexes formed by the LTR. In FEA cell cultures, progressive deletions indicated that sequences upstream of position -111
were dispensable for basal promoter activity. Deletion of an additional 35 bp produced an LTR mutant almost twice as active as the un-modified version indicating the presence of a negative regulatory element. Further removal of sequences to positions -41 or -31 resulted in mutants whose promoter activities were only about half the original while deletion of regions downstream of the transcriptional start site had no significant effect (Dorn and Derse, 1988). These results were similar to those obtained by a study of LTR deletion mutants in Cf2Th cells. This study also showed that regions downstream of the cap site were dispensable for basal transcription and that deletion of U3 sequences upstream of position -50 removed a negative regulatory element and created a deletion mutant with a much stronger promoter activity than the original LTR (Sherman et al., 1989).

During the course of this investigation, several studies were published which identified putative transcription factor binding sites in the EIAV LTR. These studies were performed in canine osteosarcoma (D17), human epithelial carcinoma (HeLa), human monocytic (U937 and THP-1), equine fibroblast (equine 6288), and human B-cell (Raji) cell lines as well as primary horse mononuclear cell cultures (PBMC). Using an LTR isolate derived from a non-infectious proviral DNA clone of EIAV, λ12, Carvalho and Derse (1993a) identified four regions protected from DNase 1 digestion by D17 nuclear extracts. Three protected regions are upstream, but within 100 base pairs, of the transcription start site while the fourth region is immediately downstream.

Based on recognition site homology, the first protected region (nucleotides -98 to -86) was believed to overlap with a binding site for the methylated DNA binding-protein (MDBP). This conclusion was supported by subsequent
electrophoretic mobility shift assays (EMSA) using D17, Raji, and THP-1 nuclear extracts. The protein-DNA complexes formed by the extracts were inhibited by the addition of an oligonucleotide containing a previously characterized MDBP binding element. In addition, deletion of sequences upstream of nucleotide -76 reduced promoter activity 38% and 58% in D17 and HeLa cells respectively, using transient gene expression assays. Subsequently, MDBP has been shown to be an isoform of histone protein H1 (Bruhat and Jost, 1995; Jost and Hofsteenge, 1992). This may reconcile this study with those previous in which a negative regulatory element was identified. The complete assembly of histone complexes would depend upon cellular conditions and could inhibit initiation of transcription from the LTR.

The second protected region (nucleotides -81 to -60) was postulated to contain two polyomavirus enhancer A factor 2 (PEA2) binding elements (Carvalho and Derse, 1993a). The protein which recognizes this element is a member of the same family of transcription factors as the Caat box binding protein (Merriman et al., 1995). An oligonucleotide containing these elements formed protein-DNA complexes with D17, equine 6288, and THP-1 but not with HeLa and Raji nuclear extracts. Formation of these complexes was inhibited by co-incubation with an oligonucleotide based on LTR nucleotides 2 to 27 which is the fourth protected region. Therefore, the binding element contained within nucleotides 2 to 27 is also a putative PEA2 binding site. Deletion and mutation analysis indicate that the second protected region behaves in a complex manner. Mutation of each site individually, PEAmut1 and PEAmut2, resulted in no change in promoter activity in D17 cells while PEAmut2 reduced activity by 75% and PEAmut1 increased activity by 170% in HeLa cells. Complete deletion of both sites (nucleotides -76 to -63) reduced activity by 40% in D17 cells, 50% in THP-1 cells and by 95% in HeLa
cells. The conclusions drawn from these results is that this region is very important for promoter activity but that different element(s) within or overlapping this region are active depending upon the cell line used. The identification of the active elements as PEA2 sites remains, at best, inconclusive.

The protected region in closest proximity to the TATA box contains, or is adjacent to, three putative binding motifs: AP-1, PU.1 and Oct. This region as a whole was found to confer TPA responsiveness upon the LTR. Mutation of the AP-1 motif (nucleotides -45 to -60) reduced promoter activity by 86%, 50%, and 95%, in D17, THP-1 and HeLa cells respectively. Nuclear extracts prepared from D17 cells formed a protein-DNA complexes with an oligonucleotide based on LTR nucleotides -40 to -67. Formation of this complex was only partially inhibited by the addition of a competitor oligonucleotide containing a consensus AP-1 site raising the possibility that this site binds different AP-1 heterodimers or multiple transcription factors. The second half of this protected region was postulated to contain a putative Oct motif overlapping a PU.1 recognition element. Mutation or deletion of this PU.1 element reduced promoter activity by 80% or 51%, respectively, in D17 cells whereas promoter activity was completely abolished in HeLa and THP-1 cells. An oligonucleotide corresponding to LTR nucleotides -30 to -58 was found to form a complex pattern of protein-DNA complexes with D17 cell extracts. Competitor oligonucleotides containing either an Oct or a PU.1 recognition element were able to change the mobility shift pattern formed by LTR oligonucleotide -30 to -58. On the basis of this data, it was postulated that in the absence of PU.1, Oct could bind the vacated element (Carvalho and Derse, 1993a). In a following study, Carvalho and Derse (1993b) showed that formation of protein-DNA complexes with an oligonucleotide based on LTR nucleotides -35 to -
46 by THP-1 and U937 cell extracts was abolished by pre-incubation with an antiserum raised against PU.1 and that PU.1 expression by HeLa cells stimulated transcription from the LTR by 10 fold. The definitive study of the PU.1 element was performed by Maury (1994) who utilized horse monocyte cell cultures and an LTR isolate, derived from the macrophage cultures infected with the Th-1 field isolate of EIAV, which contained three possible PU.1, an AP-1, and a PEA2 binding motifs. The maturation of the horse monocyte cell cultures was controlled by the withholding or the addition of adult horse sera. Mobility shift assays demonstrated that cultures which were allowed to mature to adherent macrophages formed pronounced DNA-protein complexes with the three PU.1 motifs of the LTR but none with possible AP-1 and PEA2 motifs. Cultures held as monocytes did not form these complexes at readily detectable levels. This study also showed that the complexes formed at the PU.1 sites were super-shifted by the addition of antiserum against PU.1 but not when incubated with serum against Ets-1 or Ets-2 transcription factors. Interestingly, mutation of all three possible PU.1 binding sites did not result in a drop in promoter activity under basal conditions, but eliminated transactivation by EIAV Tat. Therefore, PU.1 may directly interact with EIAV Tat. Although there are a great many curious or contradictory results in all these studies, it seems likely that provirus transcription in macrophages is controlled primarily through the expression and subsequent binding of PU.1.

Sequence variations in the LTRs of retrovirus isolates are a common phenomena and, in some cases, result in dramatic changes in the transcription factor binding motifs discussed previously. Deletion, modification, or insertion of recognition sites has been shown to affect in vitro promoter strength and replication capacity of the virus (Carvalho et al., 1993). It has often been proposed that these
sequence variations could influence the disease causing potential of different virus isolates by influencing replication capacity or cellular tropism in vivo (Clements and Payne, 1994).

The effects of LTR modification on virus phenotypes is well documented in several oncovirus systems. Rous-associated virus (RAV) is a non-acute oncovirus found in chickens. RAV-0 is benign whereas RAV-1 infection induces lymphoma and a variety of neoplasms. By exchanging regions of a pathogenic proviral RAV-1 clone with corresponding regions from RAV-0 proviral DNA, it was found that the primary determinant of disease causing potential mapped to the 5' LTR region (Brown et al., 1988). In a different study, investigators used the polymerase chain reaction technique to amplify Feline leukemia virus proviral DNA from cats with benign infections and from cats with thymic lymphomas. U3 sequences amplified from tumor cells showed considerable sequence divergence from the parent strain used for inoculation and 50% had sequence duplications of the core enhancer region. A survey of U3 sequences from benign infections showed few differences from the parental strain (Rohn and Overbaugh, 1995). Numerous experiments performed with murine leukemia viruses indicate that their long terminal repeats also confer tissue selectivity. Recombinant viruses with an LTR derived from a virus which causes T-cell leukemia preferentially infected the thymus while the same provirus backbone with an LTR derived from a virus which causes erythroleukemia preferentially infected spleen and bone marrow (Rosen et al., 1985). Of course, these examples all involve oncovirus systems in which the primary pathological effect is cell transformation. Two mechanisms by which oncoviruses induce transformation of the host cell, over-production of an adjacent cellular gene or production of a viral oncogene, are directly dependent upon the
promoter strength of the viral LTR. The affects of LTR variability on the behavior of lentiviruses is more difficult to discern as diversity in structural or regulatory gene products are most often responsible for changes in phenotype. In a study by Englund et al. (1991), three HIV-1 molecular clones isolated from a single virus stock demonstrated different replication kinetics and cytopathogenicity when propagated in a human T-cell line. The molecular clone which produced reverse transcriptase activity the most rapidly also caused the most rapid and severe loss of host cell culture viability. The viral determinant for this behavior was subsequently mapped to the LTR which was found to contain a 14 bp sequence insertion that created a third NF-κB recognition motif. The basal promoter activity of this LTR was subsequently found to be approximately four times as great as that of the LTRs from the two remaining molecular clones. To demonstrate the influence of the LTR on cellular or tissue tropism, transgenic mice were created which contained an HIV-1 provirus under the control of LTRs derived from infected neurons or from infected T-cells. Mice containing LTRs derived from neurons showed viral replication in their central nervous systems while mice containing T-cell tropic LTRs did not (Corboy et al., 1992). Thus the LTR region of retroviruses can play a very important role in tissue tropism which, in turn, can influence disease outcome.

Similar to other lentiviruses, the U3 region of the EIAV LTR demonstrates a high degree of sequence variability. Analysis of viral cDNA isolated from sequential disease episodes of a pony experimentally infected with a pathogenic isolate of EIAV demonstrated a nucleotide divergence rate for the LTR as high as 5.6% compared to a 1.43% divergence overall. In addition, analysis of proviral DNA isolated from horse peripheral blood mononuclear cell cultures (PBMCs) infected in vitro yielded a similar result, a nucleotide divergence rate for the LTR as
high as 8.4% between two different virus stocks (Carpenter et al., 1991). Both these studies also indicated that the majority of the sequence divergence was localized to a hypervariable region centered approximately 60 bp upstream of the TATA box which was prone to imperfect sequence duplications. These duplications resulted in the insertion of an additional 20 to 28 bp and the creation of LTR sequence variants. These insertions may duplicate or create transcription factor binding sites thereby changing the promoter activity of the LTR.

In a recent study, Carvalho et al (1993) examined the behavior of variant LTR isolates, amplified from FEA cell cultures persistently infected with EIAV, in U937, HeLa, and D17 cells. All these isolates contained sequence insertions in the hypervariable region that created additional AP-1, Oct, and/or PU.1 binding sites. Transient expression assays demonstrated that the LTR variants behaved differently from cell line to cell line. In U937 cells, the putative AP-1 duplication actually seems to inhibit promoter activity while successive duplications of the PU.1 motif increase it. In HeLa cells, additional PU.1 and/or AP-1 motifs result in increased activity while neither duplication has any discernible effect on activity in D17 cells. Only the creation of an Oct site results in an increase in basal promoter activity. It was speculated that these LTR variations may influence disease outcome similar to what occurs in other lentiviral systems.

Research Objectives

The goal of this dissertation research was to examine the influence of EIAV LTR variability on promoter activity, replication kinetics, cytopathogenicity, and tissue tropism. The specific research objectives are: (1) isolation and sequence analysis of multiple variant LTR isolates; (2) measurement of variant LTR promoter strength by transient gene expression in cell cultures; (3) deletion analysis
of the LTR variant exhibiting the highest promoter activity; (4) quantitation of variant LTR sequences in DNA isolated from infected horse tissues using competitive PCR; and (5) placement of an LTR variant in an infectious proviral clone and examination of its replication kinetics and cytopathogenicity by measurement of cell culture proliferation, reverse transcriptase activity, viral mRNA and viral DNA production, and immunofluorescence.
CHAPTER 2
MATERIALS AND METHODS

Virus Strains and Cell Cultures

Fetal donkey dermal (FDD), canine fetal thymic (Cf2th), and feline embryonic (FEA) cell cultures were maintained in Eagle's minimum essential media (MEM) (Sigma) supplemented with 10% fetal calf sera (Atlanta Biologicals) and 50 ug/ml Gentamycin (Sigma). FDD-adapted EIAV (AFDD) was obtained by sequential passage of Prototype virus in FDD cultures 27 times.

Preparation of Total Cellular DNA from Infected FDD Cultures

FDD cell cultures, infected with AFDD, were washed and harvested 6 days post-infection with phosphate buffered saline (PBS) solution-10 mM EDTA (PBSE). Cells were re-suspended in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.025 mM EDTA (pH 8.0), 0.1 mg/ml Proteinase K, 0.5% SDS, and incubated at 55°C overnight. The lysate was subjected to phenol and phenol/chloroform extractions followed by ethanol precipitation and centrifugation. The pellet was re-suspended in 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM NaCl, 20 ug/ml RNase A, and incubated at 37°C for one hour. The solution was extracted with phenol/chloroform and ethanol precipitated sequentially in the presence of 2.5 M NH₄Ac to remove free nucleotides. The air dried DNA pellet was re-suspended in TE buffer (pH 7.6) at 50°C overnight and quantitated spectrophotometrically.

Amplification, Cloning, and Sequencing of AFDD LTR

The 3' LTR was amplified by the polymerase chain reaction using the primer pair →ALTR5'(5'-ATTATGAAGCTTATGTATCAATGCCTCA-3') and ←ALTR3'(5'-ATAATCCAGCTGTAGGATCTCGAACA-3') which contain the restriction enzyme sites HindIII and PvuII respectively. The amplification reaction
was performed in 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 6 mM MgSO₄, 200 uM dNTPs, 2 uM primers, 0.5 ug template DNA, and 2 units Vent (exo-) polymerase (New England Biolabs) at 94°C 1 minute, and 60°C 30 seconds for 35 cycles. The reaction product was digested with HindIII and PvuII restriction endonucleases and ligated into HindIII/EcoRV digested pBluescript II KS+ (Stratagene) to form pALTR/Blue constructs. Clones were screened by sequencing using the dideoxy method as recommended by the manufacturer (United States Biochemical). Sequence comparison and alignment were performed using UGWCG software package. The sequence of AFDD LTR clone 3 (ALTR3) was numbered relative to the putative transcription start site previously identified for the LTR of EIAV molecular clone λ12 (Derse et al., 1987).

