Functional characterization of the long terminal repeat of equine infectious anemia virus

by

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INTRODUCTION

Equine infectious anemia virus (EIAV) belongs to the family Retroviridae, and is further classified under the sub-family Lentivirinea. Lentiviruses are non-oncogenic viruses that cause a variety of diseases in humans and animals. Other lentiviruses are visna virus, caprine arthritis encephalitis virus (CAEV), ovine progressive pneumonia virus (OPPV), bovine immunodeficiency-like virus (BIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV) and human immunodeficiency virus (HIV).

One of the more notable features of retroviruses is their genetic variability. Numerous virus variants may co-exist *in vivo* and it is important to realize that within an infected animal there is not a single specific genotype of virus. In HIV infection, it has been shown that there are a multitude of unique populations of closely related, yet distinct, viral genomes (19,35,70,86). It has also been shown that multiple distinct, yet related genomes exist in EIAV infected horses (1,9). Although, mutations occur throughout the lentivirus genome, only a small number will provide a selective advantage and allow the spread of a particular variant. The pressures that select for one genome population gaining a replicative advantage over another are just beginning to be understood. Factors that influence variant selection include the host immune system, cell tropism, and rate of viral replication.

The immune system was the first factor shown to have a role in selection of EIAV. Viruses isolated from successive febrile cycles were demonstrated to be

antigenically different (51,69,87). Concurrently, it was found that serum isolated from an infected animal could neutralize virus collected from an earlier febrile cycle, but not virus collected from a later febrile cycle (50,51,87).

Differences in HIV cell tropism have been found to correlate with severity of clinical disease and clinical manifestations of disease. For example, it has been shown that HIV is able to infect both T-lymphocytes and macrophages, and *in vivo* isolates often display a tropism for one particular cell type *in vitro*. Furthermore, HIV isolated from brain tissue replicates better in macrophages, while HIV isolated from peripheral blood replicates better in T-lymphocytes (2,44,52,55). In addition, HIV isolated late in the disease course was shown to exhibit a broader host range and to be more cytopathic *in vitro* than HIV recovered during asymptomatic periods (2,44,52). These studies suggest that genetic determinants of cell tropism and virulence affect the rate of virus replication and cytopathicity in particular cell types.

Lentiviruses employ a complex method of regulation, utilizing a variety of regulatory proteins that act to either up or down regulate viral replication and expression. The long terminal repeat (LTR) of lentiviruses plays an important role in regulation of transcription and gene expression (30,43,61,90,92). In addition to acting autonomously as a promoter, the LTR contains *cis* acting sequences responsive to *trans*-acting virally encoded regulatory proteins. Also within the LTR are numerous *cis*-acting enhancer sites which interact with constitutively expressed or inducible host transcription factors (24,72). These *cis*-acting sites play a role in

cell tropism in other retroviruses such as MuLV (14,16,27,29,54), and it is possible that the LTR may play some role in cell tropism and disease pathogenesis of lentiviruses.

The goal of this project is to gain insight on the role of the LTR in EIAV cell tropism and virulence. In our laboratory, we have two strains of EIAV which differ in cell tropism and virulence. A virulent field isolate, Th-1, replicates to high titers in horse macrophage cultures (HMC) and to low titers on equine dermal (ED) cells (10). The MA-1 strain was derived from the Th-1 strain by passage on ED cells. In this adaption process, MA-1 lost its ability to replicate in its natural host cell, the equine macrophage. MA-1 replicates to high titers on ED cells and to low titers on equine macrophages (10). Interestingly, this change in host cell tropism correlated with a reduction in viral virulence. Horses inoculated with the MA-1 virus seroconverted, but did not show clinical signs of disease.

To examine the genetic basis for virulence and cell tropism in EIAV, the Th-1 LTR was amplified by PCR and molecularly cloned (9). A full length proviral clone of MA-1 was derived from chronically infected cells using standard cloning procedures. Sequence comparisons between three independent Th-1 clones and the MA-1 clone revealed sequence differences located within a 66 base pair hypervarible region in the U3 region of the LTR (9). This hypervariable area of the EIAV LTR has been previously described to contain numerous negative regulatory elements and enhancer elements (28,31). The sequence differences between MA-1 and Th-1 resulted in substantial differences in the types of specific enhancer

elements present within the LTRs (9). The overall objective of this study is to determine if these sequence changes affect the transcriptional activity of the LTRs in different cell types. It is our hypothesis that the changes in the enhancer motifs may alter the interactions between enhancers and cellular transcription factors, and thus may account for differences in cell tropism between MA-1 and Th-1.

Specific Aims

1. Construct LTR CAT plasmids using LTR sequences from MA-1 and three Th-1 clones.
2. Determine optimum conditions for transfection and expression of CAT activity in HMCs and ED cells.
3. Use transient expression assays to determine promoter activity of variant LTR's in HMCs and ED cells.

LITERATURE REVIEW

Retroviruses

Retrovirus classification

Retroviruses are defined by their morphology, the structure of their RNA genomes, and their ability to synthesize DNA from a RNA template using RNA dependant DNA polymerase (reverse transcriptase) (22,60). All retroviruses have an icosahedral capsid that is surrounded by an envelope studded with proteins (22,60). Within the capsid is a probable helical nucleocapsid associated with two identical, positive-sense, non-covalently bound RNAs which make up the viral genome (22,60). Retroviruses are unique among viruses in that the RNA genome serves as a template for the synthesis of DNA through the action of reverse transcriptase (22,60). Retroviruses are divided into three sub-families based primarily upon pathogenicity. These are: Oncovirinae, the RNA tumor viruses, Spumavirinae, the foamy viruses and Lentivirinae, the slow viruses.

Oncovirinae Oncoviruses are named for their ability to cause neoplastic disease in the infected animal (22,60). The sub-family is further divided into types A through D, based upon virion morphogenesis. A-type particles are non-infectious and formed intracellularly (22,26,60). B-type particles develop their inner core in the cytoplasm and obtain their envelopes as they bud through the cell membrane (22,26,60). Their cores are eccentrically located within the virion

envelope (22,26,60). C-type morphology is the most common type displayed by oncogenic retroviruses. The virion core develops as the virion buds through the plasma membrane, giving a C shaped appearance in electron micrographs. Maturation is completed outside the cell membrane and the inner core is centrally located within the envelope. Finally, D-type particles assemble their core in the cytoplasm and bud through the cell membrane. Their core is centrally located within the envelope (22,60). Oncoviruses cause a variety of neoplastic diseases including lymphomas, leukemias, sarcomas, erythroblastosis, and mammary carcinomas (22,60). They can also cause other diseases such as anemia and neurological disorders (22,40,60).

Spumavirinae Spumaviruses are named for the "foamy" degeneration they cause in tissue culture. Morphologically, the nucleoids of spumaviruses are smaller than other retroviruses, while their surface spikes are more noticeable (26). They have been found a in variety of species including bovine, non-human primates, feline and humans. At this time, no clinical disease has been associated with the spumaviruses, but recent studies call for a re-evaluation of their pathogenic potential (8).

Lentivirinae Lentiviruses are characterized by their rod shaped virion morphology, complex genome organization, and disease characteristics (22,60). Lentiviral disease is most often characterized by its slow, chronic nature. The period between initial infection and the onset of clinical disease may be years apart. Death may or may not be the final result of infection, but those infected remain so

for life (22,33,40). The virus infects cells of the immune system. The primary host cell is the macrophage, but T-lymphocytes and B-lymphocytes are infected as well (20,22). Variability in clinical disease is often observed. Initially disease will have a acute phase characterized by rapid viral replication, viremia and fever. Acute phase varies in intensity from disease to disease with equine infectious anemia (EIA) being the most acute (18,22,40,60). Later, infection is characterized by an extremely long incubation period and chronic disease nature (40,71).

Characteristics of retroviruses

Although retroviruses vary considerably from sub-family to sub-family, some generalizations can be made in terms of the physical characteristics of their genomes, the organization of the genes therein and the proteins that these genes encode. Retroviral genomes are 8,000 to 10,000 kilobases in length and are composed of two identical positive sense RNA genomes that are non-covalently bound to each other (20,22). The 5' ends of the genomes are modified by the cellular machinery, which introduces a m^7G5'ppp5'Gmp cap. The genomic RNAs, like the cellular mRNAs, are postranscriptionally modified by internal methylation on the 6 position of occasional A residues (22). The 3' ends of the genomes are polyadenylated with a string of about 200 adenine residues (22).

Retroviral genome organization The retrovirus proviral genome has the following, basic organization listed from 5' to 3': the 5' LTR, *gag, pol, env,* and the

3' LTR (see Figure 1). In the more complex retroviruses, genes encoding regulatory proteins are found between the *pol* gene and the 3' LTR (22).

The long terminal repeats The LTRs contain non-coding sequences of DNA which are important for viral gene regulation, reverse transcription, and integration. The LTRs are divided into three regions, U3, R and U5. The U3 region is defined as the area between the initiation site of positive strand synthesis and the beginning of the R region (22,96,97). U3 contains the RNA start site, cellular transcription-factor enhancer motifs and other signals that are recognized by cellular transcription machinery (22). Two copies of the R region are present in the RNA viral genome; hence the region is named R for redundant. The R region plays an important role in reverse transcription, and is discussed in more detail below (22,60,96). The U5 region is defined as the area between the R region and the primer binding site. This region contains one of the *aat* sites that are necessary for integration (22,60).

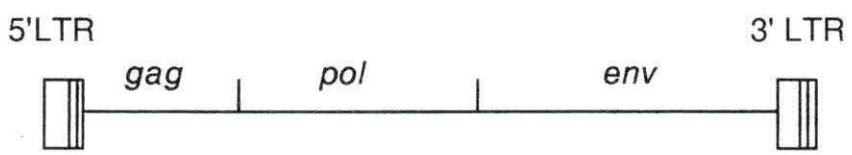


Figure 1. Organization of basic retrovirus genome showing location of 5' and 3' long terminal repeats (LTR), and the *gag*, *pol* and *env* genes.

The *gag* gene The *gag* gene is the first gene encountered moving 5' to 3' down the viral genome. The gene is transcribed as a full length RNA and translated as polyprotein that is subsequently cleaved to give rise to three to five capsid proteins (22). The three proteins made by all retroviruses are the matrix protein which is in close association with the membrane, the capsid protein which makes up the core shell, and the nucleic acid binding protein which associates with the viral genome (22).