Construction of LTR/Reporter Vectors

The pLTR/CAT expression vector, containing the 5' LTR of molecular clone λ12 upstream of the chloramphenicol acetyl transferase (CAT) gene of pSV0/CAT, was provided by Dr. Louis Schiltz. For purposes of clarity, this LTR sequence will be referred to as Prtt and the construct pPrtt/CAT. Variant LTRs from sequential horse isolates p3.2-1, p3.2-3, and p3.2-5 were obtained as SspI/DraI fragments of viral cDNA ligated into the multiple cloning region of HindII digested pUC13. These constructs were provided by Dr. Keith Rushlow. The LTR variants were removed as HindIII/MluI fragments and ligated with MluI/BamHI and BamHI/HindIII vector fragments derived from pPrtt/CAT (Fig. 3). The constructs produced (p3.2-1/CAT, p3.2-3/CAT, and p3.2-5/CAT) contain variant U3 sequences upstream of the MluI site, which includes the hypervariable region, with the remainder of the LTR being identical to Prtt. ALTR3 and ALTR4

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Figure 3. Block diagram of the EIAV LTR. The relative locations of restriction enzyme sites used in molecular cloning are indicated for the EIAV LTR in the context of the pPrtt/CAT construct (Panel A) and the molecular clone pSPEIAV44 (Panel B).
were removed and gel purified from pBluescript II KS+ and placed into
HindIII/StuI digested pPrtt/CAT to form pALTR3/CAT and ALTR4/CAT. The
constructs contain variant sequences upstream of the StuI site. The cognate LTR
(PV44) of the infectious molecular clone pSPEIAV44 (Payne et al., 1994) was
obtained as an SpeI/MluI fragment cloned into Xbal/EcoRI digested pUC13. The
LTR was removed as a HindIII/MluI fragment and ligated with MluI/BamHI and
BamHI/HindIII fragments of pPrtt/CAT to form pPV44/CAT. This construct
contains PV44 sequences upstream of the MluI site and Prtt sequences downstream.
Site-directed mutagenesis was used to create an XhoI site within the U3 region of
ALTR3. This facilitated the cloning of ALTR3 into an infectious EIAV clone. The
creation of A3(XhoI)/Alt is outlined in another section. The LTR mutant was
removed from this construct as a HindIII/HindIII fragment and placed into HindIII
digested pSVO/CAT to form A3(XhoI)/CAT. LTR fragments obtained by digestion
of pPrtt/CAT, pALTR3/CAT, p3.2-1/CAT, and p3.2-5/CAT with HindIII were gel
purified and cloned into HindIII digested pGL2-Enhancer (Promega) luciferase
reporter vector to generate pPrtt/Luc, pALTR3/Luc, p3.2-1/Luc, and p3.2-5/Luc.
All clones were verified by restriction digestion.

**Deletion Mutagenesis of pPrtt/CAT**

The LTR fragment that results from HindIII/AluI digestion of Prtt,
containing U5, R, and U3 sequences downstream of the AluI site, was modified
with a synthetic HindIII linker and subsequently inserted into HindIII digested
pSVO/CAT to form pD(HindIII/AluI)/CAT. A construct which contains no U3
sequences upstream of the AluI site. The deletion mutant pD(AluI/HincII)/CAT
was created by joining a PstI/AluI fragment, containing the first 94 base pairs of
U3, to a HincII/EcoRI fragment, containing U5, R, some U3 sequences, and a

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portion of the CAT gene, which had been inserted into *HincII/EcoRI* digested pUC13. The LTR sequences were removed from this intermediate as a *HindIII* fragment and inserted into *HindIII* digested pSV0/CAT. This construct is missing the hypervariable region and flanking sequences. Products were screened by restriction enzyme digestion and verified by sequencing.

**Construction of ALTR3/Luc Mutants.**

Deletion mutants were derived from pALTR3/Blue using a primer directed method. In brief, this procedure uses a pair of primers, which flank the region to be deleted, to amplify the remainder of the LTR and the entire vector to produce a linear molecule which is subsequently re-ligated and propagated within a bacteria host. Reactions were performed in 20 mM Tris-HCl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 6 mM MgSO₄, 200 uM dNTPs, 1-2 uM primers, 2 ng template DNA (pLTR3/Blue), and 2 units Vent (exo+) polymerase at 94°C 2 minutes, 62°C 1 minute, and 72°C 3 minutes for 25 cycles. Products were screened by sequencing and correct mutant LTR sequences were excised from pBluescript II KS⁺ with *KpnI/Sacl* and inserted into the pGL2-Enhancer vector similarly digested. Primer pairs used and their deletion products are described in Table 2.

**Transfection of FDD Cell Cultures**

Transfections were performed using the calcium phosphate precipitation technique. All plasmid DNA was purified by large scale alkaline lysis followed by centrifugation in CsCl/ethidium bromide density gradients. Ethidium bromide and cesium chloride were removed by four butanol extractions and two ethanol precipitations respectively. Purified DNA was quantitated spectrophotometrically. FDD, Cf2th, and FEA cells were plated at a density of approximately 2×10⁵ cells per well in 6 well (35 mm) culture dishes and allowed to grow for 24 hours. Cells
were washed once with serum-free MEM media and re-fed with 3 mls fresh growth media prior to transfection. A 0.250 M CaCl₂ solution (0.150 ml) was mixed with 3 ug LTR reporter vector, 6 ug pSV/β-Gal (Promega), and 1 ug pBluescript DNA in the case of basal conditions or 3 ug LTR reporter vector, 6 ug pSV/β-Gal, and 1 ug pSV/720 DNA (an EIAV Tat expression plasmid) in the case of transactivated conditions. A 280 mM NaCl, 50 mM HEPES, and 1.5 mM Na₂HPO₄·7H₂O solution (HBS) (pH 7.1) (0.150 ml) was added drop-wise under a stream of nitrogen gas. The solution was incubated at room temperature for 15 minutes and then added drop-wise to the appropriate tissue culture well. Transfection was allowed to proceed for 6 hours followed by a serum free media wash, a 3 minute glycerol shock (15%), a serum free media wash, followed by the addition of 3 mls of growth media. Cells were harvested for CAT assay 48 hours post-transfection by washing and harvesting in PBSE followed by one wash in regular PBS.

Table 2. Primer pairs and their deletion products.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Deletion Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>←LTRP1: 5'-CATACTGAGGCATTGATACAT-3'</td>
<td>pD(-233/-185)/Luc</td>
</tr>
<tr>
<td>→LTRP2: 5'-AAATGATTATAAGAGTAAAAAGAA-3'</td>
<td>pD(-181/-135)/Luc</td>
</tr>
<tr>
<td>←LTRP3: 5'-TTTATAAAACCCCTCATAAAAAC-3'</td>
<td>pD(-137/-118)/Luc</td>
</tr>
<tr>
<td>→LTRP4: 5'-ATAACCCAAAGGACTAGCTCA-3'</td>
<td>pD(-117/-101)/Luc</td>
</tr>
<tr>
<td>←LTRP5: 5'-AGGTATGAGGACTCATCAGCAA-3'</td>
<td>pD(-137/-118)/Luc</td>
</tr>
<tr>
<td>→LTRP6: 5'-CTCATGTTGCTAGGCAACTAA-3'</td>
<td>pD(-117/-101)/Luc</td>
</tr>
<tr>
<td>←LTRP7: 5'-CTAGTCTTTTGTTATGCA-3'</td>
<td>pD(-117/-101)/Luc</td>
</tr>
<tr>
<td>→LTRP8: 5'-CTAAACCGCAATAACCGCAT-3'</td>
<td>pD(-117/-101)/Luc</td>
</tr>
</tbody>
</table>

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Chloramphenicol Acetyl Transferase and Luciferase Enzyme Assays

CAT assays were performed by the acetyl-chloramphenicol diffusion method (Neuman et al., 1987). Cell pellets were re-suspended in 100 mM Tris buffer (pH 7.8) and subjected to three rounds of rapid freeze-thawing in a dry ice, ethanol bath. Insoluble cellular debris was pelleted by centrifugation (12,500×g) at 4°C for 5 minutes. The supernatant (0.050 ml) was heated to 70°C for 15 minutes, mixed with 0.200 ml of a 1.25 mM chloramphenicol (Sigma), 100 mM Tris (pH 7.8) and 0.02 uCi [14C] butyryl coenzyme A (Dupont-NEN) solution. This solution was transferred to a 7 ml liquid scintillation counting (LSC) vial, overlaid
with 5 ml Econofluor LSC fluid (Dupont), and counted in a Beckman LSC 6000 after 16 hours at 37°C. Values represent CPM per uU β-galactosidase and an average of at least three trials. Luciferase assays were performed as recommended by the manufacturer. FDD cell pellets were re-suspended in 0.200 ml reporter lysis buffer and subjected to two rounds of rapid freeze-thaw. Cellular debris was removed by centrifugation (12,500×g) at 4°C for 5 minutes. The supernatant (0.005 ml) was diluted to 0.020 ml with reporter lysis buffer, mixed with 0.100 ml luciferase assay reagent, incubated 1 minute, and counted in a Beckman LSC 4000 in single photon counting mode. Values represent CPM per nU β-galactosidase and an average of at least three trials. β-galactosidase activity was measured by mixing 0.015 ml of cell lysate with 0.300 ml of a 100 mM sodium phosphate (pH 7.5), 1 mM MgCl₂, 45 mM β-mercaptoethanol, and 2.2 mg/ml o-nitrophenyl-β-D-galactopyranoside solution at 37°C for 16 hours and measured by spectrophotometric quantitation at 420 nm as outlined in Sambrook et al. (1989). All mean values were analyzed for statistical significance using the Kruskal-Wallis one way analysis of variance based on ranks and Dunnetts method of pairwise comparison.

**Insertion of ALTR3 into an Infectious Clone**

The LTR regions of the infectious proviral clone pSPEIAV44 and AFDD3 were separately inserted into the mutagenesis vector pALTER-1 (Promega). The proviral LTR was removed as an *NruI/BamHI* fragment, which contains the entire 3' LTR and part of the *env* gene, and inserted into *SmaI/BamHI* digested pALTER-1 to form pPV44/Alt (Fig. 3). Likewise, ALTR3 was removed from pALTR3/CAT as a *HindIII* fragment and inserted into the *HindIII* site of pALTER-1 to create pALTR3/Alt. Both were subsequently subjected to site-directed mutagenesis using
the pALTER system (Promega) in order to create an XhoI site within the U3 region upstream of the hypervariable region. Template DNA (pPV44/Alt or pALTR3/Alt) was denatured by incubation in a 2 M NaOH, 2 mM EDTA solution for 5 minutes at room temperature followed by ethanol precipitation. The denatured template DNA (0.05 pmole) was incubated with 1.25 pmol mutagenic oligonucleotide LTRMOD+ (5'-TGCTGATGCTCGAGTAACCTTGTAT-3') and 0.25 pmole ampicillin repair oligonucleotide (supplied by the manufacturer), in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 50 mM NaCl at 70°C for 5 minutes and allowed to cool slowly to room temperature. This solution was then adjusted to contain 0.5 mM dNTPs, 1 mM ATP, 2 mM DTT, 10 units T4 DNA polymerase, and 2 units T4 DNA ligase followed by incubation at 37°C for 90 minutes. The reaction was cooled and used to transform competent ES1301 mutS Eschericia Coli cells. Transformed ES1301 culture was incubated overnight in LB media/125 µg/ml ampicillin/10 µg/ml tetracycline. Plasmid DNA isolated from the overnight ES1301 culture was used to transform competent JM109 bacteria cells which were subsequently plated on LB/125 µg/ml ampicillin/10 µg/ml tetracycline. Clones were screened by restriction digestion and sequencing. A mutated clone, pA3(XhoI)/Alt, was digested with XhoI/Stul and the LTR fragment was inserted into XhoI/Stul digested pPV44(XhoI)/Alt to form pPVA3/Alt. Spel and SalI were used to remove the pPVA3/Alt env/LTR fragment which was then inserted into Spel/SalI digested pSPEIAV44 to create pSPEIAVA3.

Production and Propagation of Virus Strains

Approximately 2×10⁴ feline embryonic cells (FEA) were transfected with 10 µg of pSPEIAV44 or pSPEIAVA3 DNA according to the calcium phosphate procedure discussed elsewhere. Transfected cell cultures were propagated for two
weeks in MEM/10% FCS/50 ug/ml gentamycin after transfer to 150 cm² flasks. Cell culture supernatants were then stored at -70°C and used as infectious virus stocks in subsequent studies of viral replication kinetics and cytopathogenicity. Virus stocks were titered for infectivity by the end-point dilution technique. The end-point dilution technique involves exposing fresh cell monolayers to increasing dilutions of the infectious virus stock. At some point, the virus stock will be so diluted as to be rendered no longer infectious. After several weeks of incubation, the cell monolayers are assayed for production of viral antigen which indicates infection. The greatest dilution which still renders infection is considered to contain one infectious unit. With this information, the number of infectious units per ml for the original stock can be calculated. Five-fold dilutions of the virus stocks (1×10⁻³ to 3×10⁻⁶) in a volume of 1 ml were incubated with 1×10⁴ FDD cells at 37°C for two hours. Cells were washed 3 times with PBS and then re-fed with MEM/5% FCS/50 ug/ml gentamycin and propagated for 18 days. Cell culture supernatants were subsequently assayed for viral antigen by ELISA assay. This procedure was run in triplicate for each virus stock allowing an average titer to be determined. The final titers for pSPEIAV44, pSPEIAVAd3, and AFDD were 10³, 10³.7, and 10⁴.4 infectious units per ml respectively.

**ELISA assays for viral antigen**

Cell culture supernatants (0.100 ml) were added to a standard 96 well ELISA plate containing 0.020 ml 1% Triton per well and incubated at room temperature for 1 hour with agitation. This treatment lysed the virus and allowed the viral proteins to attach to the plate. Wells were washed 3 times with PBS/0.05% Tween 20 (PBST) and blocked with 0.300 mls PBS/0.05% tween 20/5% instant milk (Blotto) for 30 minutes. After washing as stated above, plates
were dried for fifteen minutes at 37°C to irreversibly bind viral antigen and blocking protein to the plate. Anti-p26 rabbit polyclonal antiserum (0.100 ml at a 1/500 dilution in blotto) was added to wells for 1 hour. Plates were subsequently washed and incubated 1 hour with 0.100 mls of anti-rabbit peroxidase conjugated antiserum (1/1000 dilution in blotto). After washing, the plates were assayed for peroxidase activity as stated by the manufacturer by incubation with 0.1 mg/ml tetramethylbenzidine dihydrochloride, and 0.006% hydrogen peroxide in phosphate-citrate buffer (pH 5.0) for 30 minutes at room temperature followed by spectrophotometric quantitation at 655 nm.

**Time-course Infection of FDD Cell Cultures**

Approximately \(1 \times 10^6\) FDD cells at 50% to 70% confluency were incubated with AFDD, pSPEIAV44, or pSPEIAVAd3 virus cultures at a multiplicity of infection of 0.1 at 37°C for 1 hour. Cells were washed three times with PBS, split into six 6-well tissue culture plates (35 mm) and propagated in MEM/5% FCS/50 ug/ml gentamycin. Every 24 hours, wells were harvested for Northern and Southern blot analysis with the growth media saved for measurement of soluble reverse transcriptase activity and the viability of the cell culture was measured. Remaining wells were washed three times with PBS and re-fed.

**Determination of Cell Culture Viability**

The viability of the infected cell cultures as well as a mock-infected control culture was determined daily using a Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega) according to the manufacturers directions. This colorimetric procedure measures the reduction of a tetrazolium compound into a soluble formazan product by cellular dehydrogenase enzymes. The soluble formazan is subsequently released into the culture supernatant and can be
quantitated at 490 nm. This procedure measures the overall metabolic rate of the cell culture which is dependent upon the number of viable cells and their rate of growth. Assuming similar rates of growth, absorbance measurements obtained from infected cell cultures were compared to a standard curve, which plots absorbance versus cell number (as determined by standard trypan blue exclusion), for an uninfected control culture, in order to obtain the cell titer for the infected cell culture. Specific experimental conditions were as follows. Cell monolayers were washed and overlaid with 1 ml fresh growth media to which 0.200 ml of a MTS/PMS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt and phenazine methosulfate respectively) solution was added. Cultures were allowed to incubate at 37°C for 4 hours after which the media was quantitated at 490 nm.

**Reverse Transcriptase Analysis**

One of the most accurate methods to quantitate retrovirus infections is by measuring reverse transcriptase activity. Virus was precipitated from cell culture supernatants by the addition of 0.250 ml of a 40% polyethylene glycol solution and incubation overnight at 4°C with gentle agitation. This solution was then spun at 12,500xg at 4°C for 15 minutes to pellet the virus. After complete removal of the supernatant, the virus pellet was lysed by re-suspension in 0.040 ml of solubilizing buffer (50 mM Tris (pH 7.8), 20% glycerol, 0.8 M NaCl, 0.5% Triton X-100, and 0.5 mM phenylmethylsulfonylfluoride) and incubated on ice for 10 minutes. Solubilized virus (0.020 ml) was added to 0.180 ml of reaction buffer (52 mM Tris (pH 7.8), 10 mM MgCl, 5 mM DTT, 0.20 mM dATP, 0.9 ug poly(rA)-oligo(dT), and 52 uCi [3H]dTTP) and incubated at 37°C for 1 hour. Yeast tRNA was added to a final concentration of 0.1 mg/ml followed by 1 ml of ice cold 10% trichloroacetic
acid in order to precipitate nucleic acid. The solution was kept on ice for 30 minutes followed by filtration through a grade 25 glass microfiber filter. Filters were then washed three times with 1 ml of ice cold 5% TCA, 1 ml 70% ice cold ethanol, and 1 ml ether. Dried filters were counted in 7 ml of Liquisint liquid scintillation cocktail in a Beckman LSC 6000 for 1 minute. Final values reflect subtraction of background counts.

**Isolation and Purification of Poly-adenylated RNA**

Cell cultures were harvested by incubation in PBSE for 10 minutes at 37°C followed by three washes in PBS. Cell pellets were re-suspended in 0.400 ml denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sodium sarcosyl, and 0.1 M 2-mercaptoethanol) and incubated on ice for 10 minutes. Sequentially, 0.04 ml sodium acetate (pH 5.2) and 1 ml phenol (pH 4.0)/chloroform/isoamyl alcohol (25/24/1) were added and the solution incubated on ice for 30 minutes followed by centrifugation at 12,500×g for 15 minutes at 4°C. RNA was precipitated from the aqueous solution and washed in 70% ethanol. Poly-adenylated (Poly-A) RNA was purified from the total RNA by using a MicroFastTrack mRNA isolation kit (Invitrogen) and quantitated.

**Isolation and Purification of DNA**

Cell cultures were harvested by incubation in PBSE for 10 minutes at 37°C followed by three washes in PBS. Cells were re-suspended in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.025 mM EDTA (pH 8.0), 0.1 mg/ml Proteinase K, 0.5% SDS, and incubated at 55°C overnight. The lysate was subjected to phenol and phenol/chloroform extractions followed by ethanol precipitation and centrifugation. The pellet was re-suspended in 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM NaCl, 20 μg/ml RNase A, and incubated at 37°C for one hour. The
solution was extracted with phenol/chloroform and ethanol precipitated sequentially in the presence of 2.5 M NH₄Ac to remove free nucleotides. The air dried DNA pellet was re-suspended in TE buffer (pH 7.6) at 50°C overnight and quantitated.

**Northern Blot Analysis**

Sequentially, 0.6 ug poly-A RNA was re-suspended in 40 mM MOPS (pH 7.0), 10 mM sodium acetate, 0.2 mM EDTA, 50% formamide, and 6% formaldehyde, incubated at 60°C for 15 minutes, and allowed to cool on ice. Glycerol, xylene cyanol, and bromophenol blue were added to final concentrations of 10%, 0.08%, and 0.08% respectively. Samples were applied to a 1.5% agarose gel that contained 6% formaldehyde. Electrophoresis was conducted in 40 mM MOPS (pH 7.0), 10 mM sodium acetate, 0.2 mM EDTA at 25 volts for 4.5 hours. Upon completion of electrophoresis, the gel was washed in 1×SSC (0.15 M sodium chloride, 0.015 sodium citrate) and the RNA transferred to a Nytran membrane (Schleicher and Schuell) by the capillary method in 10×SSC overnight. The membrane was washed in 5×SSC for 5 minutes and then cross-linked by exposure to 120 mJ/cm² of UV light in a Stratagene Stratalinker. The blot was pre-hybridized in 50% formamide, 5×SSC, 0.1% SDS, 200 µg/ml sonicated herring sperm DNA, and 5×Denhardt's reagent at 42°C for 2 hours followed by hybridization, in fresh pre-hybridization solution plus approximately 10 ng/ml denatured probe (1×10⁷ CPM/ml), overnight at 42°C. Probe prepared using a Rediprime DNA labeling system (Amersham) with pSPEIAV44 as the template DNA and Redivue [³²P]dCTP (Amersham). Washes were performed twice in 6× SSC, 0.5% SDS at room temperature for 15 minutes, twice in 1×SSC, 0.5% SDS at 37°C for 15 minutes, once in 0.1×SSC, 0.1% SDS at 65°C for 30 minutes, and once
in 0.1×SSC at room temperature for 15 minutes. The damp membrane was inserted between two plastic transparency sheets and exposed to X-OMAT AR (Kodak) X-ray film for 24 to 48 hours.

**Southern Blot Analysis**

Cellular DNA (5.0 ug) was digested to completion with *StuI*, which cuts the EIAV genome in the LTR U5 region exclusively. Glycerol, xylene cyanol, and bromophenol blue were added to final concentrations of 10%, 0.08%, and 0.08% respectively and the sample was subjected to electrophoresis in a 0.8% agarose gel in TAE for 16 hours at 20 volts. Upon completion of electrophoresis, the gel was soaked once in 0.25 M HCl for 10 minutes, twice in 1.0 M NaCl, 0.5 M NaOH for 15 minutes, and twice in 1.5 M NaCl, 0.5 M Tris (pH 7.4). The fractionated DNA was then transferred to a Nytran membrane (Schleicher and Schuell) by the capillary method in 10×SSC overnight. The membrane was washed in 5×SSC for 5 minutes and then cross-linked by exposure to 120 mJ/cm² of UV light in a Stratagene Stratalinker. The blot was pre-hybridized in 50% formamide, 6×SSC, 0.5% SDS, 200 μg/ml sonicated herring sperm DNA, and 5×Denhardt’s reagent at 42°C for 2 hours followed by hybridization, in fresh pre-hybridization solution plus approximately 10 ng/ml denatured probe (1×10⁷ CPM/ml), overnight at 42°C. Probe prepared using a Rediprime DNA labeling system (Amersham) with pSPEIAV44 as the template DNA and Redivue [³²P]dCTP (Amersham). Washes were performed twice in 7×SSC, 0.5% SDS at room temperature for 15 minutes, twice in 1×SSC, 0.5% SDS at 37°C for 15 minutes, once in 0.1×SSC, 0.5% SDS at 65°C for 30 minutes, and once in 0.1×SSC at room temperature for 15 minutes. The damp membrane was inserted between two plastic transparency sheets and exposed to X-OMAT AR (Kodak) X-ray film for 72 hours.
**Immunofluorescent Slide Staining**

Cell monolayers were washed once in PBS and harvested in 1 ml PBSE. Cell suspensions were streaked onto gelatin coated slides and allowed to air dry. Cell smears were fixed by incubation in acetone/formaldehyde fixing solution for 30 seconds at room temperature and air dried. Fixed slides were incubated in blocking solution (PBS with 5% BSA) for 1 hour followed by incubation in PBS plus rabbit polyclonal antiserum against EIAV protein gp90 (1/50 dilution) for 2 hours. Smears were washed for 10 minutes in PBS/tween 20 (0.5%) 3 times and re-blocked in PBS/BSA for 1 hour followed by incubation with anti-rabbit fluorescein conjugate diluted 1/100 in PBS for 2 hours. All of these steps were performed at 4°C. Following final washing (3 times in PBS/tween 20), cells were photographed using a fluorescent light source at 400× magnification.

**Competitive PCR**

Reactions were performed primarily by Pramod Vijayagopal of Dr. Ding Shih's laboratory using horse tissue genomic DNA kindly provided by Dr. Susan Payne. Genomic DNA was isolated from liver, lung, kidney, and lymph node tissues of a horse euthanized during an acute disease episode after being experimentally infected with the Wyoming strain of EIAV. Samples were isolated and purified by Dr. Payne using Qiagen DNA purification columns according to the manufacturers directions and supplied to this investigator in a lyophilized form. The control plasmids, pD1/GL2 and pD3/GL2, were constructed from LTR variants p3.2-1 and p3.2-3 which were removed from pSV0 as HindIII fragments and cloned into the HindIII site of pGL2-Enhancer. Deletion of 58 bp from the U5 regions of these LTRs was accomplished using the primer directed method outlined elsewhere and the primer pair ←LTRP21 (5'-AGAAGGGACTCAGACCGCA-3') and →
LTRP22 (5'-GTTTGTCTGTTCGAGATCCTA-3') which delete LTR sequences between nucleotides 32 and 90. This deletion allows control plasmid amplification products to be separated from tissue DNA products electrophoretically. The oligonucleotide primer →P-3 (5'-TAAAATGTAGTTCTCCTATAGTTCCG-3') is identical to a unique region (nucleotides -111 to -84) of variant LTR 3.2-3 and will only amplify similar LTR variants. Primer →P-1 (5'-ATTGTGACGCGAGTTCCCCATTGG-3') is identical to a unique region (nucleotides -84 to -58) of variant LTR 3.2-1 and will only amplify similar variants. The primer ←P-C (5'-GTAGGATCTCGAACAGACAAAC-3') is complementary to a region at the end of the LTR (nucleotides 112 to 91) and will recognize both types of LTR variants. Reactions were performed in 20 mM Tris-HCl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 6 mM MgSO₄, 200 uM dNTPs, 200 uM [α-35S]dATP, 1 uM primers (→P-1 and ←P-C or →P-3 and ←P-C), 150 ng infected tissue DNA (liver, lymph node, lung, or kidney), 300 to 0.003 pg control plasmid DNA (pD1/GL2 or pD3/GL2), and 2 units Taq polymerase at 94°C (60 seconds) and 60°C (30 seconds) for 35 cycles. Reactions were resolved on 8% non-denaturing polyacrylamide gels in 1×TBE at 50 volts for 5 hours. Gels were fixed in 7% acetic acid for 20 minutes followed by vacuum drying and exposure to X-omat AR film for 24 hours at room temperature.
CHAPTER 3

RESULTS AND DISCUSSION

Comparative Analysis of Variant LTR Sequences

The prototype LTR, Prtt, was obtained from a proviral DNA clone of EIAV, λ12. (Rasty et al., 1990). It contains a number of possible transcription factor binding sites including a PU.1 an AP-1, and two PEA2 motifs. These sites have been identified using a variety of established cell lines such as human monocytic (U937), cervical carcinoma (Hela), canine osteosarcoma (D17), and equine fibroblasts (equine 6288), as well as primary horse macrophage cultures (Carvalho and Derse, 1993a; Carvalho and Derse, 1993b; Carvalho et al., 1993; Maury, 1994). Although the EIAV LTR may form unique transcription factor complexes within each different cell type, these motifs influence promoter activity in some manner in almost every cell culture in which the LTR has been studied. Therefore, it is likely that some of these same motifs influence the promoter activity of the LTR within FDD, Cf2Th, and FEA cells as well.