The *pol* gene The *pol* gene encodes the viral reverse transcriptase, protease and integrase proteins that are essential for retroviral replication. The *pol* proteins are translated from full length mRNA and translation occurs either as a read through or frame shift mutation (22,60).

The *env* gene As the name suggests, the *env* gene codes for proteins that are inserted into the viral envelope. The *env* polyprotein is translated from a singly spliced mRNA and cleaved into two proteins which are post-translationally modified by glycosylation (22,60). The larger of the two *env* proteins is the surface glycoprotein which functions in cell receptor recognition and contains type specific epitopes (22,60). The surface glycoprotein is complexed with the smaller transmembrane protein which spans the viral membrane and anchors the entire complex.

Retroviral regulatory genes In addition to the genes outlined above, a number of retroviruses require regulatory proteins for successful replication. Examples of these are human T-cell leukemia virus (HTLV) and HIV. For the

purposes of this discussion, only the regulatory proteins identified in HIV will be mentioned. This is fitting because of all the retroviruses, HIV has the most genes identified (22). Two regulatory genes absolutely required for HIV replication are *tat* and *rev*, which are both transcribed from multiply spliced mRNAs (22,24,62,63). *Tat* encodes a viral transactivating protein which interacts with sequences in the LTR downstream of RNA start site, thereby increasing transcription levels 50 to 200 fold (5,24). *Rev* regulates expression of viral gene products. Rev facilitates the transport of unspliced and singly spliced message out of the nucleus, allowing for the expression of viral *env*, *pol* and *gag* products in the cytoplasm (15,24).

Less well characterized regulatory genes include *nef* and *vif*. Nef is a 27 kilodalton myristilated protein which is believed to act in a negative regulatory fashion (24). Nef is associated with the cytoplasmic membrane and it is proposed that it belongs to the guanine nucleotide binding family of signal transducing proteins (39). *Vif* encodes for a protein that is believed to be necessary for viral infectivity (22,24).

Retrovirus replication

Retrovirus replication proceeds in the following order: attachment, penetration, reverse transcription, integration, RNA synthesis, mRNA processing, protein synthesis, assembly, budding and processing. Although this series of events is somewhat similar to other virus replication schemes, there are a few differences that need to be pointed out. Firstly, the replication cycle can be

considered in two distinct phases which are divided at the integration step. In the first phase all processes are carried out autonomously by the virus, whereas in the second phase, processes are carried out by the cell machinery. Secondly, once the virus genome is integrated into the host cell chromosome, it is a stable entity which can be passed to progeny cells during cell division. Thirdly, while many retroviruses do not require viral gene products for expression of the provirus, lentiviruses do. This requirement adds a level of control to lentiviral replication as well as a level of complexity, an aspect that will be discussed in detail later. Finally, in most cases, retroviral infections are fairly benign. The infected cell continues to function, while producing progeny virions.

Attachment Attachment of retroviruses requires a cell surface receptor which associates with the virion envelope glycoprotein and initiates penetration. This reaction is quite specific and antibodies to either the cell receptor or the viral envelope protein will neutralize virus infectivity (22). Retroviruses are very resistant to superinfection because newly synthesized envelope glycoproteins associate with the cell surface receptor thereby preventing attachment and penetration of external virions (22,60).

Penetration and uncoating The process of penetration and uncoating of retroviruses is not well understood. It is thought that after the surface protein binds to its receptor, the cell membrane and the viral envelope fuse, enabling the virion core to enter the cell. Fusion is probably mediated by the hydrophobic amino acids at the amino terminus of the smaller transmembrane protein (22).

Reverse transcription Once the virion core is inside the cell, the process of reverse transcription begins. Reverse transcription is initiated at the 5' end of the genome by binding of a tRNA lysine to the primer binding site (PBS) located just 3' to the 5' LTR (22,96,97). Negative-strand DNA synthesis proceeds 5' to 3' from the PBS, through the U5 and R regions of the LTR until there is no more RNA to serve as a template (22,96,97). This is called a strong stop. The newly synthesized DNA "jumps to" the 3' end of the genome for continued synthesis of negative-strand DNA. This "jump" occurs due to the RNAse H activity of reverse transcriptase which frees the DNA from its RNA template. The complement base pairing of the R regions, present on both ends of the genome, insures that correct positioning is maintained during the jump. Once the "jump" has occurred, negative strand synthesis continues until the 5' end of the genome is reached.

The initiation of positive-strand DNA synthesis is not entirely understood (22). There appears to be a specific cleavage of the RNA template 5' of the U3 region, within the polypurine tract. The cleavage results in a small piece of RNA which serves as a primer for the synthesis of the positive-strand DNA. Synthesis of the positive-strand DNA proceeds until the 5' end of the negative-strand DNA is reached. This is called positive-strand strong stop. At this point, another "jump" is required. The positive-strand DNA contains the 18 base pair PBS sequence which can now base pair to the PBS at the 3' end of the negative-strand DNA. At this point positive-strand synthesis continues to its logical end. Finally, the tRNA primer

is removed by RNAse H action of reverse transcriptase and a double stranded DNA molecule is the final result.

Integration Integration of the double stranded viral DNA into the host genome is the next step in the virus replication cycle. The process of integration is not entirely understood, although recent research has shed more light on the process (22,96,97). Initially, the core structure containing viral DNA and integrase proteins enter the nucleus. The integrase protein then removes two bases from both ends of the viral genome, resulting in 3' OH ends. The viral endonuclease makes a random, staggered (4 to 6 bases) cut in the host genome, resulting in 5' phosphorylated overhangs (22,97). Integration occurs by joining the 5' overhangs of cell DNA with the 3' overhangs of viral DNA. Cellular DNA repair machinery fills in the gaps left by the integration process and displaces the mismatched base pairs (22,97).

There are a few common features of integration shared by all retroviruses. First, both viral and cellular DNA are changed. The viral DNA has been shortened, usually by two base pairs whereas 4 to 6 base pairs of cellular DNA have been duplicated. The integration site in the viral DNA is very specific, always joining cellular DNA at the same place on the viral sequence. In contrast, integration along the cellular DNA is random, although some reports claim that integration occurs in areas of the genome not associated with chromatin (84,98,99).

Transcription The retrovirus LTR contains sequences that are recognized by the cellular transcription machinery as well as by viral and cellular

transcription factors. Because the LTR is the only promoter present in the retrovirus, it controls transcription of all virus genes. Initially the provirus is transcribed into a single RNA precursor, which is then processed in one of two ways. Full length RNAs are used either as genomes for new virions, or as mRNAs for the Gag and Pol proteins (22,24). The second option is that the full length RNA is spliced in a number of ways to give rise to subgenomic mRNAs which encode viral envelope and regulatory proteins (22,24).

The mRNA products are transported out of the nucleus into the cytoplasm where they are translated into proteins. The full length mRNAs which encode the *gag* and *pol* products are translated on free ribosomes (22,60). The spliced transcripts are translated on membrane bound polyribosomes, which allows these proteins to be post-translationally modified in the golgi apparatus.

Assembly and Release Virion assembly is a poorly understood aspect of retrovirus biology (22). Electron microscopy studies have revealed two patterns of assembly. The first, which is typical of C type virions, is that the assembly of capsid and budding proceed simultaneously (22). In the second form, typical of B- and D-type virions, the core assembles in the cytoplasm and then buds through the cell membrane. In both cases, a rearrangement of the capsid to a more electron dense form is observable shortly after, or during, virion release.

The complexity of retroviral replication allows for regulatory control mechanisms at multiple stages in virus replication. As a stably integrated provirus, replication is controlled by the eukaryotic regulatory mechanisms. Therefore, an

understanding of eukaryotic gene regulation is important in understanding mechanisms of retrovirus regulation and control.

Eukaryotic Gene Control

Introduction

The processes by which genotype is converted to phenotype are multifaceted and occur at many levels and time points during the lifetime of a cell. These processes can be placed under the broad subject of gene regulation. The study of eukaryotic gene regulation has made great gains in the last few years allowing for a better understanding of the mechanisms that give rise to the seemingly infinite diversity of eukaryotic cells. Control is exerted at every step in the making of a protein: transcription, mRNA processing, translation, and post-translational modification. To cover all of the these steps, even in a basic sense, is beyond the scope of this review. Therefore only transcriptional control will be addressed. Discussion in this section will begin with an example of the steps in the initiation of transcription in a generic promoter. More detailed discussion of promoters, enhancers, and transcription factors will follow in order to explain how these factors, when acting in concert, give rise to a level of gene control that is amazingly subtle and precise.

Initiation of transcription Initiation of transcription is a primary control point of gene regulation. The initiation of transcription begins with the association

of RNA polymerase to DNA. There are three functionally distinct types of RNA polymerase found in eukaryotic cells, RNA polymerease I, II and III. RNA polymerase I makes ribosomal RNA, RNA polymerase II makes mRNA, and RNA polymerase III makes small RNAs such as transfer RNA (6). Each of the RNA polymerases is complex of large and small proteins. There are two large proteins, approximately 200 kilodaltons (Kd) and 140 Kd (6), as well as up to 10 smaller proteins between 90 Kd and 10 Kd in size (6). At this point in time, it has not been possible to reconstitute active RNA polymerase from purified sub-units.

The steps involved in binding the RNA polymerase II complex to DNA are shown in Figure 2. The first step is the binding of the transcription factor TFIID to the TATA box (Figure 2a and 2b). Next, TFIIA binds upstream of TFIID (Figure 2c), then TFIIB binds downstream of the TFIID/TATA box complex (Figure 2d). Only after this point is RNA polymerase able to bind to the complex. After RNA polymerase II binds (Figure 2e), TFIIE binds downstream from the polymerase (Figure 2f). It is thought that at this point, transcription begins and the transcription factors are released (6,100).

Promoters In the most simplistic terms a promoter can be defined as a sequence of DNA that can initiate transcription. Promoter sequences are located immediately (~25 bases) up stream of the RNA start site and are typically around 100 base pairs in length (6,34). Promoters have directionality, meaning they can only promote in one direction, and are modular in nature (6,34,65). A basic promoter will contain a TATA box, which is found in most eukaryotic

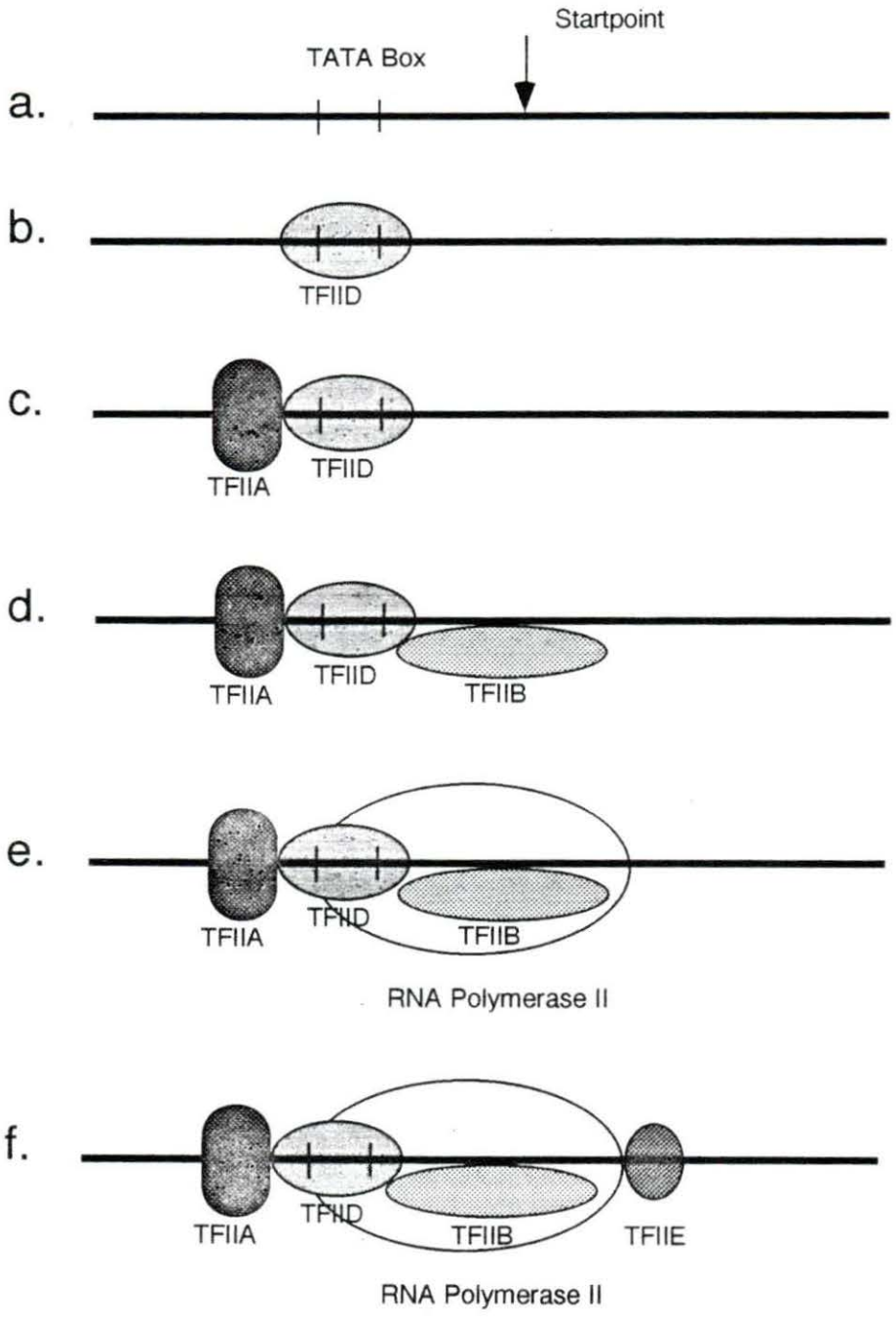


Figure 2. Diagram of basic transcription, showing the sequential steps in binding RNA polymerase II.

promoters(6,88), and a variable number of upstream promoter elements. The main function of the TATA box is to accurately initiate transcription, while the upstream promoter elements increase the rate of transcription (6,65). Two of the best characterized promoter elements are the CAAT box and the GC box. Interestingly, while the promoter as a whole functions unidirectionally, the CAAT and GC box can function in either direction within the promoter (6). It must be stated that none of the examples mentioned are uniquely essential for promoter function. Promoters may lack TATA boxes, or have no CAAT or GC boxes. On the other hand, there may be multiple copies of each (6,34). However, the frequency of initiation of transcription is strongly influenced by the presence of these upstream promoter elements. It has been suggested that these elements may function to bring the RNA polymerase into the general area of the start-point but do not actually align the enzyme for accurate initiation (6). Accurate initiation depends upon the TATA box, and promoters that lack a TATA box will also lack a unique start site (6,66).

Enhancers Enhancers are short sequences of DNA (30 to 100 base pairs) which, in the presence of transcription factors, are able to significantly increase the transcriptional efficiency of nearby promoters (6,65,66). Trying to draw clear distinctions between promoters and enhancers is becoming more difficult. However, enhancers differ from promoters in that their position does not need to be fixed to an area near the RNA start site. In addition, enhancers can function in either orientation (6,65,66).

Enhancers are able to activate transcription independent of the their position, orientation, and distance from the target promoter. For example, enhancers can increase transcription even if they are 10 kilobases away from the target promoter (66) and are able to function even when they are located downstream of the RNA start site (6,66). The modular units that make up an enhancer sequence are known as enhansons. Enhansons are short, eight to ten base sequences which bind transcription factors (34). The efficiency with which enhancers regulate transcriptional activity depends upon the spatial array of enhansons within the enhancer as well as the availability of the transcription factors (6,34,95). Interestingly, enhanson sequences found in enhancers are also found in promoters; an example of how the distinctions between enhancers and promoters are becoming less clear.

Transcription factors Transcription factors are soluble proteins that are able to bind to DNA and regulate transcription. In the very broadest terms, transcription factors can be divided into several groups. One group would be the subunits of RNA polymerase. These are not promoter specific, but they are required to initiate transcription. Another group are the sequence specific transcription factors that bind to specific enhanson sequences in promoters and enhancers. This group includes factors that play a role in the basic transcription complex, i.e.TFIID which binds to the TATA box, as well as inducible or tissue specific transcription factors. Table 1 lists examples of known transcription factors and the sequences they bind to.

Table 1. List of known transcription factors and the consensus sequences (6).

Factor	Consensus	Module	Distribution
TFIID	TATAAA	TATA box	general
CTF/NF1	GGCCAATCT	CAAT box	general
SP1	GGGCGG	GC box	general
Oct-1	ATTTGCAT	Octamer	general
Oct-2	ATTTGCAT	Octamer	lymphoid
NFκB	GGGACTTTTCC	κB	lymphoid
H2-TF1	GGGACTTTTCC	κB	general
ATF	GTGACGT	ATF	general

The sequence specific transcription factors can be further divided into regulatory and non-regulatory transcription factors. The non-regulatory transcription factors are ubiquitous in cells and consequently are available for any promoter or enhancer with the correct sequence module (53,65). Examples of this are: TFIID, CTF/NF1, Oct-1 and SP1. The role of these transcription factors appears to be in the general enhancement of transcription. The regulatory

transcription factors include those factors that respond to changes in the environment (inducible transcription factors) and those that are active only at a specific time during development or only in specific tissues (temporal and tissue-specific transcription factors). The regulatory transcription factors play an important role in the regulation of gene expression and how genotype is expressed as phenotype in a given cell or tissue.

Induction of an active transcription factor can occur through several possible routes. For example, a transcription factor may be in an inactive state until some environmental stimulus, such as hormones or heat, induce the activation of the transcription factor (65,95). The activated transcription factor is now able to bind to the specific enhanson sequence in either the enhancer or promoter sequence. Alternately, a repressor protein may bind, or sterically hinder, access of the transcription factor to an enhanson sequence. Upon outside stimulus, the repressor protein is altered making it inactive (65). Consequently, the already active transcription factor is able to bind to the enhansons. It is also possible that an environmental stimulus induces the synthesis of a transcription factor. In this scenario, it is easy to imagine a cascade of transcription factors, where one transcription factor induces the production of another transcription factor and so on, culminating in an array of proteins produced in response to the outside stimulus.

Retrovirus gene regulation

The simplest retroviruses, such as the murine leukemia virus, make only two mRNA transcripts and encode no regulatory proteins. Consequently these retroviruses are completely dependent on cellular transcription factors for control of viral gene expression. On the other hand, HIV has the most complex genome of any known retrovirus. HIV makes several species of singly and multiply spliced transcripts that encode at least nine proteins, three of which have known regulatory function. Because HIV is the most studied of the retroviruses, it will be used as a model for complex retroviral regulation.

HIV gene expression can be divided into two simple categories: early and late gene expression. Early expression is characterized by the presence of small, completely spliced viral RNAs in the cytoplasm. In the late phase, cytoplasmic viral mRNAs are composed primarily of incompletely spliced viral mRNAs which encode for viral enzymatic and structural proteins as well as the genomic RNA (15,25). Early gene transcription is initiated by the combined interaction of cellular transcription factors with the LTR, resulting in a low level of full length proviral mRNA transcripts. These transcripts are fully spliced in the nucleus and only the small (about 2 kb) viral mRNAs, which encode the regulatory proteins Tat and Rev, are found in the cytoplasm. Tat localizes in the nucleoli (25) where it is able to *trans*-activate HIV LTR-dependent expression by 50 to 1000 fold (24). Tat associates with a *cis* acting target sequence in the LTR which is called the *trans*-activation response element, or TAR region. The TAR region forms a

complex RNA secondary structure that is present in all nascent transcripts and the interaction between Tat and TAR culminates in the up regulation of transcription.

The increased transcription induced by Tat results in accumulation of a second regulatory protein, Rev, which is responsible for the shift from early to late gene expression (15,24). Rev is a 19 kilodalton phosphorylated protein that is localized in the nucleoli of expressing cells (22,24). Rev is responsible for the transport of incompletely spliced message from the nucleus to the cytoplasm (15,24,36). Rev acts through a sequence on the RNA of singly and unspliced message located in the *env* gene which is termed the Rev response element, or RRE (22,42). Rev interacts with the RRE, either alone or through an intermediate, to bring about the exportation of singly and unspliced viral mRNAs from the nucleus to the cytoplasm (15,42,62). This allows for translation of viral structural proteins. Along with the increase of singly and un-spliced message, there is a concurrent decrease of fully spliced message. Thus, Rev down regulates its own expression. The shift from early gene expression to late is an abrupt process suggesting that the level of Rev in a cell must reach a critical threshold before the shift can occur (22,24). In summary, in a resting cell, lentivirus expression is greatly restricted and few virus proteins are expressed. Upon activation and proliferation of infected cells, a variety of cellular transcription factors interact with enhansons in the viral LTR and induce the expression of viral regulatory genes Tat and Rev. During the early phase, Tat up regulates expression of itself and Rev, until a time that enough Rev has accumulated to shift to the late phase of

expression. In this phase, viral structural genes are expressed and mature virions are produced. It is therefore clear that inducible cellular transcription factors play a major role in lentivirus replication and pathogenesis. Less information is available on the role of cell-specific transcription factors in other lentiviruses, including equine infectious anemia virus.