Sequence variations within the EIAV LTR that result in duplication or modification of these sites is well documented. These variations can influence the promoter strength of the LTR in a cell-line dependent manner (Carpenter et al., 1991; Carvalho et al., 1993) and it has been suggested that they may play a role in influencing cellular tropism of the virus in vivo. In addition, the LTR directly influences the quantity of viral mRNA, the levels of which, in turn, influence the replication kinetics or replication capacity of the virus. While a higher replication rate may result in a more virulent sub-type of virus, increasing the severity of disease episodes, a lower relative rate of replication, resulting in attenuation, may cause the virus to establish less severe, persistent infections. Due to the lack of a
pathogenic molecular clone, this study relied primarily on in vitro experiments, examining the effects of LTR variation on promoter strength, replication, and cytopathogenicity, in order to implicate LTR sequence variation in virus pathology.

The first step of this investigation involved the sequence analysis of multiple LTR variants from a variety of sources. ALTR3 and ALTR4 were amplified from the cellular DNA of FDD cell cultures infected with FDD-adapted virus (AFDD), a virus stock that demonstrates accelerated cytopathogenic effects in FDD cell cultures, using the polymerase chain reaction. Six of the positive clones identified were sequenced and their homology analyzed by the software program Pileup (Genetics Computer Group). This program generates an alignment of multiple DNA sequences representing their maximum homology. The six AFDD LTR sequences analyzed were highly homologous with a maximum variability of only 1.6 percent. AFDD LTR clone 3, ALTR3, and clone 4, ALTR4, were chosen for subsequent functional studies as they represent the majority of LTR sequence variants within the AFDD strain. These sequences were aligned and compared with Prtt (Fig. 4). Alignments were based on maximum sequence homology and numbered relative to the ALTR3 sequence. The hypervariable region of ALTR3 differs from the corresponding Prtt region in that it contains a 20 bp sequence insertion, upstream of the putative transcription start site of ALTR3 beginning at position -58, as well as an additional adenine residue at position -51. ALTR3 also contains three transitions relative to Prtt; a thymine to cytosine at -135, an adenine to guanine at -164, and a thymine to cytosine at position -3. These mutations may have a significant effect on transcription factor binding sites or regulatory elements previously identified in the prototype LTR. The large sequence insertion causes imperfect duplications of the putative PU.1 and AP-1 binding sites and the
Figure 4. Sequence comparison of variant LTR isolates. Alignment of LTR variants isolated from FDD-adapted virus, (ALTR3 and ALTR4), molecular clone λ12 (Prtt), molecular clone pSPEIAV44 (PV44), and three sequential isolates from an infected pony (p3.2-1, p3.2-3, and p3.2-5). Alignments were based on maximum sequence homology and numbered relative to the transcription start site of ALTR3. Transcription factor recognition sites, TATA box, and TAR element, are also shown.
-210
ALTR3:  TGTGGGGTTT TTATGAGGGG TTTTATAAAT GATTATAAGA GTAAAAGGAA
ALTR4:   A
3.2-1:      A
Prtt:       A
PV44:       A
3.2-3:      A
3.2-5:      A

-160
ALTR3:  AGTTGCTGAT GCTCTCATAA CCTTGCAAA CCCAAAGGAC TAGCTCATGT
ALTR4:   T
3.2-1:      T
Prtt:       T
PV44:       T
3.2-3:      T
3.2-5:      T

-110
ALTR3:  TGCTAGGCAA CTAAAACGCA ATAA -----------------------------------------------CCGCAT
ALTR4:   PEA2
3.2-1:      PEA2
Prtt:       PEA2
PV44:       PEA2
3.2-3:      PEA2
3.2-5:      PEA2

-80
ALTR3:  TGTGAGCCG AGTTACCACAT TGTGAGCGCA TTAAACTTCC TGTTTTTACA
ALTR4:   AP-1
3.2-1:      AP-1
Prtt:       AP-1
PV44:       AP-1
3.2-3:      AP-1
3.2-5:      AP-1

-30
ALTR3:  GTATATAAGT GCTTGATTTG CACAATCGG GCACTCAGAT TCTGGGCTCT
ALTR4:   +1
3.2-1:      +1
Prtt:       +1
PV44:       +1
3.2-3:      +1
3.2-5:      +1

21
ALTR3:  GAGTCCCTTC TCTGCTGGGC TGAAAAGGCC TTTGTAATAA ATATAATTCT

21
ALTR3:  CTACTCAGTC CCTGTCTCTA GTTTGCTCTGT TCGAGATCCT ACA

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additional adenine at position -51 brings the original AP-1 site closer to a consensus recognition sequence (TGASTMA) (Carvalho and Derse, 1993a; Carvalho and Derse, 1993b; Diamond et al., 1990; Maury, 1994). ALTR4 is very similar to ALTR3 except it retains an adenine residue at position -164, a thymine at position -135, and is missing three thymine residues at positions -79 to -81. Based on sequence analysis, the AFDD LTR contains a number of mutations that could create additional transcription factor binding sites or modify pre-existing ones.

The cognate LTR, PV44, of pSPEIAV44, an infectious molecular clone of EIAV produced from the virulent cell culture stock PV (Payne et al., 1994), was also compared to the Prtt, ALTR3, and ALTR4 sequences. It contains an insertion of 21 bp, relative to Prtt, within the hypervariable region but this insertion causes the formation of a possible PU.1 site without the creation of a new AP-1 site as seen in AFDD LTR sequences. The insertion also interrupts one of the putative PEA2 sites (GACCGCA) (Carvalho and Derse, 1993a).

Additional variants, 3.2-1, 3.2-3, and 3.2-5 were isolated during sequential disease episodes experienced by an experimentally infected pony (Payne et al., 1987). Although derived from the same host, variant 3.2-1 is more homologous to ALTR3 and ALTR4 while variants 3.2-3 and 3.2-4 are more homologous to PV44. Isolate 3.2-1 contains a 20 bp insertion/duplication almost identical to that of ALTR3 and ALTR4 with the exception of adenine to cytosine and thymine to guanine base pair transitions at positions -74 and -59 respectively. With the exception of the insertion, 3.2-1 is identical to Prtt. Therefore, like ALTR3 and ALTR4, it contains imperfect duplications of the putative AP-1 and PU.1 binding sites but, like Prtt, its original AP-1 site is less homologous to the consensus recognition sequence. Variant 3.2-3 contains an insertion almost identical to that of
PV44 with the exceptions of an additional adenine residue at the beginning and a cytosine to thymine transition 4 bp downstream of the additional adenine. Neither of these mutations would create or interfere with any of the recognition motifs identified in the discussion of PV44. Other than the insertion, 3.2-3 is identical to Prtt. Variant 3.2-5 is a very unique variant in that it contains two significant insertions. It contains a 21 bp insertion between positions -86 and -87 which is very similar to that of PV44 with one significant exception. A thymine to cytosine transition 9 nucleotides into the insertion creates a second possible PU.1 recognition motif. The transition restores the TTCC core binding motif found in the original PU.1 site. In addition, 3.2-5 contains 5 bp identical to nucleotides -78 to -74 of ALTR3 followed by additional thymine and adenine nucleotides. The remainder of variant 3.2-5 is identical to Prtt. Therefore, variant 3.2-5 may contain up to three PU.1 and two AP-1 binding sites in close proximity to the TATA box.

In order to facilitate the cloning of ALTR3 into the proviral clone pSPEIAV44, an A  

\[ \text{XhoI} \]

site was produced by site-directed mutagenesis in a region believed to be devoid of any transcription factor binding motifs. A3(XhoI) was included in subsequent transient gene expression studies to verify that this mutation had no effect on the promoter strength of the LTR.

As can be seen by these comparisons, the EIAV LTR shows considerable heterogeneity even when isolated from the same host. The greatest region of disparity is centered approximately 70 nucleotides upstream of the TATA box. It is interesting to note that LTR variants, despite the source, can be separated into two broad classes based upon their sequence homology and transcription factor motifs. Those that contain two PEA2, AP-1, and PU.1 sites such as ALTR3, ALTR4, and 3.2-1 and those that contain only one PEA2 and AP-1 but three possible PU.1
binding sites such as 3.2-3, 3.2-5, and PV44. Of course, this is a very loose
classification as small mutations can destroy possible binding sites such as the third
PU.1 site or create additional AP-1 sites as seen in variants PV44, 3.2-3, and 3.2-5
respectively. The Prtt LTR, which is the shortest functional LTR isolated to date,
would fall into neither category.

Relative Promoter Strength of Variant LTR Sequences

To assess the impact of sequence variation within the AFDD LTR on its
promoter strength, transient gene expression assays were performed using FDD,
Cf2Th, and FEA cell cultures. Variant LTR sequences ALTR3, ALTR4,
A3(Xho1), 3.2-1, Prtt, PV44, 3.2-3 and 3.2-5 were placed upstream of the
chloramphenicol acetyl transferase, CAT, gene of pSV0/CAT (Gorman et al., 1982)
and transfected into cells by the calcium phosphate precipitation technique. Values
represent the mean of at least six trials, normalized with β-galactosidase activity,
with standard error and statistical significance as indicated (Table 3).

In FDD cell cultures, variant 3.2-5 was the least active promoter under basal
conditions. Despite their slightly higher activity, Prtt, PV44, and 3.2-3 did not
behave significantly different from 3.2-5 while ALTR3, ALTR4, A3(Xho1), and
3.2-1 were 5 to 9 times as active. The high activity of ALTR3, ALTR4, A3(Xho1),
and 3.2-1 relative to the remainder of the variants indicates that the duplicated AP-1
motif, common only to these four variants, or an unidentified overlapping motif is
responsible for the increase in promoter strength. Although these four variants also
share a duplicated PU.1 site, it seems unlikely that this motif is responsible for their
increased promoter strength as PV44, 3.2-3 and 3.2-5 also contain duplicated PU.1
motifs. Variant 3.2-5 contains an additional possible AP-1 site, but it is less
homologous to the consensus recognition sequence and appears to be inactive. The
lack of any discernible difference in activity between ALTR3, ALTR4, and A3(XhoI) indicates that the small sequence differences between these variants are irrelevant. Although highly homologous in sequence, ALTR4 was almost twice as active as 3.2-1. Therefore, LTR variants which contain similar sequence insertions tend to behave approximately the same \textit{in vivo} but smaller mutations can create significant differences. In the case of ALTR4 and 3.2-1, the additional adenine residue and guanine to thymine transition, nucleotide positions -51 and -50 respectively, in ALTR4 create a more optimal AP-1 recognition sequence which may be responsible for increasing its promoter strength relative to 3.2-1. It is also possible that the thymine to cytosine transition at nucleotide -3 may influence activity in an unidentified manner. When co-transfected with the EIAV Tat expression plasmid pSV720 (Schiltz \textit{et al.}, 1992), the constructs showed different stimulation of expression over basal levels. Prtt, PV44, 3.2-3 and 3.2-5 showed considerable stimulation, 17 to 25 fold, but ALTR3, ALTR4, A3(XhoI), and 3.2-1 showed only a 5 to 10 fold stimulation making them approximately twice as active as Prtt, PV44, 3.2-3 and 3.2-5 under transactivated conditions. In general, the magnitude of transactivation was inversely proportional to the basal activity. This effect causes the large differences in activity between LTR sequences under basal conditions to become less distinctive when under transactivated conditions. A similar effect has also been observed in studies of transactivation with the HIV-1 LTR (Antoni \textit{et al.}, 1994). Although less distinctive under transactivated conditions, there were still significant differences in levels of activity between different LTR variants. For example, 3.2-1 remains more than twice as active as Prtt.
Table 3. Variant LTR activity in FDD cell cultures.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal</th>
<th>Transactivated</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pALTR3/CAT</td>
<td>1744 ± 428s</td>
<td>8178 ± 457s</td>
<td>5</td>
</tr>
<tr>
<td>pALTR4/CAT</td>
<td>1921 ± 240s</td>
<td>10050 ± 817s</td>
<td>5</td>
</tr>
<tr>
<td>pA3(Xhol)/CAT</td>
<td>1658 ± 318s</td>
<td>8995 ± 673s</td>
<td>5</td>
</tr>
<tr>
<td>p3.2-1/CAT</td>
<td>1105 ± 264s</td>
<td>11293 ± 792s</td>
<td>10</td>
</tr>
<tr>
<td>pPrtt/CAT</td>
<td>242 ± 31</td>
<td>5406 ± 227</td>
<td>22</td>
</tr>
<tr>
<td>pPV44/CAT</td>
<td>343 ± 78n</td>
<td>5800 ± 579n</td>
<td>17</td>
</tr>
<tr>
<td>p3.2-3/CAT</td>
<td>351 ± 76n</td>
<td>6664 ± 559n</td>
<td>19</td>
</tr>
<tr>
<td>p3.2-5/CAT</td>
<td>215 ± 58n</td>
<td>5448 ± 580n</td>
<td>25</td>
</tr>
</tbody>
</table>

In FEA cell cultures, only the promoter activity of LTR variants 3.2-1, Prtt, 3.2-3, and 3.2-5 were measured (Table 4). Variants 3.2-1 and 3.2-5 were the most active promoters in this cell type producing twice as much CAT activity as Prtt. Although the average activity of 3.2-3 was higher than that of Prtt, it did not behave significantly different. The only sequence motif common to these three variants that is not found in Prtt is the duplicated PU.1 motif. Based on this comparison, it seems likely that the PU.1 motif, or an overlapping one, is being recognized by a transcription factor expressed in FEA cells. The second PU.1 site duplication found in 3.2-5 may be inactive either due to sequence differences or due to its location further upstream from the TATA box. One of the most striking differences between the results obtained in FEA cells and results obtained in FDD cells is the magnitude of transactivation. Co-transfection with pSV720 resulted in as much as a 90 fold increase in activity in FEA cells as apposed to the maximum increase of
only 25 fold seen in FDD. Without the quantitation of cellular Tat levels, it is difficult to discern whether this effect represents a difference in the mechanism of transactivation or whether it is an experimental artifact caused by the expression levels of EIAV Tat being different between the two cell lines. Similar to what was seen in FDD cells, the magnitude of transactivation was inversely proportional to the level of basal activity.