Equine Infectious Anemia Virus

Disease characteristics

History Equine infectious anemia was one of the first viral diseases described. In 1843 Lingnee described the disease *anhemie du cheval* which he thought was a nutritional disorder caused by the artificial diet of horses (58). Later, EIA was reported as being contagious (3) and the infectious agent was implicated to be virus in 1904 when Carre and Vallee showed that the agent was filterable (12). In 1976 it was shown that EIA was caused by a non-oncogenic retrovirus, or a lentivirus (4,17).

Clinical signs and pathological lesions One of the hallmarks of lentiviruses is the slow, chronic nature of the disease process. In some cases, the time between initial infection and the first signs of clinical disease may be years apart (46,73). Death may or may not occur after this extended period of viral quiescence. This is true for most lentiviruses, though EIAV can be an exception. The disease process of EIAV follows one of three forms. The first is the acute form

of the disease, where clinical signs are observed 1 to 4 weeks after initial infection. Attacks of fever can occur at 1 to 4 day intervals or fever may be continuous (46,89). Symptoms include debility, depression, excessive thirst and perspiration. Epistaxis and sublingual and nasal hemorrhages are occasionally observed (33). Viral replication occurs at a very high rate and death of the animal is the end result.

The second, more classic form is chronic disease. Clinical signs of disease, characterized by anorexia, fever, incoordination, depression, debility and anemia, are usually seen within one month of infection (33,46). The animal will recover within three to five days after the initial signs of disease. Subsequently, another round of viral replication occurs, resulting in a relapse of clinical signs. Cycling between disease recrudescence and quiescence may continue for up to a year. Eventually, cycling becomes less frequent and less severe, however these animals are still seropositive and low levels of virus can sometimes be recovered. The administration of immunosuppressive drugs such as corticosteroids can elicit another round of viral replication and clinical recrudescence (18,46). The third form of EIAV is subclinical. In this case, the infected animal is found to be seropositive, but no clinical signs of disease are observed.

The gross pathological lesions of EIA include lymphadenopathy, anemia, emaciation, accentuated hepatic lobular architecture, edema, and hemorrhages (89). Microscopically, there is lymphoid and reticuleodothelial hyperplasia in lymphoid tissue and lymphoproliferative changes with interstitial lymphocytic infiltration in most organs (89).

Transmission Transmission of EIAV is accomplished through exchange of body fluids, namely blood. The transmission most often occurs by a mechanical mechanism such as hypodermic needles or other instruments that may come in contact with blood. Blood sucking insects, mainly Tabanids, have also been implicated as vectors (47,89). Insect vectors act in a purely mechanical fashion in disease spread. To date no insect has been shown to be able to harbor the virus for any length of time (33). There is evidence that EIAV can be transmitted vertically from mares to their foals, however the actually route of transmission is not clear (33).

Virus structure and replication

Morphology EIAV ranges from 90 to 200 nm in diameter (37). Virions have a smooth envelope with a pleomorphic core that is 40 to 80 nm in diameter (37). It has been observed that maturation occurs during the budding process with the formation of the electron dense core.

EIAV genome The genome of EIAV is approximately 9000 bases in length and is organized in typical retrovirus fashion (85,93). It contains the three major structural genes, *gag, pol* and *env* which encode the core proteins, polymerase proteins, and the envelope proteins respectively. Located between the 3' end of the *pol* gene and the 3' LTR are three small open reading frames (ORFs), designated S1, S2 and S3, which are believed to code for regulatory proteins (85). S1 encodes the transactivating protein, Tat, and S3 encodes the putative Rev

protein (31,94). The function of S2 gene product has not yet been determined, but it is suspected to act in some regulatory capacity.

Transcription of EIAV Similar to the other lentiviruses, EIAV has a complex transcription pattern, with several multiply spliced RNAs. However, the transcription pattern of EIAV is not as well understood as in other lentiviruses, notably HIV. Transcription patterns differ depending upon the type of cells used for Northern blot analysis. For example, in EIAV infected fetal donkey cells and equine kidney cells, three major transcripts of 8.2, 3.5 and 1.5 kilobases (Kb) were detected (81). In canine thymus cells, Northern blot analysis identified transcripts of 8.2, 5.0, 4.0, 2, and 1.8 Kb in size (74).

The 8.2 kb transcript represents genomic RNA (74,81). The 3.5-4.0 kb transcript is singly spliced and most likely encodes the envelope glycoproteins (81). The 1.5 kb transcript is presumably a doubly spliced message containing sequences from the 5' end of the genome and sequences from the S1, S2 and S3 open reading frames (81). As detection methods improve, more mRNA transcripts are being discovered. A recent study detected no less than seven viral mRNA transcripts in EIAV infected canine cells (75). These included genomic length transcripts, as well as singly, doubly and triply spliced transcripts. The complexity of transcription patterns may play a role in regulation of virus gene expression.

EIAV proteins The *gag* and *pol* encoded proteins are translated from full length genomic mRNA. *Gag* proteins are initially translated as a large precursor protein (Pr 55) which is subsequently cleaved to yield four proteins. These are,

from largest to smallest, the capsid protein p26 (235 amino acids), the matrix protein p15 (124 amino acids), the nucleocapsid protein p11 (76 amino acids), and p9 whose function is unknown (93). The capsid protein is hydrophobic and forms the core shell of the virus. The matrix protein is associated with the viral membrane through a sequence in its amino terminus (80). The nucleocapsid protein is basic and is associated with the RNA genome. The p9 protein's function is unknown, but it is internal and hydrophobic (11). These proteins are in the *gag* open reading frame in the following 5' to 3' order: p15, p26, p11 and p9.

As stated previously the *pol* and *gag* open reading frames are translated from the same full length mRNA. The *pol* open reading frame overlaps the *gag* open reading frame by 251 bases and is in a different reading frame than the *gag* open reading frame (93). It is most likely that translation of *pol* occurs as a result of a ribosomal frame shift, as reported for certain retroviruses (48). The EIAV *pol* region codes for the viral protease, reverse transcriptase and the integrase proteins. The function of these proteins are to cleave the gag and pol precursor proteins from one another, to reverse transcribe the RNA genome to DNA, and to cleave DNA prior to proviral integration (22).

The *env* gene products are translated from a singly spliced mRNA. Initially the mRNA is translated as a large precursor protein which is subsequently cleaved into the two envelope products. The precursor protein has a predicted molecular weight of 97,078 daltons (85) and contains a 21 amino acid signal peptide at the amino terminus which is later removed (85). The EIAV surface glycoprotein, gp90,

contains 424 amino acids and has 12 potential glycosylation sites (82,85). The transmembrane glycoprotein, gp45, consists of 415 amino acids and contains four glycosylation sites. Gp90 is associated with gp45 on the virus envelope, with gp45 serving as an anchor. The interaction between these proteins is not especially strong and gp90 is frequently lost (22). The complex is important for receptor binding and virus-cell fusion. In addition, the majority of neutralizing antibodies are made to gp90 (22).

As mentioned above, S1 encodes the EIAV Tat protein which is approximately 83 amino acids long (31) and is able to increase the transcription level of the EIAV LTR by approximately twenty fold (31). The area in the LTR that responds to Tat spans the transcriptional start site (+1 to +25) (32), and like other retroviral transactivators, Tat works at the transcriptional level. Interestingly, the *tat* gene is initiated at a non AUG codon (31,32,94).

The S3 open reading frame is thought to encode the EIAV Rev protein of 105 amino acids (31). This has not been proven at this time, but S3 is analogous in terms of size and placement in the genome to other lentivirus *rev* genes such as HIV (62,63). S2 encodes a protein of potentially 160 amino acids (31) whose function is unknown at this time.

EIAV LTR Similar to other retroviruses, EIAV gene expression is governed by sequences located in the LTR and by the cellular environment (28). The LTR contains a transcriptional promoter that is controlled by upstream *cis*-acting elements (96). The lengths of LTR sequences vary depending upon the

virus isolate. Reported lengths of the EIAV LTR are 302 base pairs (28) and 322 base pairs (9). The EIAV LTR is divided into 3 regions, U3 , R, and U5, as stated above. For the 322 base pair LTR, the U3 region of the EIAV LTR is 211 base pairs, the R region is 71 base pairs, and the U5 is 40 base pairs (28). The functions of these regions have been described above. The U3 region of the EIAV LTR contains the hypervariable region and the TATA box (Figure 3). The EIAV LTR is also responsive to a virus encoded *trans*-activating protein, Tat. EIAV Tat has been shown to greatly increase EIAV LTR-directed chloramphenicol acetyltransferase activity in transient expression assays (28,31,32). Tat interacts either directly or through an intermediate with virus mRNAs. Tat interacts with a region called the Tat-responsive element or TAR region. The EIAV TAR region maps to nucleotides +1 to +25 which forms a stem and loop structure in EIAV mRNAs (13). Interestingly, the EIAV TAR region is much smaller and structurally simpler than the HIV TAR region which contains bases spanning a region from +1 to +60 (13).

Variation

One of the more notable features of retroviruses is their genetic variability. Retroviruses use three enzymes in their replication scheme, reverse transcriptase, RNA polymerase II, and DNA polymerase. Random mutations arise from the activity of any of these enzymes. However, errors by DNA polymerase are very rare due to it's proofreading ability. The error rate of RNA polymerase II is

postulated to be around $1x10^{-5}$ per generation, plus or minus a factor of three (21). The error rate of EIAV reverse transcriptase is unknown, but a wide range of error frequencies has been reported for various retroviruses. For example, HIV has a reported error rate of $5.9x10^{-4}$ nucleotides, while reverse transcriptase from avian myeloblastosis and murine leukemia viruses have much lower average error rates of $5.9x10^{-5}$ and $3.3x10^{-5}$ respectively (57,83). Because of the high mutation rate, numerous virus variants may co-exist *in vivo.* It is important to note that within an infected animal there is not a single population of virus with one specific genotype of virus but a collection of populations with slightly different genotypes. It has been shown in HIV infections that there are indeed a multitude of unique populations of closely related, yet distinct, viral genomes (19,35,70,86). Through the use of the polymerase chain reaction (PCR) it has also been demonstrated that distinct, yet related genomes exist in EIAV-infected horses (1,9). Although mutations occur throughout the virus genome, only a small number provide a selective advantage and allow the spread of a particular variant. The pressures that select for one genome population gaining a replicative advantage over another are just beginning to be understood. Factors that influence variant selection include, but are not limited to, the host immune system, cell tropism and rate of viral replication.

Persistence

EIAV establishes a chronic relapsing disease that persists in infected horses for life. The mechanisms of persistence comprise a important aspect of the

disease pathogenesis. Persistence can be attributed to at least three characteristics of the virus biology. These are circulating immune complexes, antigenic variation, and the integration of the virus genome into the host chromosome.

The circulating immune complexes that occur during the course of an EIAV infection may provide a mechanism for EIAV persistence. Over 90% of virus in the serum of an infected animal exists in the form of immune complexes and these have been shown to be infectious (67). The immune complexes are able to block neutralizing antibody (46). The natural host cell of EIAV is the macrophage, and it is possible that macrophage phagocytosis of immune complexes containing infectious virus creates a unique opportunity for the virus to re-infect macrophages and persist in the infected animal.

One of the most salient features of EIA is the cyclical nature of the chronic disease. During this stage the animal experiences fever, anemia, viremia and other clinical signs, punctuated by periods of clinical quiescence (51,68,77,87). It has been shown experimentally that an infected horse will develop neutralizing antibodies to a predominant population of virus, yet another population of antigenically distinct virus will emerge to circumvent the host immune response (51,77,87). This action of circumventing the immune system through antigenic variation is also a method of viral persistence.

The last mechanism of persistence is the act of integrating the viral genome into the host cell chromosome. After this occurs, the viral genome is replicated

every time the host cell divides. As discussed above, lentiviruses have evolved many mechanisms to regulate viral expression. In addition, EIAV Tat is encoded by at least three transcripts in either a polycistronic or monocistronic form (75). This suggests that the expression of EIAV Tat may be subject to several levels of postranscriptional control.

Sequence comparisons between different strains of EIAV have shown that the majority of the differences occur in the long terminal repeats and in the S3 open reading frame (the putative *rev* gene) (1,9). Sequence changes within these two genes could easily alter either the efficiency of viral transcription or the ability of the virus to make the shift from an early phase replication to a late phase replication. Thus, variablity in the viral regulation sequence likely contribute to EIAV persistence and pathogenesis.

MATERIAL AND METHODS

Construction of EIAV LTR CAT Plasmids

Isolation of EIAV LTR fragments

A pUC 19 plasmid containing a 327 base pair fragment consisting of the 5' LTR of MA-1 and 5 base pairs of flanking sequence was digested with *Hin*dIII digestion, following manufacturer's instructions, to release the 327 base pair fragment. Briefly, 10 μg of DNA was incubated 1 to 2 hours at 37°C in 50 ul containing 10 units of *Hin*dIII, 1 mM Tris, pH 7.9, 1 mM $MgCl_2$, 50 mM NaCl, and 0.1 mM dithiothreitol (DTT). The reaction was stopped with 20 mM ethylenediaminetetraacetic acid (EDTA) and the DNA was electrophoresed through 2% agarose in TAE buffer (40 mM Tris base, 52 mM sodium acetate and 1 mM EDTA). The gel was stained in 150 mM ethidium bromide and the DNA was visualized under ultraviolet light. An agarose plug containing the 327 base pair fragment was excised from the gel and DNA was isolated by electroelution. DNA was precipitated overnight at -20°C in 2 M ammonium acetate and 2.5 volumes of 95% ethanol. DNA was pelleted by centrifugation at 10,000 x g for 30 minutes, washed once in 70% ethanol, dried by vacuum centrifugation for 10 minutes, and resuspended in 1X TE buffer (10 mM Tris-HCL, pH 8.0, 1 mM EDTA). The concentration and size of the LTR fragment was confirmed by agarose gel electrophoresis.

The Th-1 LTRs were prepared in a similar manner, except that they were excised from pGEM3Z plasmids using the restriction enzyme *Xba*I.

Construction of CAT plasmids

The transient expression vector pCAT Basic (Promega Biotec) contains the prokaryotic gene chloramphenicol acetyltransferase (CAT) and no eukaryotic promoter or enhancer sequences. Putative regulatory sequences can be cloned upstream of the CAT gene and following transfection of DNA into appropriate cells, levels of CAT activity can be measured in crude cell extracts. Studies have shown that levels of CAT activity are proportional to the relative strength of the promoter (38).

Preparation of vector DNA Two μg of pCAT Basic was digested with either *Hin*dIII or *Xba*I and digestion confirmed by agarose gel electrophoresis. The DNA was extracted with an equal volume of a 1:1 phenol:chloroform mixture and was ethanol precipitated, pelleted, washed, and dried as described above. Because of the possibility of vector recircularization during ligation, the 5' phosphate groups were removed using calf intestinal alkaline phosphatase (CIAP). One μg of digested vector was incubated 30 minutes at 37°C with CIAP buffer, (50 mM Tris-HCl, pH9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine) and 0.25 U of CIAP (1 U/μl Boehringer Mannheim Biochemicals). Another 0.25 U of CIAP was added, the reaction was allowed to incubate an additional 30 minutes, and stopped by addition of 25 μl of H_20, 5 μl STE buffer (150 mM NaCl, 10 mM Tris, 1

mM EDTA), 2.5 μl 10% sodium dodecyl sulfate (SDS) and incubation at 65°C for 15 minutes. The DNA was phenol:chloroform extracted, ethanol precipitated, pelleted, washed, dried and resuspended in TE buffer. The concentration and size of the vector was confirmed by agarose gel electrophoresis.

Ligation and transformation Long terminal repeats were ligated to pCAT Basic under the following conditions. In a 5 μl reaction, 20 to 40 ng of vector, 50 to 100 ng of insert, 1 μl of 5X ligation buffer (supplied by the manufacturer), and 1 μl of bacteriophage T4 ligase were incubated overnight at 12°C, and then stored at 4°C until use.

Transformation of competent cells was done according to Hanahan (41). Briefly, 1 ml of an overnight culture of JM109 cells was inoculated into 30 ml of SOB (bacto-tryptone 20 g/l, bacto-yeast extract 5g/l, NaCl 5 g/l, 250 mM KCl, 10 mM $MgCl_2$) (64) and cells were grown to log phase (1 to 1.5 hours). Cells were pelleted by centrifugation, resuspended in 8.0 ml TFB [10 mM MES(2-[N-morpholino]ethanesulfonic acid), 45 mM $MnCl_2 \cdot 4H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 100 mM RuCl and 3 mM hexamminecobalt chloride] and incubated 10 to 15 minutes on ice. Cells were pelleted, resuspended in 2 mls of SOB buffer and incubated 10 minutes on ice. Seventy μl of N,N-dimethlyformamide (DMF) was added, cells were incubated on ice 5 minutes, 4 μl 2-mercapthoethanol was added, cells were iced 10 minutes and then 70 μl DMF was added. Two μl of ligated DNA, diluted up to 10 μl with 8 μl of TFB buffer, was added to 200 μl of cells and incubated on ice for 30 minutes. The cells were heat shocked for 1.5 minutes at 42°C, placed on ice for 1 to 2 minutes, and diluted to 1.0 ml with SOC (SOB+20 mM glucose)

broth. The cells were allowed to incubate with shaking for 1 hour at 37°C and were plated on YT (bacto-tryptone 16 g/l, bacto-yeast extract 10 g/l, NaCl 5 g/l, agarose 15 g/l) containing 100 μg/ml carbenicillin.

Identification of recombinant clones Recombinant colonies were identified by three methods: colony blot hybridization, restriction enzyme analysis, and sequence analysis.

For colony blot hybridization, colonies were transferred onto nitrocellulose filters. The filters were soaked in 10% SDS for 5 minutes, 1.5 M NaCl, 0.5 M NaOH for 5 minutes, 1 M Tris pH 7.4, 1.5 M NaCl for 15 minutes and finally in 1M Tris pH 7.4 for 15 minutes. Filters were dried in a vacuum oven for 45 minutes at 80°C, and washed in 2X SSPE (1X SSPE=0.18 M NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM Na_4 EDTA), 0.1% SDS overnight at 55°C. The filters were prehybridized in 3X SSPE containing 200 μg/ml sheared salmon sperm DNA, 5X Denhardt solution (64), 0.1% SDS and hybridized in the same solution to an EIAV LTR-specific probe. The probe was labelled with [α^{32}P]-dCTP to a specific activity greater than 10^8 dpm/μg using the random hexamer primer method (Boehringer Mannheim Biochemicals). Hybridization was carried out overnight at 60°C. The filters were washed in 2X SSPE, 0.1% SDS for 30 minutes at 60°C, 0.1X SSPE, 0.1% SDS for 30 minutes at 60°C and exposed to film at -70°C with an intensifying screen for 12 to 24 hours.

Hybridization positive colonies were picked, grown overnight in NZY broth (NaCl 5 g/l, $MgCl_2$ 2 g/l, N-Z amine 10 g/l, yeast extract 5 g/l) containing 100 μg/ml ampicillin and plasmid DNA was isolated by alkaline lysis (64). Restriction

enzyme digestions were performed to determine the size of the insert and the orientation of the insert with respect to the CAT gene. The size of the LTR was determined by digestion of MA-1 or Th-1 clones with either *Hin*dIII or *Xba*I, respectively. The orientation of the Th-1 LTRs with respect to the CAT gene was determined by digestion with *Afl*III which cuts once in each LTR and once in pCAT Basic resulting in different sized fragments, depending on orientation. The same principal was used to determine the orientation of the MA-1 LTR except that *Eco*R1 and *Stu*1 were used. All enzyme digestions were performed according to manufacturer's instructions. Size and concentration of DNA was determined by agarose gel electrophoresis.

Sequence analysis LTR CAT plasmids were sequenced using methods for sequencing double stranded DNA (Promega Biotec). Primers on either side of the multiple cloning site were synthesized on an ABI 380B DNA synthesizer using published sequences of pCAT Basic. The 5' primer has the sequence TGTGGGGTTTTTATGAGGGG and the 3' primer sequence is GTAGGATCTCGAACAGACAAAC. Primer placement allowed the determination of the complete nucleotide sequence of the LTR insert, as well as the orientation of the LTRs (Figure 3).