Table 4. Variant LTR activity in FEA cell cultures.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal</th>
<th>Transactivated</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>p3.2-1/CAT</td>
<td>270 ± 35s</td>
<td>11489 ± 1919n</td>
<td>55</td>
</tr>
<tr>
<td>pPrtt/CAT</td>
<td>127 ± 29</td>
<td>14789 ± 2427</td>
<td>90</td>
</tr>
<tr>
<td>p3.2-3/CAT</td>
<td>223 ± 38n</td>
<td>15012 ± 3459n</td>
<td>67</td>
</tr>
<tr>
<td>p3.2-5/CAT</td>
<td>304 ± 31s</td>
<td>14390 ± 2564n</td>
<td>47</td>
</tr>
</tbody>
</table>

In Cf2Th cells, LTR variants 3.2-1 and Prtt exhibited the strongest promoter activities showing more than twice the activity of 3.2-3 which was the weakest promoter in this cell type (Table 5). Variants ALTR3, ALTR4, A3(Xhol), and PV44 were not used in this study. Based on sequence homology, the most likely motif responsible for the increased activity produced by 3.2-1 and Prtt is a PEA2 recognition site. There are two such possible sites in both Prtt and 3.2-1 while 3.2-3 and 3.2-5 have only one. The second site in the latter variants being interrupted by sequence insertions. Although it demonstrates lower activity, p3.2-5 does not differ significantly from Prtt. Variant 3.2-5 might be forming a transcription factor complex different from both Prtt and 3.2-3 but what this difference might be is
unknown. Although the magnitude of transactivation in Cf2Th cells was not as pronounced as in FEA cells, it still tended to be larger than the magnitude of transactivation in FDD cells. The maximum increase in activity in Cf2Th cells was 38 fold whereas it was only 25 fold in FDD cells. As discussed previously, without a quantitation of cellular Tat levels it is impossible to conclude whether this result represents a difference in the mechanism of transactivation or is merely the result of more Tat being expressed in Cf2Th cells relative to FDD cells.

Table 5. Variant LTR activity in Cf2Th cell cultures.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal</th>
<th>Transactivated</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>p3.2-1/CAT</td>
<td>284 ± 39n</td>
<td>6366 ± 630n</td>
<td>22</td>
</tr>
<tr>
<td>pPrtt/CAT</td>
<td>236 ± 53</td>
<td>4985 ± 572</td>
<td>21</td>
</tr>
<tr>
<td>p3.2-3/CAT</td>
<td>112 ± 13s</td>
<td>4263 ± 509n</td>
<td>38</td>
</tr>
<tr>
<td>p3.2-5/CAT</td>
<td>160 ± 20n</td>
<td>4738 ± 516n</td>
<td>30</td>
</tr>
</tbody>
</table>

In combination, these results indicate that LTR variants behave differently in a cell line dependent manner. Thus LTR variation could strongly influence the adaptation of a virus quasi-species to a particular cell line in vitro and, by analogy, influence cellular or tissue tropism in vivo. Based on the results obtained from FDD cell cultures, which showed dramatic differences in activity levels under basal conditions, it can also be speculated that LTR variation could influence the virulence of a virus sub-type by increasing or decreasing its replication rate. The identities of the transcription factors that actually interact with the LTR variants in these cell types remain to be determined conclusively but some likely possibilities
have been discussed based upon previous studies, sequence analysis, and basal promoter strengths. Although the differences in promoter strength between variants became less pronounced under transactivated conditions, they often remained significant. The magnitude of transactivation was also dependent upon cell type but it is difficult to conclude whether this represented a difference in mechanism or was an experimental artifact.

**Deletion Analysis of the LTR**

Reporter constructs containing deletions within the U3 region of Prtt, pD(Aelu/HincII)/CAT, pD(HindIII/HincII)/CAT, and pD(HindIII/Aelu)/CAT were created using existing restriction enzyme sites in order to localize regions predominately responsible for transcriptional activity. Prtt is the most highly characterized EIAV LTR. Previous deletion studies, performed in a variety of cell lines, with this variant indicated that virtually all the active enhancer elements were located proximal to the hypervariable region (Carvalho et al., 1993; Carvalho et al., 1993; Dorn et al., 1988; Sherman et al., 1989). In order to confirm these observations, these three Prtt deletion mutants were transfected via the calcium phosphate precipitation technique into FDD, FEA, and Cf2Th cells. Values represent an average of three trials, normalized with β-galactosidase activity, with a standard error and statistical significance as indicated. The deletion mutants are missing either the hypervariable region and flanking sequences, pD(Aelu/HincII)/CAT (nucleotides -48 to -117), the remainder of the U3 region, pD(HindIII/Aelu)/CAT (nucleotides -118 to -210), or both regions, pD(HindIII/HincII)/CAT (nucleotides -48 to -210) (Fig. 5).
Figure 5. Alignment of Prtt LTR deletion mutants. The LTR regions of reporter constructs ALTR3/CAT and Prtt/CAT are shown aligned with deletion constructs derived from Prtt/CAT. Deletion constructs are missing the hypervariable region and flanking sequences (pD(AluI/HincII)/CAT), U3 sequences upstream of the hypervariable region (pD(HindIII/AluI)/CAT), and removal of both regions (pD(HindIII/HincII)/CAT). Putative cis-elements are also identified.
In FDD cell cultures, deletion of sequences encompassing the hypervariable region, as indicated by pD(AluI/HincII)/CAT, resulted in greater than a 10 fold decrease in basal activity when compared to Prtt (Table 6). In addition, deletion of these sequences seemed to affect transactivation by EIAV Tat. When co-transfected with pSV720, this mutant showed less than a 4 fold stimulation making it 78 fold less active than Prtt under transactivated conditions. Deletion of upstream U3 sequences, as shown by pD(HindIII/AluI)/CAT, exhibited a less pronounced but significant, 2 to 3 fold, drop in basal activity while showing a 42 fold stimulation upon transactivation. Strangely, deletion of almost the entire U3 region, as shown by pD(HindIII/HincII)/CAT, also resulted in a 10 fold decrease in activity under basal conditions but the magnitude of transactivation was restored to 25 fold.

Table 6. Prtt deletion mutant activity in FDD cell cultures.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal</th>
<th>Transactivated</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPrtt/CAT</td>
<td>250 ± 17</td>
<td>6583 ± 616</td>
<td>26</td>
</tr>
<tr>
<td>pD(AluI/HincII)/CAT</td>
<td>23 ± 5s</td>
<td>84 ± 15s</td>
<td>4</td>
</tr>
<tr>
<td>pD(HindIII/AluI)/CAT</td>
<td>99 ± 10s</td>
<td>4169 ± 494s</td>
<td>42</td>
</tr>
<tr>
<td>pD(HindIII/HincII)/CAT</td>
<td>25 ± 2s</td>
<td>629 ± 130s</td>
<td>25</td>
</tr>
</tbody>
</table>

The results obtained with pD(AluI/HincII)/CAT and pD(HindIII/AluI)/CAT indicate that in FDD cells, enhancer elements close to the hypervariable region have the greatest effect on transcription rate, but enhancer element(s) further upstream also exert a significant influence. The dramatic decrease in the magnitude
of transactivation caused by deletion of the hypervariable region might have been caused by the removal of a recognition element(s) recognized by a cellular factor(s) that either directly interacts with Tat or is indirectly required for Tat function.

In FEA cells, none of the deletion construct activities differed significantly from Prtt under basal conditions. The deletion mutant pD(Alul/HincII)/CAT exhibited only 35% less activity than Prtt (Table 7). Thus the hypervariable region may exert far less influence on basal expression in FEA as opposed to FDD cells. This deletion construct still contains an intact PU.1 motif directly upstream of the TATA box, however, and transient gene expression assays with full-length LTR variants indicated that PU.1 motifs were being recognized by a factor in FEA cells. Therefore this PU.1 motif may be responsible for maintaining the relatively high basal transcription rate. Similar to what was observed in FDD cells, deletion of the hypervariable region decreased the magnitude of transactivation from 54 fold, as seen with pPrtt/CAT, to only 6 fold, as shown by pD(Alul/HincII)/CAT. The simplest explanation for this result would be that EIAV Tat indirectly requires a DNA cis-element in order to function efficiently but results obtained with pD(HindIII/HincII)/CAT contradict this theory. Removal of almost the entire U3 region, pD(HindIII/HincII)/CAT, dropped the basal activity only 29% compared to Prtt and, similar to what was observed in FDD cells, this deletion did not seem to affect the magnitude of transactivation, as a 26 fold induction in activity was seen under transactivated conditions. Deletion of upstream U3 sequences, pD(HindIII/Alul)/CAT, actually increased activity under basal conditions by 38%.

A previous study performed using FEA cell cultures also indicated a possible negative regulatory element in this region (Dorn and Derse, 1988). This deletion did not have any discernible effect on the magnitude of transactivation.
Table 7. Prtt deletion mutant activity in FEA cell cultures.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal</th>
<th>Transactivated</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPrtt/CAT</td>
<td>173 ± 30</td>
<td>9410 ± 820</td>
<td>54</td>
</tr>
<tr>
<td>pD(AluI/HincII)/CAT</td>
<td>112 ± 28n</td>
<td>631 ± 233s</td>
<td>6</td>
</tr>
<tr>
<td>pD(HindIII/AluI)/CAT</td>
<td>239 ± 50n</td>
<td>7951 ± 1408n</td>
<td>33</td>
</tr>
<tr>
<td>pD(HindIII/HincII)/CAT</td>
<td>123 ± 31n</td>
<td>3153 ± 929s</td>
<td>26</td>
</tr>
</tbody>
</table>

Cf2Th cells transfected with pD(AluI/HincII)/CAT yielded results similar to those obtained in FEA cells (Table 8). None of the deletion constructs exhibited behavior significantly different than that of Prtt. There was a 35% drop in activity under basal conditions and a virtual elimination of induction by transactivation. Deletion of upstream U3 sequences, pD(HindIII/AluI)/CAT, also lowered basal activity by 35% but did not affect transactivation as activity was induced 16 fold in the presence of Tat. Deletion of both regions, pD(HindIII/HincII)/CAT, resulted in only a 29% drop in basal activity, but unlike the behavior observed in FDD or FEA cell cultures, the deletion also resulted in a loss of response to transactivation.

Table 8. Prtt deletion mutant activity in Cf2Th cell cultures

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal</th>
<th>Transactivated</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPrtt/CAT</td>
<td>181 ± 24</td>
<td>3453 ± 570</td>
<td>19</td>
</tr>
<tr>
<td>pD(AluI/HincII)/CAT</td>
<td>115 ± 20n</td>
<td>152 ± 9s</td>
<td>1</td>
</tr>
<tr>
<td>pD(HindIII/AluI)/CAT</td>
<td>118 ± 23n</td>
<td>1937 ± 256s</td>
<td>16</td>
</tr>
<tr>
<td>pD(HindIII/HincII)/CAT</td>
<td>129 ± 56n</td>
<td>432 ± 54s</td>
<td>3</td>
</tr>
</tbody>
</table>
The identity of the actual cis-element(s) involved in the behavior of the three deletion mutants can only be speculated upon using previously published data. The results obtained in these studies indicate that the transcription factor complexes formed with the EIAV LTR are quite different between cell lines. Deletion of U3 sequences encompassing the hypervariable region and flanking sequences, illustrated by pD(Alu/HincII)/CAT, had a profound impact on promoter activity in FDD but not FEA or Cf2Th cells. Even deletion of virtually the entire U3 region, as shown by pD(HindIII/HincII)/CAT, had no significant effect on basal activity in FEA or Cf2Th cells. There are three feasible explanations for these results. The intact PU.1 motif in proximity to the TATA box may be sufficient to maintain transcription at a relatively high rate. Studies with full-length LTR variants, however, indicated that the PU.1 motifs were only actively recognized in FEA cells. It is also possible that the presence of the TATA box itself is sufficient to maintain basal transcription in these cell lines and that upstream enhancer elements influence transcription in only a minor way. The last possible explanation is that unidentified enhancer elements in the R and U5 regions of the LTR are being recognized by factors in these cell lines. Another interesting observation made during these studies was that transactivation was abrogated in FDD, FEA, and Cf2Th cell lines upon deletion of the hypervariable region and flanking sequences, but upon deletion of the entire U3 region, induction of transcription by Tat was restored in FDD and FEA but not in Cf2Th cells. Using horse PBMC cultures, Maury (1994) showed that the PU.1 sites in the EIAV LTR were essential for transactivation but had little affect on basal activity. She also demonstrated that sequences 5' to the PU.1 core motif were required for efficient recognition of this...
motif by cellular factors. Therefore, it is possible that FEA and FDD cells simply require a recognition element in proximity to the TATA box for transactivation. As already discussed, an intact PU.1 element is present in all three deletion mutants, however, it is only two base pairs downstream of the HindII site used in the construction of the deletion mutants. Deletion of sequences between the Alul and HindII sites and their subsequent blunt-end ligation, as described for the creation of pD(Alul/HindII)/CAT, may have removed nucleotides 5' to the PU.1 site essential for its recognition. The ligation of the HindII site to the synthetic HindIII linker sequence, as described in the creation of pD(HindIII/HindII)/CAT, may restore the essential nucleotides. The study by Maury (1994) also showed that Cf2Th cells did not require the PU.1 motifs for basal activity nor transactivation. Therefore, while the PU.1 motif may be sufficient to restore Tat responsiveness in FDD and FEA cells, Cf2Th cells seem to require a different element altogether which would explain why induction by EIAV Tat was not observed in Cf2Th cells transfected with pD(HindIII/HindII)/CAT as well as pD(Alul/HindII)/CAT. To date, no studies have shown that EIAV Tat recognizes any DNA cis-elements. Therefore, any interaction an element would have with EIAV Tat must be an indirect one via the cellular factor which recognizes it.