Construction of chimeric LTR CAT plasmids

Th-1.152 and Th-1.11 are deleted at the 3' end and consequently lack the TATA box needed for the accurate initiation of transcription. Therefore, it was

```
MA-1        TGTGGGGTTT  TTATGAGGGG  TTTTATAAAT  GATTATAAGA  GTAAAAAGAA   50
Th-1.111
Th-1.11
Th-1.152

MA-1        AGTTGCTGAT  GCTCTCATAA  CCTTGTATAA  CCCAAAGGAC  TAGCTCATGT  100
Th-1.111
Th-1.11                                         G
Th-1.152

                        **  **
MA-1        TGCTAGGCAA  CTAAACCGCA  ATATCCTGTA  GTTCCTCATT  TGCGTTCCGC  150
Th-1.111                                          A   ATA
Th-1.11
Th-1.152                                        -------  ------

                    ↓                           *****
MA-1        ATTTGTGACG  TGTTAAGTTC  CTGTTTTTAC  AGTATATAAG  TGCTTGTATT  200
Th-1.111
Th-1.11                                         _______  __________
Th-1.152                                  __    _______  __________

                    U3 | R
MA-1        CTGACAATTG  GGCACTCAGA  TTCTGCGGTC  TGAGTCCCTT  CTCTGCTAGG  250
Th-1.111                                                        G
Th-1.11     __________  __________  __________  __________  __________
Th-1.152    __________  __________  __________  __________  __________

                                                R | U5
MA-1        CTGAAAATGC  CTTTGTAATA  AATATAATTC  TCTACTCAGT  CCCTGTCTCT  300
Th-1.111          G
Th-1.11     __________  __________  __________  __________  __________
Th-1.152    __________  __________  __________  __________  __________

MA-1        AGTTTGTCTG  TTCGAGATCC  TA    322
Th-1.111      _______   __________  __
Th-1.11     __________  __________  __
Th-1.152    __________  __________  __
```

Figure 3. Nucleotide sequences of MA-1 and Th-1 LTRs showing location of U3, R and U5 regions. Dashed line indicates the internal deletion in Th-1.152. Solid lines indicate 3' deletions in clones Th-1.11 and Th-1.152. The hypervariable region is bounded by a CAAT and TATAA box designated by asteriskes. Arrow indicates *Af*lII site.

necessary to construct chimeric LTRs by using the 3' end of the Th-1.11 LTR and ligating it to the 5' end of Th-1.152 and Th-1.11. This was accomplished by taking advantage of an *Af*lII site present in all of the LTRs (Figure 4). Ligation, transformation and screening was performed by methods described above. Structure of chimeric LTRs was confirmed by DNA sequence analysis.

Control plasmids

Control plasmids included: pCAT Control (Promega Biotec), pCH110 (Pharmacia LKB Biotechnology Inc.) and pRS-Etat-M (kindly provided by Dr. David Derse, Frederick Cancer Research Facility). pCAT Control contains the CAT gene under the control of the SV40 strong promoter and enhancer and was used to monitor transfection effciency as well to standardize relative promoter and enhancer strength of variant LTRs. Transfection efficiency was also monitored by co-transfecting with pCH110 which contains the β-galactosidase gene under control of the SV40 early promoter. The pRS-Etat-M plasmid contains the EIAV *tat* gene under the control of the Rous sarcoma virus promoter (31) and was used to optimize the sensitivity of the CAT assay. Studies in our lab indicated that MA-1 LTR CAT activity in cells co-transfected with pRS-Etat-M is comparable to activity in cells chronically infected with EIAV. Large scale plasmid preparations were made using ion exchange chromatography (Qiagen).

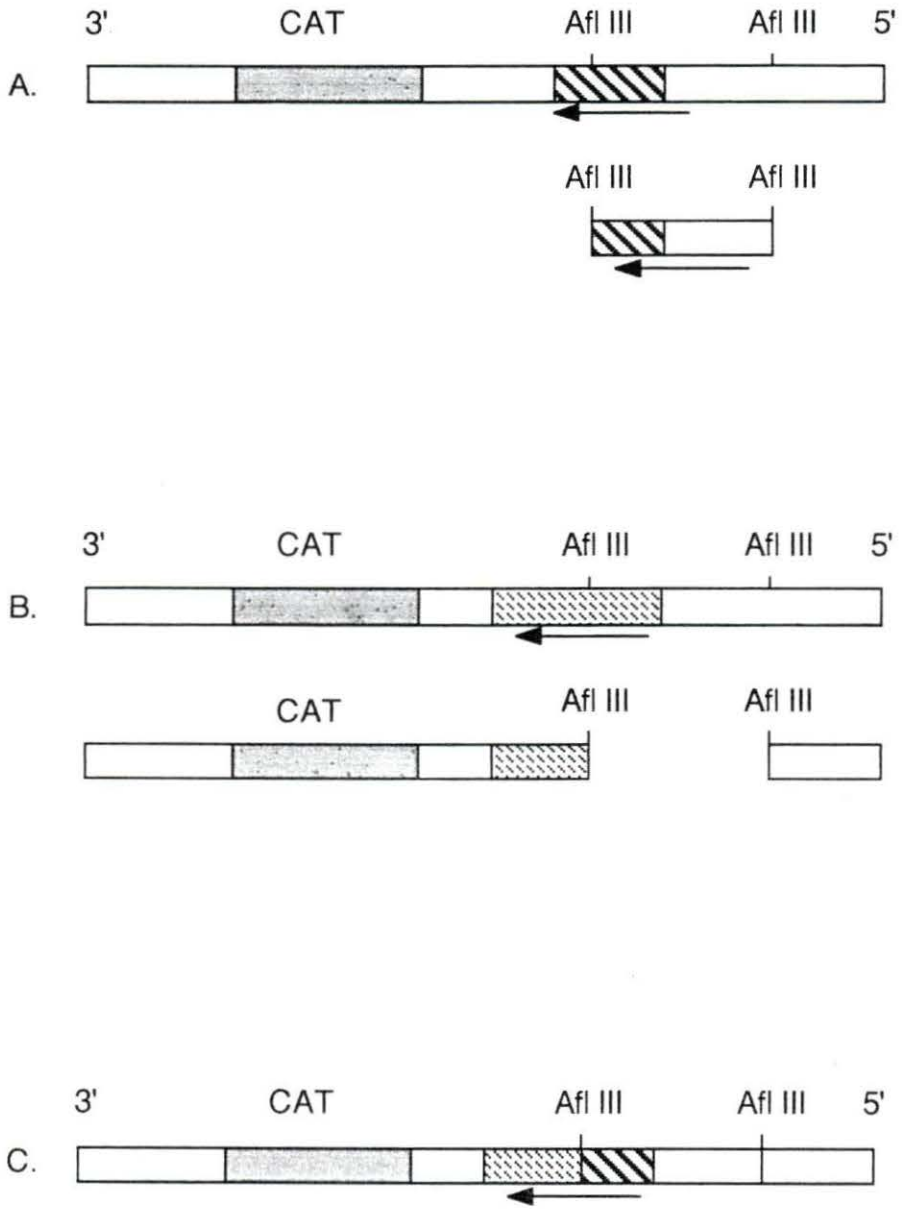


Figure 4. Construction of chimeric LTRs. (A) pTh-1.11 and pTh-1.152 were digested with *Afl*III and the fragment containing the 5' end of the LTR (hatched lines) was isolated. (B) pTh-1.111 was digested with *Afl*III and the fragment containing the CAT gene (gray fill) and 3' end of the LTR (broken hatched lines) was isolated. (C) Structure of chimeric plasmid following ligation.

Optimization of Transfections.

Cell culture

Equine dermal cells Equine dermal cells (ED, ATCC# CCL57) were grown in Dulbecco minimum essential medium (DMEM) supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml) and 20% fetal calf serum (Gibco BRL). Cells were subcultured at weekly intervals and could be maintained *in vitro* for up to 30 passages.

Horse macrophage cultures Primary horse macrophage cultures (HMC) were established from peripheral blood mononuclear cells (PBMC) using methods similar to those previously described (10). Horse leukocytes were collected by plasmaphoresis and cells were allowed to sit 1 hour at room temperature to allow red blood cells settle out. Leukocytes were decanted and mixed 1:1 in Hanks' balanced salts (HBSS) supplemented with 2% fetal calf serum. Mononuclear cells were separated by centrifugation on Ficoll-Hypaque (Sigma Chemical Co.), cells were washed twice in HBSS and seeded into 60 mm dishes at a concentration of $2x10^8$ cells per dish in DMEM supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), 20% normal horse serum and 10% newborn calf serum. The following day, non-adherent cells were removed by washing twice with HBSS. Cells were washed twice every 12 hours and allowed to incubate 36 to 48 hours at 37°C with 5% CO_2 before transfection.

Effect of macrophage activation It is known that cellular transcription factors play a role in regulation of HIV LTR (49,76). Certain of these factors are not expressed continuously but only during cellular activation. To determine if the EIAV LTR is responsive to similar inducible transcription factors, CAT assays were performed using HMCs stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA), or 5 μg/ml *E. coli* lipopolysaccharide (LPS). Preliminary studies indicated that stimulation resulted in a significant increase in tumor necrosis factor at 24 to 48 hours after stimulation (S. Carpenter, M. Wannemuehler, unpublished observations).

Transfections

Initial studies were conducted to determine optimum methods of transfection for each cell type. Methods tested were calcium phosphate DNA precipitation ($CaP0_4$) and two cationic lipid formations Transfectace™ (Gibco BRL) and Lipofectin™ (Gibco BRL). Transfections were performed on nearly confluent monolayers of cells in 60 mm culture dishes. For $CaPO_4$ precipitation, DNA, H_20 and $CaCl_2$ (125 mM final concentration) were mixed in a final volume of 250 μl and added drop wise to 250 μl of 2X Hepes ([N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]]) buffer (HBS) pH 7.04. The precipitate that formed was placed on the cell monolayers and allowed to incubate for 3 to 5 hours. The cells were then washed in phosphate buffered saline (PBS) and glycerol shocked with

1.5 ml of 15% glycerol in HBS for 1 to 2 minutes at room temperature. Cells were washed twice in PBS and incubated 48 hours at 37°C in 5% $C0_2$.

Transfectace™ is a liposome formulation of the cationic lipid dimethyl dioctadecylammonium bromide (DDAB), and dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. The Transfectace™ reagent was used according to the manufacturers instructions. Briefly, DNA was diluted in DMEM to a volume of 100 μl and combined with 12 μl of Transfectace™ in 100 μl DMEM. The mixture was allowed to incubate 5 to 15 minutes at room temperature, diluted to 1.0 ml in DMEM and added to cells. Cells were incubated for 5 hours at 37°C in 5% CO_2 and 1 ml of DMEM containing 40% fetal calf serum was added. Cells were incubated overnight, media was replaced, and the cells were incubated another 24 hours until harvested.