**Analysis of ALTR3 Deletion Mutants**

Oligonucleotide directed deletion mutagenesis was performed on ALTR3 to further localize LTR cis-elements active in FDD cell cultures and to study the impact of mutations within the hypervariable region on promoter strength. Deletion sizes varied depending on their location within the LTR. Large deletions were created in regions where no transcription factor binding sites had previously
Figure 6. Alignment of ALTR3 deletion mutants. Deletion mutants were created from the reporter construct ALTR3/Luc using a primer directed method as outlined in Materials and Methods. The LTR regions of the resulting deletion constructs are shown aligned with the LTR region of pALTR3/Luc with deleted sequences as indicated. Putative cis-elements for the EIAV LTR are also shown.
been identified while smaller deletions, of sequences in or near the hypervariable region, were produced to remove putative motifs identified in previous studies or by sequence analysis (Fig. 6). These deletion mutants were placed upstream of the \textit{luc} gene of the pGL2-Enhancer vector and the promoter strengths measured under both basal and transactivating conditions. The pGL2-Enhancer vector and the \textit{luc} reporter gene were chosen for subsequent experiments because of increased sensitivity which would help clarify small differences in activity between deletion mutants. Constructs were transfected into FDD cell cultures via the calcium phosphate precipitation technique. Values represent a mean of three trials, normalized with \( \beta \)-galactosidase activity, with a standard error and statistical significance as indicated (Table 9).

\textbf{Table 9}. ALTR3 deletion mutant activity in FDD cell cultures.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal</th>
<th>Transactivated</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pD(-233/-185)/Luc</td>
<td>1572 ± 391s</td>
<td>29698 ± 5517n</td>
<td>19</td>
</tr>
<tr>
<td>pD(-181/-135)/Luc</td>
<td>1830 ± 499s</td>
<td>32805 ± 9095n</td>
<td>18</td>
</tr>
<tr>
<td>pD(-137/-118)/Luc</td>
<td>3180 ± 475n</td>
<td>39209 ± 4500n</td>
<td>12</td>
</tr>
<tr>
<td>pD(-117/-101)/Luc</td>
<td>4400 ± 1061n</td>
<td>47418 ± 2677n</td>
<td>11</td>
</tr>
<tr>
<td>pD(-94/-87)/Luc</td>
<td>1406 ± 405s</td>
<td>35819 ± 7368n</td>
<td>25</td>
</tr>
<tr>
<td>pD(-85/-80)/Luc</td>
<td>2128 ± 592s</td>
<td>38321 ± 4792n</td>
<td>18</td>
</tr>
<tr>
<td>pD(-77/-61)/Luc</td>
<td>3968 ± 1418n</td>
<td>42487 ± 5499n</td>
<td>11</td>
</tr>
<tr>
<td>pD(-57/-43)/Luc</td>
<td>1594 ± 363s</td>
<td>23947 ± 2148n</td>
<td>15</td>
</tr>
<tr>
<td>pD(-46/-31)/Luc</td>
<td>2003 ± 237s</td>
<td>21254 ± 1553s</td>
<td>11</td>
</tr>
</tbody>
</table>

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The results showed that every deletion caused some loss of promoter strength under basal conditions but that not all decreases were significant. There was no indication of a negative regulatory element as seen in previous deletion studies. While the activities of some deletion constructs, pD(-117/-101)/Luc, pD(1/26)/Luc, and pD(32/90)/Luc, were only slightly lower, 21% to 38%, than that of the full-length construct, pALTR3/Luc, the majority of the constructs demonstrated more dramatic, approximately 50% to 75%, reductions in activity. Sequences flanking the hypervariable region seemed to be very important, as shown by pD(-94/-87)/Luc, pD(-85/-80)/Luc, pD(-57/-43)/Luc, and pD(-46/-31)/Luc, while deletion of 17 base pairs within the large insertion unique to the FDD-adapted LTR, as indicated by pD(-77/-61), resulted in only a 40% decrease in promoter activity. Surprisingly, the removal of sequences near the beginning of the U3 region, demonstrated by pD(-233/-185)/Luc and pD(-181/-135)/Luc, also had a large impact on basal transcription rates resulting in 76% and 72% drops in activity respectively. Computer homology searches indicated that this area contains imperfect sequence repeats which differ from an NF-κB consensus binding sequence (GGGRHTYYHC) (Lenardo and Baltimore, 1989) by only one base pair. Deletion of PU.1, pD(-46/-31)/Luc, or PU.1 and AP-1, pD(-57/-43)/Luc, sites immediately upstream of the TATA box resulted in 70% and 76% drops in activity respectively. Therefore, the PU.1 element is very important for basal transcription levels. It is impossible to conclude whether the AP-1 site is inactive or whether it
behaves synergistically with the adjacent PU.1 site. Removal of the duplicate AP-1 and PU.1 sites, pD(-77/-61)/Luc, had a less dramatic effect resulting in a 40% decrease in activity. Deletion of one, pD(-85/-80)/Luc, or both, pD(-94/-87)/Luc, PEA2 sites decreased activity 68% and 79% respectively indicating an important role for these elements in the control of the LTR in FDD cells. Deletion of large portions of the R region, pD(1/26)/Luc, had a modest impact on basal activity resulting in a 38% decrease. It is possible this region contains a recognition element but no other studies have indicated one in this region. Deletion of the remainder of the R and U5 regions, pD(32/90), decreased activity by 21% this difference was not significant.

None of the deletions within the U3 region affected the degree of transactivation as significantly as the large deletion mutant, pD(AluI/HincII)/CAT, of the Prtt LTR. Under transactivating conditions, all deletion mutants showed significant stimulation except pD(1/26)/Luc which deletes the putative EIAV transactivation response element (TAR) (Derse et al., 1993). Deletion mutants that demonstrated a dramatic loss of basal promoter activity, such as pD(-233/-185)/Luc and pD(-94/-87)/Luc, showed considerable stimulation by EIAV Tat, approximately 19 and 26 fold increases respectively. Mutants that demonstrated basal promoter activity levels near that of the full length ALTR3, such as pD(-117/-101)/Luc and pD(32/90)/Luc, were stimulated only 11 and 7 fold respectively. As seen in the previous expression study with LTR variants, the degree of stimulation by transactivation tended to be inversely proportional to the level of basal activity. This causes the significant variation in promoter activity between deletion mutants under basal conditions to become less distinctive under transactivated conditions. Only in the case of pD(-46/-31)/Luc, which deletes the PU.1 motif closest to the
TATA box and dramatically reduces basal activity, does this effect not seem to apply as induction by Tat for this mutant was only 11 fold. It is possible that this deletion may affect the mechanism of transactivation as discussed in the previous study of Prtt deletion mutants.

These results indicate a large number of cis-elements spread throughout the U3 region that act in concert to control transcription from the EIAV LTR and which is similar to results obtained from deletion studies of the Bovine immunodeficiency virus and Visna-maedi virus LTRs (Fong et al., 1995; Hess et al., 1989). The most significant elements are clustered near the hypervariable region and include the PU.1 and PEA2 recognition sites although there appears to be an important element(s) in the upstream regions of U3 as well. It is unlikely that each deletion resulted in the loss of a recognition element despite the fact that they all demonstrated less activity than the full-length LTR. Instead, this may indicate a requirement for correct spacing between existing elements. For example, LEF imparts a 110° turn in the HIV LTR which is believed to allow factors bound to upstream regions to interact with those in proximity to the TATA box. This mechanism requires correct spacing between the LEF recognition site and both upstream and downstream elements (Jones and Peterlin, 1994). In the Mouse mammary tumor virus LTR, exact spacing is required between glucocorticoid response elements. The exact spacing allows the two elements to lay in proximity to one another when the LTR is assembled with the nucleosome complex (Wolffe, 1994). It is interesting to note, that Carvalho and Derse (1993) identified an MDBP element in the EIAV LTR. MDBP has subsequently been identified as a histone H1 homologue (Bruhat and Jost, 1995). Spacing may therefore also be important between elements in the EIAV LTR upon assembly into chromatin.
Quantitation of Variant LTR Sequences in Infected Horse Tissues

As shown previously, variant LTR behavior is dependent upon cell type. The LTR also contains a large number of putative regulatory cis-elements that may allow the virus to respond to a variety of different cellular environments. This indicates that LTR variation may play a role in cellular adaptation or tropism in vivo. Quantitation of two different types of variant LTR sequences in several horse tissues was performed using the technique of competitive PCR. Total DNA from liver, lymph node, kidney, and lung tissue of an experimentally infected horse was obtained from Dr. Susan Payne. Primer P-1 was designed to recognize and amplify LTR variants homologous to 3.2-1 while primer P-3 was designed to recognize and amplify LTR variants homologous to 3.2-3. In addition to either P-1 or P-3 and horse tissue DNA, each amplification reaction contained either purified 3.2-1 or 3.2-3 DNA at decreasing concentrations. Both external controls, 3.2-1 and 3.2-3, contained an internal deletion allowing their amplification products to be separated from the amplification products of the horse DNA by polyacrylamide gel electrophoresis.

This study indicated that variant LTR sequences homologous to 3.2-3 were by far the most prevalent in all tissues examined (Fig. 7). Amplification of variants homologous to 3.2-1 could not be achieved in any tissue even upon extension of the reaction to 50 cycles. Distribution of viral DNA was somewhat similar between tissues, with liver and lymph node demonstrating a transition from control amplification products to horse DNA amplification products between reactions containing 3.0 and 0.3 pg external control and 150 ng tissue DNA. The transition for kidney tissue occurred at 3.0 pg external control while lung tissue, obtained by lung lavage, demonstrated less viral DNA with the transition occurring between
Figure 7. Quantitation of LTR variants in infected horse tissue DNA. Competitive PCR reactions were run with primers specific for variant LTR sequences homologous to 3.2-1, as shown by P-1, or with primers specific for variant LTR sequences homologous to 3.2-3, as shown by P-3. In addition to 150 ng of tissue DNA, reactions contained purified plasmid DNA containing either 3.2-1 or 3.2-3, each containing a small deletion, in decreasing amounts, from 300 pg to 0.03 pg. C1 reactions contained genomic DNA isolated from FDD cell cultures infected with virus derived from the molecular clone pSPEIAVAd3 in the case of P-1 reactions or from FDD cell cultures infected with virus derived from the molecular clone pSPEIAV44 in the case of P-3 reactions. C2 reactions did not contain tissue DNA. The tissues used were: (Panel A) liver, (Panel B) kidney, (Panel C) lymph node, and (Panel D) lung (isolated by lung lavage).
reactions containing 0.3 and 0.03 pg external control DNA. Assuming $6 \times 10^6$ kb per diploid genome, and 5.3 kb per plasmid control, the number of LTR sequences per liver cell is between 2.4 and 24. Numerous studies have shown that EIAV infects macrophages almost exclusively. Macrophages in the liver (Kupffer cells) account for about 10% of the cells by number (Rice et al., 1989). Assuming a 100% infection rate and two LTR regions per provirus, the results indicate that each liver macrophage contains between 12 and 120 copies of the EIAV proviral genome.

These experiments yielded some very unexpected results. It was very surprising that LTR variants homologous in sequence to 3.2-1 could not be amplified from any infected horse tissue even with extended cycles. It is not very likely that this is the result of poor amplification as the primer P-1 recognized and amplified the control plasmid. An additional primer, different in sequence from primer P-1 but specific for 3.2-1, was also tested and failed to amplify any products from tissue DNA. In these particular tissues therefore, sequences homologous to 3.2-1 were present in quantities so low, if they were present at all, they can be considered irrelevant. Although this experiment was performed with tissue DNA from a single animal that was euthanized during its first disease episode shortly after infection, it can certainly be speculated that there is no strong selective pressure for LTR variants between tissues and that LTR sequences homologous to 3.2-3 are sufficient to enable a pathogenic virus strain to cause acute disease. This probably indicates that LTR variants similar to 3.2-3 are the most active promoters in vivo and confer a selective advantage upon their own viral genotype. It remains to be determined however, if LTR regions homologous to 3.2-3 are required for pathogenicity. It is also not known if more divergent LTR sequences would appear
if the host were allowed to live through multiple disease episodes. These experiments also indicated a very high number of proviruses per macrophage based upon data for liver tissue (the only tissue for which the percentage of macrophage cells was available). Another study used hybridization techniques and calculated a proviral copy number of between 6 and 60 per macrophage cell (Rice et al., 1989). There are likely explanations for the two-fold discrepancy such as the inherent inaccuracy of both experimental techniques and differences in the level of viremia or percentage of cells infected between the subject horses. Previous studies using immunofluorescence, and hybridization have shown that the liver and lymph node are primary sites for EIAV replication. Kidney and lung tissue, while positive for EIAV replication and antigen, were not previously shown to be primary sites of replication (McGuire et al., 1971; Sellon et al., 1992). In this study however, kidney tissue contained the highest proviral copy number of all. Once again, this discrepancy may be due to the wide range of error inherent in the experiments themselves or differences in the horse subjects. It may also be the result of differences in the level of virus expression as the previous studies measured viral mRNA and antigen production. It is possible that while a high percentage of kidney macrophages are infected they may not be expressing the virus. This explanation is supported by two studies which show that EIAV replication is restricted to mature tissue macrophages and does not occur in circulating monocytes although they do contain proviral DNA (Maury, 1994; Rice et al., 1989, Sellon et al., 1992).