Lipofectin™ is a liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoly phosphatidylethanolamine (DOPE) in membrane filtered water. The Lipofectin™ reagent was used according to the manufacturers instructions. Briefly, DNA and the Lipofectin™ reagent were diluted separately in water, each to a final volume of 50 μl. They were then combined and allowed to incubate for 15 minutes at room temperature. Nearly confluent cells were washed twice in serum free media and 3 ml of serum free media was added to the cells. The DNA Lipofectin™ complex was added and the cells were allowed to incubate for 5 to 24 hours at 37°C in 5% CO_2. After incubation, the appropriate media with serum was added and the cells were allowed to incubate 48 hours until harvested.

Determination of CAT Activity

Preparation of cell lysates

At two days post-transfection, cells were rinsed twice in PBS, pH 7.4, and 1.5 ml of PBS containing 0.5 mM EDTA were added. Cells were harvested by scraping with a rubber policeman, washed once in ice cold PBS, resuspended in 100 μl of 0.25 M Tris-HCl, pH 7.8, and lysed by three rounds of freeze-thawing in a dry ice/ethanol bath. Cell debris was pelleted and the lysate was collected. Samples from each lysate were analyzed for protein concentration and β galactosidase activity to determine transfection efficiency. The lysate was incubated at 65°C for five minutes, centrifuged and the supernatant fraction was collected and analyzed for CAT activity.

Protein concentration determination

Protein concentrations were determined using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co.). Briefly 5 μl of lysate was serially diluted in a microtiter plate and mixed with 200 μl of working reagent. The reaction was incubated for 30 minutes at 37°C. Optical density (609 nm) was determined using a Molecular Devices Kinetic Microplate Reader. BSA standards were included in each assay and results were expressed as micrograms of protein per ml of lysate.

β-galactosidase activity

Transfection efficiency was determined by measuring β-galactosidase activity. Briefly, 10 μl of lysate in a microtiter plate was mixed with 40 μl 0.25 M Tris pH 7.8, 150 μl of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$ and 0.05M β-mercapthoethanol) and 40 μl of ONPG [(o-nitrophenyl b-$_D$-galactose) 4 mg/ml in 0.1M $NaPO_4$] were added. This reaction was incubated for 1 hour at 37°C and the optical density measured on a spectrophotometer at 408 nm. Results were expressed as U/ml lysate (Boehringer Mannheim Biochemicals).

CAT assay

To analyze CAT activity, cell lysates were incubated 1 to 4 hours at 37°C in a 100 μl volume of 0.25 M Tris-HCL (pH 7.8) containing 1.0 μCi ^{14}C-chloramphenicol (50 mCi/mmol, NEN Research Products) and 0.8 mM acetyl coenzyme A. The reaction was stopped and the chloramphenicol extracted with 500 μl ethyl acetate. The organic layer was dried in a vacuum centrifuge, taken up with 20 μl ethyl acetate and spotted on to silica gel thin layer chromatography plates (Kodak). This was run in chloroform:methanol (95:5) and the separated, acetylated forms were detected by autoradiography. The acetylated and non-acetylated forms were quantitated by cutting out the spots and determining the amount of radioactivity in a liquid scintillation counter. Results were expressed as either amount acetylated relative to pCAT Control, or as percent conversion.

Data analysis

For the presentation of data, values were expressed as mean relative CAT activity. Analysis of variance procedure was used to determine significant differences between ED cells, HMCs, and PMA treated HMCs and to determine significant differences between LTRs within a cell type.

RESULTS

Construction of EIAV LTR CAT Expression Vectors

The overall strategy for construction of the EIAV LTR CAT expression vectors was to excise LTR specific fragments from plasmid clones and to ligate the fragments into pCAT Basic. Plasmid p481 contains the 321 base pair MA-1 LTR insert in pUC 19 which was excised with *Hin*dIII (Figure 5, lane 1). Plasmids p24.111, p14.11 and p24.152 contain the Th-1 LTRs (302, 182 and 165 base pairs, respectively) in pGEM, and LTR specific sequences were excised with *Xba*I digestion (Figure 5, lanes 2-4). Individual LTR sequences were isolated by gel electrophoresis and electroelution (Figure 5, lanes 5-8). The LTRs were then cloned into the transient expression vector pCAT Basic at the *Hin*dIII site in the case of the MA-1 LTR and at the *Xba*I site in the Th-1 LTRs (Figure 5, lanes 9-12).

Orientation of LTR inserts with respect to the CAT gene was accomplished by restriction enzyme digestion using *Afl*III which cuts asymetrically in the LTRs and once in pCAT Basic. Depending upon the orientation of the LTR, digestion with *Afl*III results in the excision of different size fragments. For example digestion of pTh-1.152 LTR CAT in the forward orientation released a 526 base pair fragment, while digestion of pTh-1 LTR CAT in the reverse orientation released a 403 base pair fragment (Figure 6). Similar methods were used to identify LTR CAT clones for each LTR (data not shown).

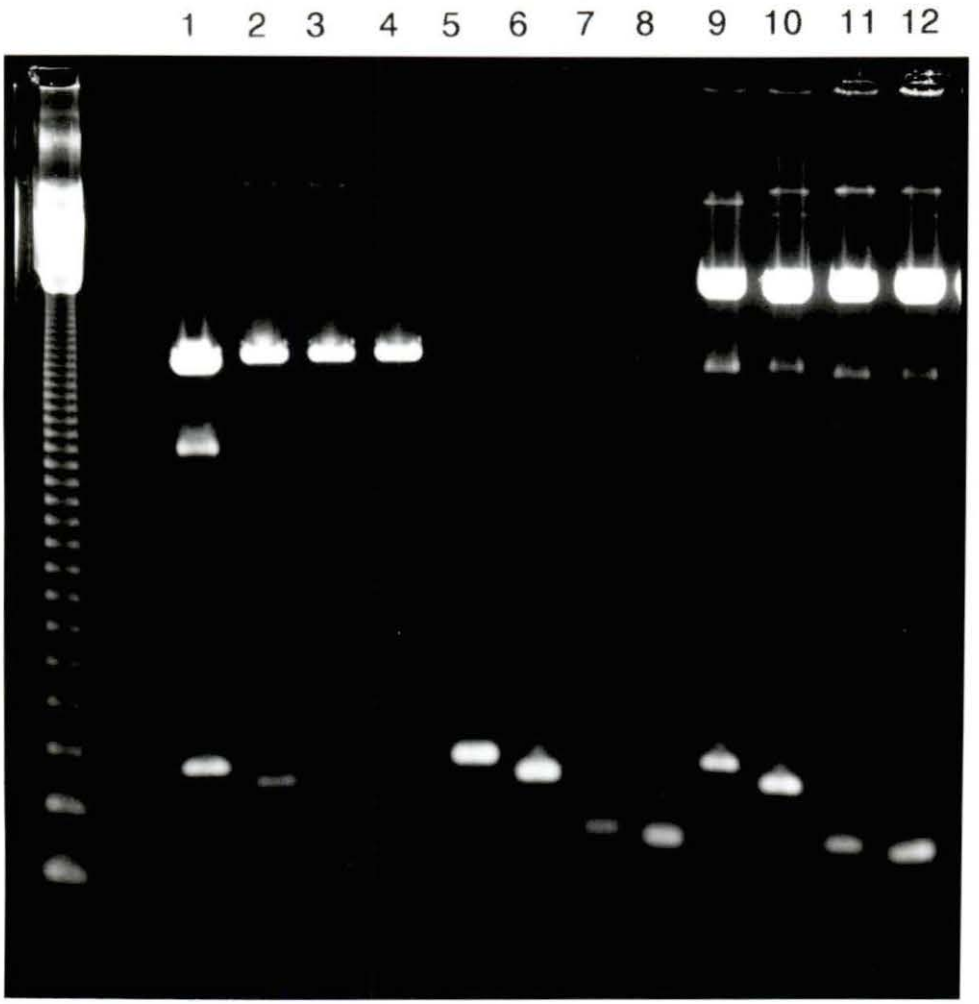


Figure 5. MA-1 LTR was excized from pUC 19 by Hind III digestion (lane 1). Th-1.111, Th-1.11 and Th-1.152 LTRs were excized from pGEM by Xba I digestion (lanes 2-4). MA-1 and Th-1 LTRs were isolated by electroelution (lanes 5-8) and cloned into pCAT Basic (9-12).