Progress of Infection in FDD Cell Cultures

To assess the impact of the LTR on replication capacity and cytopathogenicity, ALTR3 was cloned into an infectious molecular clone of EIAV,
pSPEIAV44. This was accomplished by creating an \textit{XhoI} site within ALTR3 as well as within the 3' LTR of pSPEIAV44. The portion of ALTR3 between the \textit{XhoI} and \textit{StuI} sites was subsequently used to replace the corresponding portion of the cognate LTR of pSPEIAV44. Infection of FDD cell cultures by virus derived from the resulting clone pSPEIAVAd3 as well as the original pSPEIAV44 and AFDD virus strains were monitored by cell proliferation assays, measurement of reverse transcriptase activity, Northern and Southern blot analysis, and immunofluorescence.

Cell viability was determined daily for all three infections as well as uninfected cultures by measuring the reduction of a tetrazolium compound into a formazan product that was released into the cell culture media and quantitated. This procedure measures the overall metabolic activity of the cell culture which is directly proportional to the number of viable cells, assuming a similar rate of growth. Absorbance measurements obtained during the initial days of infection continually increased until the cultures reached confluency after which the measurements tapered off to a low point around day 9 (Fig. 8). Measurements obtained from uninfected cell cultures and those infected with the pSPEIAV44 and pSPEIAVAd3 virus strains slowly rose from the low point on day 9 until the maximum propagation time of FDD cell cultures was approached around day 19 when they dropped severely. Microscopic examination of the cell cultures infected with the pSPEIAV44 and pSPEIAVAd3 virus strains gave no indication of any cytopathogenic effects during the life-span of the cultures. Absorbance measurements obtained from cell cultures infected with the cytopathogenic AFDD strain reached a low point at day 13. Microscopic examination of the cell culture
Figure 8: Cell culture viability for infected and mock-infected FDD cell cultures. Assays were performed during the course of the experiment according to procedures outlined in Materials and Methods.
Figure 9. Visual appearance of FDD cell cultures. Both mock-infected (Panel A) and AFDD infected (Panel B) FDD cell cultures were stained and photographed ten days post-infection. Bar represents a length of 50 uM.
during this period showed an extreme deterioration of the cell culture monolayer and a dramatic reduction, greater than 6 fold by day 13, in viable cells (Fig. 9). As the infection progressed, the monolayer was slowly regenerated by cells evidently resistant to the cytopathic effects of the virus. Absorbance measurements from the culture during this period steadily increased from day 13 to day 18 after which the values dropped, as seen in all cell cultures, on day 19.

Based on these observations, it can be concluded that the AFDD LTR alone does not confer the cytopathogenicity of the original AFDD strain upon a molecular clone. Additional studies such as the measurement of reverse transcriptase production, Northern and Southern blot analysis, and immunofluorescence, were performed to determine if the replacement of the LTR affected replication kinetics or viral phenotype in any way. The subsequent experiments also served to further characterize the behavior of the cytopathogenic FDD-adapted virus strain.

Analysis of reverse transcriptase activity was performed by harvesting cell culture supernatants every 24 hours and precipitating the virus with polyethylene glycol. Solubilized viral pellets were mixed directly with the template oligonucleotide and $[^3H]dTTP$. Values represent incorporated CPM minus background.

Daily measurement of reverse transcriptase activity in the cell culture media showed similar profiles for both the pSPEIAV44 and pSPEIAVAd3 strains. Both strains show activity steadily increasing during the course of infection to a maximum peak of activity on day 17 followed by a rapid decline in activity as the maximum propagation time of FDD cell cultures is approached (Fig. 10). However, even though the curve profiles are similar, cell cultures infected with pSPEIAVAd3 consistently exhibited more reverse transcriptase activity than twice
Figure 10. Reverse transcriptase production by infected FDD cell cultures. Production of reverse transcriptase by FDD cell cultures, infected with pSVEIAVAd3, pSVEIAV44, or AFDD virus strains, was monitored using the procedures outlined in Materials and Methods.
the activity as pSPEIAV44. FDD cell cultures infected with AFDD produced cultures infected with pSPEIAV44. On day 17, pSPEIAVAd3 showed almost a peak of reverse transcriptase activity by day 14 after which activity steadily decreased for the remainder of the infection. Although the activity profile of AFDD was similar to the profiles demonstrated by the pSPEIAV44 and pSPEIAVAd3 virus strains, reverse transcriptase production by AFDD was accelerated relative to the other virus strains in that its maximum peak of activity occurred three days earlier in infection than the corresponding peaks of activity demonstrated by the pSPEIAV44 and pSPEIAVAd3 virus strains. In addition, AFDD never produced the same levels of activity as the virus strains pSPEIAV44 and pSPEIAVAd3. Its maximum level of activity during the course of infection, day 14, was 3 fold less than the maximum level achieved by pSPEIAVAd3 on day 17.

Assuming reverse transcriptase activity is directly proportional to virion production and is an accurate measurement of replication capacity, these results indicate that the FDD-adapted virus LTR increased the replication capacity of the molecular clone. The difference in activity produced by cultures infected with pSPEIAVAd3 and those infected with pSPEIAV44 was not due to unequal levels of proviral DNA as indicated by subsequent Southern blotting. The study also showed that the cytopathogenic effects of AFDD are relatively independent of its replication capacity. Therefore, the cytopathogenicity of the AFDD strain is not caused by the excessive production and secretion of virions. The actual pattern of reverse transcriptase production by AFDD infected cultures is difficult to reconcile with the previous study on cell culture viability. Activity produced by AFDD began to drop several days in advance of the severe monolayer deterioration
witnessed by day 13. It is possible that the visible signs of monolayer deterioration may lag behind widespread host cell death by a considerable amount of time. Although it is impossible to determine from these studies the degree or even the actual existence of this lag time, a large time difference between visual cytopathogenic effects and the actual onset of cell death may help explain reverse transcriptase production, or the lack thereof, by AFDD infected cell cultures late in infection. It is possible that the second peak of activity produced between days 13 and 15 by AFDD infected cultures preceded yet another round of monolayer deterioration that was simply never witnessed due to the life-span of the culture being reached at day 19.

Analysis of viral mRNA during the course of the infections by Northern blot indicated little difference between the pSPEIAV44 or pSPEIAVAd3 strains at the mRNA level. Northern blot analysis of total RNA from infected cell cultures showed three characteristic EIAV specific mRNA bands: an 8.2 kb band, the unspliced mRNA which produces the \textit{gag} and \textit{pol} gene products as well as serving as virion RNA; a 3.2 kb band, a singly-spliced mRNA species that produces the \textit{env} as well as the \textit{S2} open reading frame gene products and EIAV Tat; and a 1.5 kb band, consisting of multiply-spliced mRNA species that produce the EIAV Tat, Rev, and the non-characterized Ttm proteins (Derse \textit{et al.}, 1993; Rasty \textit{et al.}, 1990). Upon poly-A RNA selection, the 1.5 kb viral mRNA species decreased to virtually undetectable levels (Fig. 11). Both the pSPEIAV44 and pSPEIAVAd3 infections show a low abundance of viral mRNA early in the infection, detectable only by day 6, which increases during infection, illustrated by day 12, and then rapidly decreases as the host cell culture reaches the end of its life-span, shown by day 18. Both infections show a more intense 3.2 kb band relative to the 8.2 kb band. The
Figure 11. Analysis of viral mRNA by Northern blot hybridization. Poly-A RNA isolation from infected FDD cell cultures and Northern blot hybridization was performed as outlined in Materials and Methods. Location and identification of molecular weight size markers is indicated at the left as is a mock-infected control (lane B). (Panel A) Analysis of viral mRNA produced by cultures infected with pSPEIAVAd3 on day 6 (lane 1), day 12 (lane 2) and day 18 (lane 3) post-infection. (Panel B) Analysis of viral mRNA produced by cultures infected with pSPEIAV44 on day 6 (lane 4), day 12 (lane 5) and day 18 (lane 6) post-infection. (Panel C) Analysis of viral mRNA produced by cultures infected with AFDD on day 6 (lane 7), day 12 (lane 8) and day 18 (lane 9) post-infection. Markers at right indicate the approximate sizes of the three viral mRNA species detected.
AFDD infected cell cultures show a dramatic increase, at least 10 fold, in the overall amount of viral mRNA relative to the pSPEIAV44 and pSPEIAVAd3 infections. This strain produced a large amount of viral mRNA detectable by day 6, which then slightly decreased during the course of infection, as indicated by day 12 and day 18. Both the 3.2 kb and 8.2 kb bands showed a similar intensity.

Based upon these results, it can be concluded that despite an increased basal transcription activity, the AFDD LTR had no detectable influence on viral mRNA production by the molecular clone and that neither pSPEIAVAd3 nor pSPEIAV44 produced the large amount of viral mRNA demonstrated by cultures infected with AFDD. In actuality, the technique of Northern blotting is prone to a great deal of variation between trials and between individual samples. This inherent inaccuracy of the technique coupled with the fact that these blots are based upon only one trial makes the strict quantitative interpretation of these results questionable. Although there is no doubt that AFDD produces far more viral mRNA than pSPEIAVAd3 and pSPEIAV44, it cannot be concluded with any reasonable certainty whether there are small quantitative differences in viral mRNA levels produced by pSPEIAVAd3 and pSPEIAV44.

Southern blotting was performed in order to monitor the relative levels of proviral DNA present in infected cell cultures. Total cell DNA was isolated and purified from infected cell cultures and digested to completion with Stul. This cleaves EIAV proviral DNA once in each LTR. Upon complete digestion, a roughly 8.2 kb EIAV proviral DNA band should be produced. Examination of the results obtained from this experiment showed that cultures infected with pSPEIAVAd3 and pSPEIAV44 contained approximately the same amount of proviral DNA, relative to one another, throughout the course of infection (Fig. 12).
Figure 12. Analysis of cellular DNA by Southern blot hybridization. Total cellular DNA was isolated from infected FDD cell cultures, digested to completion with Stul, and subjected to Southern blot analysis according to the procedures outlined in Materials and Methods. The location and size of molecular weight markers are indicated at the left as is a mock-infected control (lane B). (Panel A) Cellular DNA isolated from cultures infected with pSPEIAVAd3 on day 6 (lane 1), day 12 (lane 2), and day 18 (lane 3) post-infection. (Panel B) Cellular DNA isolated from cultures infected with pSPEIAV44 on day 6 (lane 4), day 12 (lane 5), and day 18 (lane 6) post-infection. (Panel C) Cellular DNA isolated from cultures infected with AFDD on day 6 (lane 7), day 12 (lane 8), and day 18 (lane 9). Marker at right indicates the approximate size of digested proviral DNA.

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Both cultures showed a somewhat weak, approximately 8.2 kb band by day 6 post-infection which gradually increased in intensity during the course of infection as illustrated by results obtained on days 12 and 18. Cultures infected with AFDD once again produced markedly different results, exhibiting a 8.2 kb band by day 6 which increased in intensity even more during the course of infection, as illustrated by results from day 12, and then decreased slightly as the end of the culture lifespan was approached at day 18.

Once again, it must be stated that precise quantitative interpretation of these blots is questionable due to their own inherent imprecision and the lack of multiple trials. Clearly, AFDD infected cultures contained considerably more proviral DNA than cultures infected with pSPEIAVAd3 and pSPEIAV44. This finding throws considerable doubt upon the theory that the accelerated cytopathogenicity exhibited by AFDD is caused by the production of increased amounts of viral mRNA. Taken together, the results obtained from the Northern and Southern blots seem to indicate that AFDD proviral DNA produces amounts of mRNA comparable to that produced by pSPEIAVAd3 or pSPEIAV44 but that AFDD infections result in considerably more proviral DNA. Cytopathogenic retrovirus strains that exhibit increased amounts of proviral DNA have been extensively studied and this phenotype is believed to result from the loss of host cell immunity to super-infection by the virus strain. Changes in the virus-host cell recognition process can result in the inability of the virus to down-regulate its own host cell receptor later on in infection.

In order to determine if the increased amounts of proviral DNA and viral mRNA produced by FDD cultures infected with AFDD were due to super-infection or simply due to a larger percentage of host cells being infected,
immunofluorescence studies were performed to determine the percentage of cells expressing EIAV antigen within a culture. In brief, samples of cell cultures at various stages post-infection were fixed on slides and incubated with a rabbit polyclonal antiserum raised against a peptide corresponding to gp90, the surface protein of EIAV. Subsequent incubation with a Fluorescein conjugated antiserum against rabbit IgG and microscopic examination under both visible and blue light allowed the determination of the percentage of cells containing gp90 which is a strong indication that the cells are infected with EIAV (Appendix A).

Cultures infected with AFDD showed a high percentage, 25%, of fluorescent cells as early as day 6 which increased during the infection to approximately 40% on day 12 and 36% on day 18. Cultures infected with pSPEIAVAd3 yielded no detectable fluorescence until day 12 when approximately 10% of the host cells expressed antigen. By day 18, this percentage had risen sharply to 50%. Cultures infected with pSPEIAV44 gave similar results, not demonstrating a strong amount of fluorescence until day 18 at which time 40% of the host cells expressed antigen.

The large percentage of fluorescent cells detected in cultures infected with AFDD is in agreement with the previous results obtained from Northern and Southern blotting. These results indicate that, to some extent, increased amounts of proviral DNA and viral mRNA detected in AFDD infected cultures, relative to cultures infected with pSPEIAVAd3 and pSPEIAV44, was due to a higher percentage of host cells being infected. This does not reconcile all results, however. Whereas cultures infected with pSPEIAVAd3 and pSPEIAV44 contained a large percentage of fluorescent cells later in infection but Northern and Southern blot analysis still indicated lower levels of proviral DNA and viral mRNA relative
to cultures infected with AFDD. It must be noted, however, that samples harvested on day 18 post-infection were used to represent the late stages of infection. By this time, cell cultures were approaching the end of their life-span and both cell viability and reverse transcriptase production were dropping.

Overall, these studies indicate that replacement of the LTR region of a molecular clone, pSPEIAV44, by the corresponding region of AFDD failed to confer cytopathogenicity upon the chimeric molecular clone, pSPEIAVAd3, but did affect replication capacity, as shown by measurements of cell proliferation and reverse transcriptase production. Detailed examination of the infections by these and a variety of other techniques revealed several significant insights into the behavior of the AFDD virus strain. Immunofluorescence studies as well as Northern and Southern blot analysis agreed that the AFDD infects a large percentage of the host cell culture which exhibit high levels of viral mRNA, proviral DNA, and surface protein. Oddly, reverse transcriptase production by these cultures was very low. It is possible that this represents a defect in proper virion assembly and release. This defect may also result in the super-infection of the host cell as Southern blot analysis indicates that even late in infection, when cultures infected with pSPEIAVAd3 and pSPEIAV44 are releasing far more reverse transcriptase activity and have infected comparable percentages of host cells relative to cultures infected with AFDD, they do not exhibit comparable levels of proviral DNA. Of course, the lack of induction seen at the viral mRNA and proviral DNA level in cultures infected with pSPEIAVAd3 and pSPEIAV44 as infections progressed is also noteworthy. The low levels of viral mRNA and DNA indicated in the blots may be deceiving as samples were taken on day 18 post-infection when cell culture viability and reverse transcriptase production were
dropping. It still seems unlikely however, that the levels would drop so dramatically in such a short period of time.

Although far from conclusive, a possible description of the AFDD replication cycle in FDD cell cultures can be constructed. Initial infection of the cultures results in widespread infection and the rapid appearance of infectious virions in the culture supernatant. As the levels of virions rises the majority of host cells become super-infected resulting a decrease in intact virion production and host cell death. Subsequently, cells that had escaped super-infection rapidly proliferate and, once again, high levels of infectious virions begin to accumulate in the supernatant resulting in a continuing cycle of virus production followed by host cell super-infection and death.
Prior to this investigation, very little information was known about the EIAV LTR and how transcription of the EIAV genome was regulated. Preliminary studies had shown a high degree of LTR sequence variation between virus isolates obtained from sequential disease episodes of an infected pony (Payne et al., 1987). Comparison of the variant LTR isolates indicated that the sequence variation was primarily located in a specific region approximately 60 base pairs upstream of the TATA box. This region of the LTR, later dubbed the hypervariable region, was prone to sequence insertions or duplications. Deletion studies performed in FEA cell cultures showed that cis-elements in proximity to the hypervariable region had a dramatic impact on basal transcription (Dorn and Derse, 1988). Examined together, these experiments indicated that elements critical for the assembly of the basal transcription complex might be duplicated or changed in certain LTR variants. It was further postulated, that LTR sequence variation may increase the replication capacity of the virus influencing its pathology or allow the virus to adapt to new cellular environments thereby influencing cellular adaptation or tropism. Macrophages are a very heterogeneous cell type varying considerably in phenotype between different locations in vivo (Weinberg and Athens, 1993). These microscopic and antigenic differences must reflect differences at the molecular level. LTR heterogeneity may allow the virus to infect the many different sub-populations of macrophages resulting in a more extensive infection of the host and the high level of viremia required to cause acute disease. In the absence of a pathogenic molecular clone, experiments designed to test these theories were performed primarily in vitro.
Sequence comparison between the highly characterized Prtt LTR sequence and LTR variants isolated from diverse sources such as an infected pony, a tissue culture-adapted strain of EIAV, and an infectious EIAV molecular clone, indicated a number of sequence differences that could affect promoter strength. The most significant differences occurred in the hypervariable region of the EIAV LTR and resulted in imperfect duplication of the putative AP-1 and PU.1 or interruption of the PEA2 transcription factor motifs identified within Prtt (Carvalho and Derse, 1993a; Carvalho and Derse, 1993b). Variant LTR sequences from a tissue culture-adapted strain, ALTR3 and ALTR4, as well as one variant from an infected pony, 3.2-1, contained duplications of the putative PU.1 and AP-1 sites while retaining both PEA2 sites. The cognate LTR of an infectious molecular clone, PV44, and an additional horse isolate, 3.2-3, contained a duplication of the PU.1 element but this mutation interrupted one PEA2 motif. The final horse isolate, 3.2-5, contained two additional putative PU.1 motifs and possibly an additional AP-1 site as well.

Similar to the two previous LTR variants discussed, it contained an insertion which interrupted the PEA2 motif. Based on this analysis, most LTR variants can be placed into two general classes based upon sequence homology which differ significantly in the transcription factor elements they contain.

Transient gene expression studies performed in FDD cell cultures using these same LTR variants indicated significant disparities between basal promoter activities overall but similar activity levels between variants belonging to the same family. Variants ALTR3, ALTR4 and 3.2-1 all showed dramatically higher levels of activity than Prtt, PV44, 3.2-3, and 3.2-4. This indicates that AP-1 and PEA2 binding motifs may be very important for LTR promoter strength in FDD cell cultures. These differences became less pronounced when cells were co-transfected.
with an EIAV Tat expression plasmid but were still significant. Deletion mutants created from Prtt, pD(Alu/HincII)/CAT, and pD(HindIII/Alu)/CAT, which remove the hypervariable region and U3 sequences upstream of the hypervariable region respectively, indicated that elements in proximity to the hypervariable region as well as in the upstream regions of the U3 were important for basal transcription. Complete removal of the hypervariable region also decreased the magnitude of transactivation. Similar assays were performed in FEA cell cultures but with only the three horse isolates, Prtt, and the Prtt deletion mutants. The three horse isolates were all more active promoters than Prtt but the differences in the basal levels of activity, while significant, were not as dramatic as in FDD cell cultures. These results implicate the PU.1 site in LTR behavior in FEA cells. Under transactivating conditions the differences in activity between variants became insignificant. Deletion of the hypervariable region had far less an impact on basal transcription than that seen in FDD cell cultures and removal of upstream U3 sequences actually increased activity indicating the presence of a possible negative regulatory element. Similar to the effect in FDD cells, removal of the hypervariable region decreased the magnitude of transactivation. In Cf2Th cells, Prtt and 3.2-1 were the most active promoters under basal conditions although the differences in activity levels between these two variants and the remaining two were not as dramatic as seen in FDD cell cultures. Under transactivating conditions, no variants maintained a significantly higher activity. These results implicate the PEA2 sites in regulating the LTR in Cf2Th cells. Deletion of both the hypervariable region and upstream U3 sequences had only a moderate impact on basal transcription while deletion of the hypervariable region abrogated transactivation completely.
In conclusion, LTR variants behave differently depending upon cell type. Therefore, LTR variation may play a role in tissue adaptation or tropism. Although highly speculative, cis-elements important in controlling basal transcription from the LTR were putatively identified, and the requirement of a cis-element, in proximity to the TATA box, for efficient transactivation was demonstrated. It is impossible to discern whether the cell dependent behavior of the LTR variants reflects differences in the tissue sources or differences in species origin of these cell lines. FEA and Cf2Th cell lines were chosen on the basis of their ability to support replication of EIAV, their use in previous studies, and the unavailability of non-dermal equine cell lines. Because of their non-equid origins, additional studies in these cell lines could not be justified despite the interesting observations made. Peripheral blood macrophages could be obtained but not in large quantities, required extensive purification, and were difficult to maintain. Although attempted, transient transfection with reporter plasmids could never be achieved. Subsequently, the remaining experiments were all performed FDD cell cultures.

To further localize the cis-elements within the EIAV LTR active in FDD cell cultures, a more extensive deletion analysis was performed using the AFDD LTR as a template. These deletion mutants revealed that sequences proximal to the hypervariable region, which includes the AP-1, PU.1, and PEA2 motifs originally identified within the prototype LTR, were highly important for basal transcription. Deletion of the additional AP-1 and PU.1 sites created by the sequence insertion had only a moderate effect on basal transcription indicating that other sequence variations contribute to the increased promoter strength of the AFDD LTR. Deletions within the first 100 base pairs of the U3 region also had a dramatic impact on basal promoter strength. No specific cis-elements within this area of the
EIAV LTR have previously been identified nor did previous deletion studies indicate that this area had as great an effect on basal transcription as seen here (Carvalho and Derse, 1993a; Carvalho et al., 1993). None of the deletions had as dramatic an impact on the magnitude of transactivation as did the Prtt deletion mutant pD(AluI/HincII)/CAT. This may indicate that a specific cis-element is not required but that any active element in proximity to the TATA box is sufficient for efficient transactivation to occur.

These studies indicate that in FDD cell cultures, the EIAV LTR may contain two core enhancer units; one centered within the hypervariable region and proximal to the TATA box, the other within the first 80 base pairs of the U3 region. The presence of multiple enhancer elements may allow the LTR to respond to a wide variety of cellular environments. Of course, none of the elements or the factors that recognize them have been definitively identified here. More extensive studies, such as DNase 1 protection and mobility shift assays, were not performed. While the deletion mutant experiments were being conducted, extensive studies on the transcription factor complexes formed with EIAV LTR variants were published making the results presented here merely confirmatory. Any future studies would require a repetition of these assays in a monocytic cell line or, preferably, in primary horse macrophage cultures with an emphasis on identifying the unknown upstream elements should they be active in a monocyte/macrophage cell type.

In order to implicate LTR variability in cellular adaptation in vivo, competitive PCR was performed with tissue DNA isolated from an acutely infected horse using primer pairs specific for two different types of LTR variants. These assays indicated that liver, lymph node, and kidney tissue were all infected at roughly equivalent levels with virus possessing an LTR homologous to horse
isolate 3.2-3. Lung tissue, isolated by lung lavage, was infected at lower levels but also contained LTR sequences homologous to 3.2-3. Sequences homologous to 3.2-1 could not be amplified even under extended conditions. In this case, LTR heterogeneity was neither required for the onset of acute disease nor was it even detected at significant levels. It would be interesting to use this system to analyze LTR heterogeneity in an infected horse over a longer time period. The host in this case was euthanized during the first acute disease episode and there may not have been sufficient time allowed for LTR divergence to arise.

Replication of the FDD-adapted strain, AFDD, of EIAV in FDD cell cultures presents a unique system for studying the viral determinants of replication capacity or cytopathogenicity. Both the *env* and LTR regions are likely candidates to contain these determinants as sequence comparison of EIAV isolates indicate that these regions are prone to a high degree of sequence variability and that both these regions influence replication capacity or cytopathogenicity in other lentivirus systems (Brown *et al.*, 1988; Carpenter *et al.*, 1991; Dewhurst *et al.*, 1990; Englund *et al.*, 1991; Golub *et al.*, 1990; Payne *et al.* 1987). In a recent study, Cook *et al.* (1995) replaced a region of *env*, which codes for the surface protein gp90, of the infectious molecular clone, pSPEIAV44, with the corresponding region from AFDD proviral DNA. The chimeric clone produced did not show any cytopathogenic effects when replicated in FDD cell cultures.

A similar result was obtained in this study in which the LTR region of AFDD was placed into pSPEIAV44. The resulting molecular clone, pSPEIAVAd3, did not display any discernible cytopathogenic effects when replicated in FDD cell cultures although it did demonstrate a higher replication capacity than pSPEIAV44. Assuming reverse transcriptase activity correlates directly with virus production,
reverse transcriptase assays performed on infected cell culture supernatants indicated that pSPEIAVAd3 produced approximately twice as much virus as cultures infected with pSPEIAV44. Cultures infected with AFDD showed the least amount of virus production of all, indicating that the cytopathic nature of AFDD is relatively independent of its virion production. Strangely, Northern and Southern blot analysis indicated enormous amounts of viral mRNA and DNA in AFDD infected cell cultures and immunofluorescence studies indicated large amounts of surface protein antigen as well. Therefore the AFDD strain seems capable of super-infecting the host culture and possesses a deficiency in virion maturation and egress. Which of these characteristics results in its accelerated cytopathogenicity is impossible to determine. Any future studies on this virus will have to confirm these results and analyze, as of yet, untested regions of the genome.
REFERENCES


envelope protein gene region of Equine infectious anemia virus is not an important determinant of tropism \textit{in vitro}. \textit{J. Virol.} 66, 4085-40096.


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APPENDIX

Permission Letter

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VITA

Charles R. Madden was born on April 11, 1967 to Edward and Leona Madden in Hays, Kansas. Charles attended local public schools for his primary and secondary education, graduating from Hays High School in May of 1985. He attended a local college, Fort Hays State University, for two years before moving to Manhattan, Kansas where he received a bachelor of science degree in biochemistry from Kansas State University in May of 1989. It was at Kansas State University that Charles became interested in basic research after investigating a novel recombination event occurring in mitochondrial DNA of male sterile wheat in the lab of Dr. Charles Hedgecoth. In August of 1989, Charles was awarded a Louisiana Board of Regents Graduate Fellowship and entered the graduate program in biochemistry at Louisiana State University. In the Spring of 1990, he joined the lab of Dr. Ding Shih and began working on his dissertation research. In the Spring of 1991, Charles met and subsequently fell in love with Lisa Fernandes who became his wife on July 17, 1993. Charles continued to pursue his doctoral degree, successfully defending his dissertation research on October 24, 1996. Upon graduation, Charles will continue working as a research associate in the lab of Dr. W. A. Deutsch.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Charles R. Madden

Major Field: Biochemistry

Title of Dissertation: Characterization of Variant Equine Infectious Anemia Virus Long Terminal Repeat Sequences

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

10-24-96