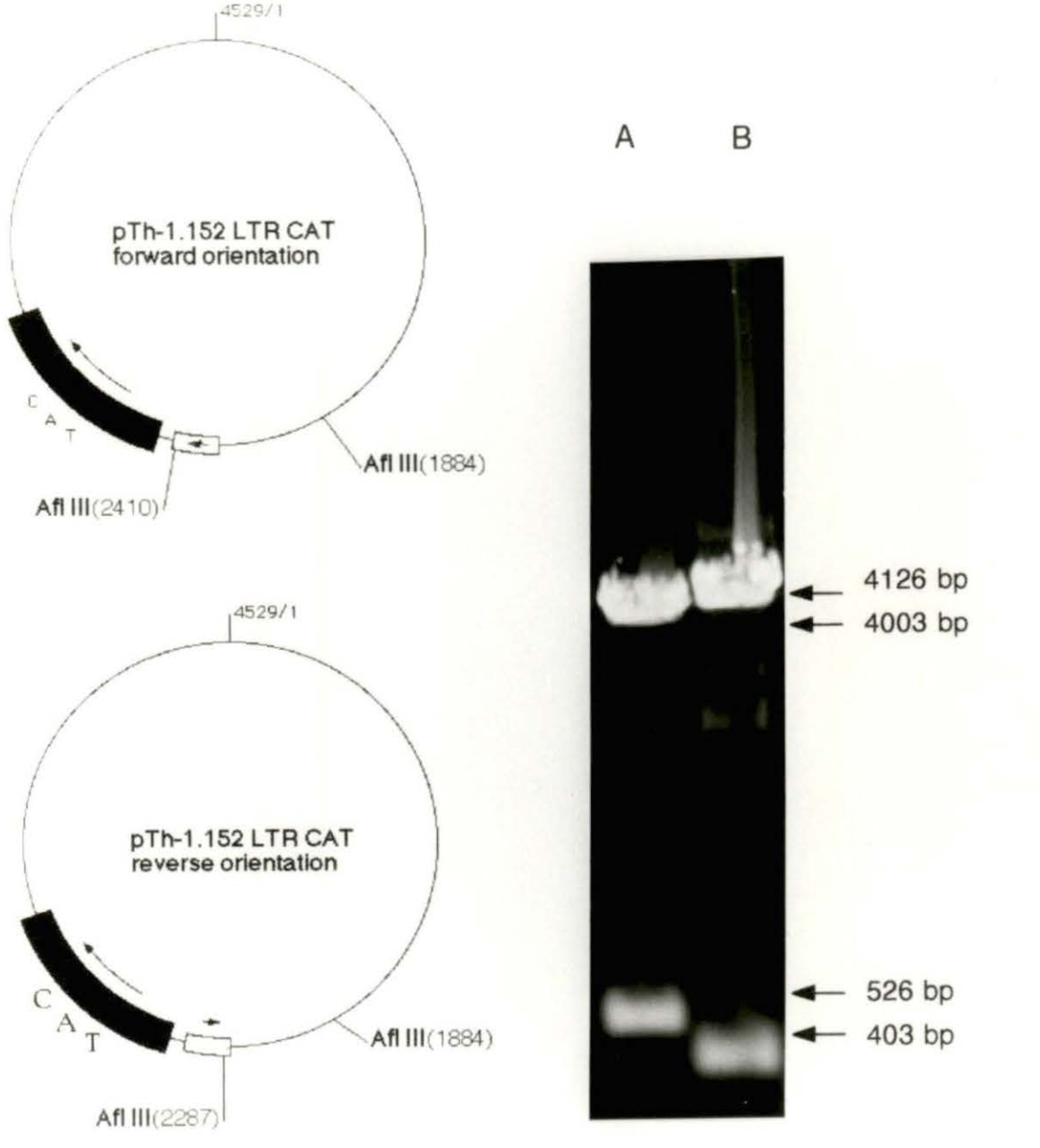


Figure 6. *Afl*III digestion of pTH-1.152 LTR CAT to determine orientation of insert. (A) In the forward orientation digestion produces bands of 4003 bp and 526 bp. (B) In the reverse orientation digestion produces bands of 4126 bp and 403 bp.

The Th-1.152 and Th-1.11 LTRs are deleted at their 3' end, most likely due to an artifact of PCR cloning. To accurately test differences in the hypervariable region of these clones, chimeric plasmids were made between the Th-1.111 LTR and Th-1 152 and Th-1.11 (Figure 4). pTh-1.111 LTR CAT was digested with *Afl*III and the fragment containing the 3' end of the LTR and the CAT gene was isolated and used as a vector. pTh-1.152 LTR CAT and pTh-1.11 LTR CAT were digested with *Afl*III, the fragments containing the hypervariable region were isolated by electroelution and ligated to the Th-1.111 LTR CAT vector fragment. *Afl*III cuts at A'C Pu Py G T, so there are two specific sequences that it may recognize, A C G/A C/T G T. The two *Afl*III sites in the LTR CAT plasmids each have different sequences, resulting in a forced cloning in the correct orientation. Ligated DNA was transformed into *E.coli*, positive colonies were identified by colony blot hybridization and analyzed by restriction enzyme digestion and DNA sequencing using methods mentioned above.

In conclusion, six plasmids were selected and large scale DNA preps were made. These plasmids were designated pMA-1 LTR CAT, pTh-1.111 LTR CAT, pTh-1.11 LTR CAT, pTh-1.152 LTR CAT and chimeric LTRs pTh-1.11x302 and Th-1.152x302. These plasmids all contain their designated LTR in the correct orientation to drive the CAT gene.

Optimization of Transfection Conditions.

β-galactosidase activity

The control plasmid pCH110 containing the β-galactosidase gene under control of SV40 promoter sequence was co-transfected with LTR CAT plasmids in all transfections. Levels of β-galactosidase activity in cell lysates were determined in order to compare transfection efficiency in both cell types. Results indicated that β-galactosidase activity in lysates from both ED cells and HMCs were at the limits of assay sensitivity (data not shown). In order to compare LTR directed promoter activity in different cell types, results were expressed as relative CAT activity. Relative CAT activity is the ratio of acetylated ^{14}C chloramphenicol in test lysates to acetylated ^{14}C chloramphenicol in lysates transfected with pCAT Control.

Transfections in equine dermal cells

ED cells were tested using calcium phosphate DNA precipitation and Transfectace™. Cells were seeded in 60 mm plastic tissue culture dishes at 1×10^6 cells per dish and allowed to attach overnight. The following day duplicate cultures were co-transfected with 10 μg of pMA-1 LTR CAT and 5 μg of pRS-Etat-M. CAT assays were performed on the lysates and results were expressed as the percent conversion of the non-acetyated form of chloramphenicol to mono- and di-acetylated forms (Figure 7). In replicate assays Transfectace™ resulted in percent conversions of 49.1% and 73.6% respectively while calcium phosphate

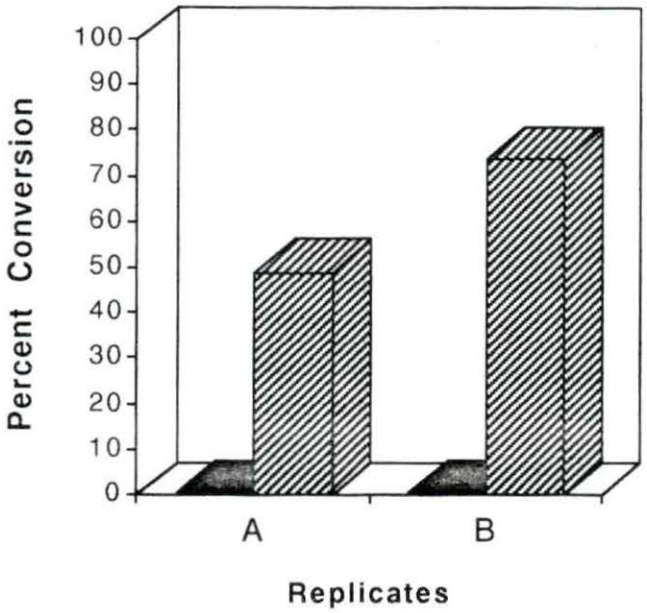


Figure 7. Comparisons of different transfection methods in ED cells. Replicate plates (A or B) were transfected with pMA-1 LTR CAT (10μg) and pRS-Etat-M (5μg) using $CaPO_4$ DNA precipitation (black bars) or Transfectace™ (hatched bars). Cells were harvested 40-48 hours post transfection, and assayed for CAT activity. Results are expressed as the percent of ^{14}C-chloramphenicol converted to acetylated forms.

DNA precipitation resulted in much lower percent conversion of 0.47% and 0.39%. Consequently all subsequent transfections with ED cells were performed using Transfectace™.

pRS-Etat-M is a potent transactivator of EIAV LTR. To detect slight variances in enhancer activity among the LTR CAT plasmids it was necessary to use limiting amounts of pRS-Etat-M. ED cells were co-transfected with 10 fold serial dilutions of pRS-Etat-M and 10 μg of pMA-1 LTR CAT or 10 μg pTh-1.111 LTR CAT. Results indicated that the linear range of transactivation occurred using between 1 and 10 ng of pRSEtat-M (Figure 8). Consequently all subsequent transfections with ED cells were done using 1 ng of pRS-Etat-M per transfection.

Transfections in horse macrophage cultures

Transfection efficiency in HMC was tested using calcium phosphate DNA precipitation, Transfectace™ and Lipofectin™. PBMC were seeded at $2x10^6$ cell per cm^2 in 60 mm tissue culture dishes. Macrophages were allowed to adhere overnight and non-adherent cell were washed away. Cells were transfected with either 10 μg or 30 μg of pMA-1 LTR CAT and 5 μg of pRS-Etat-M. Results were expressed as percent conversion of the non-acetyated form of chloramphenicol to mono- and di-acetylated forms (Figure 9). Results indicated that the calcium phosphate method of transfection gave the highest transfection efficiency in HMC. However, the overall level of CAT activity was 10 to 20 fold less than in ED cells.

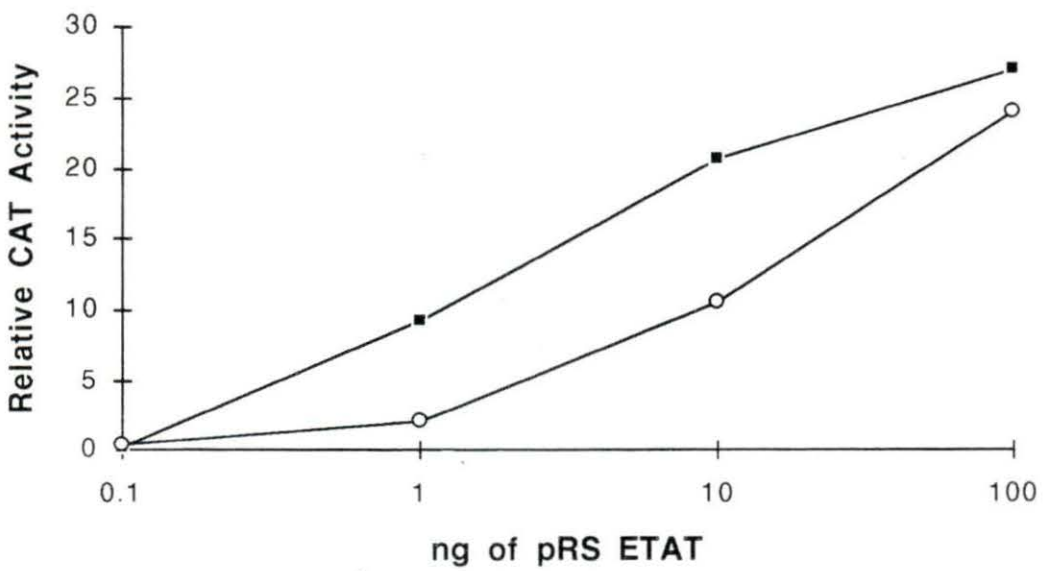


Figure 8. Titration of pRS-Etat-M in ED cells. ED cells were transfected with 10 fold serial dilutions of pRS-Etat-M and 10μg pMA-1 LTR CAT (closed squares) or pTh-1.111 LTR CAT (open circles). Cells were harvested 40-48 hours post transfection and assayed for CAT activity as described in text.

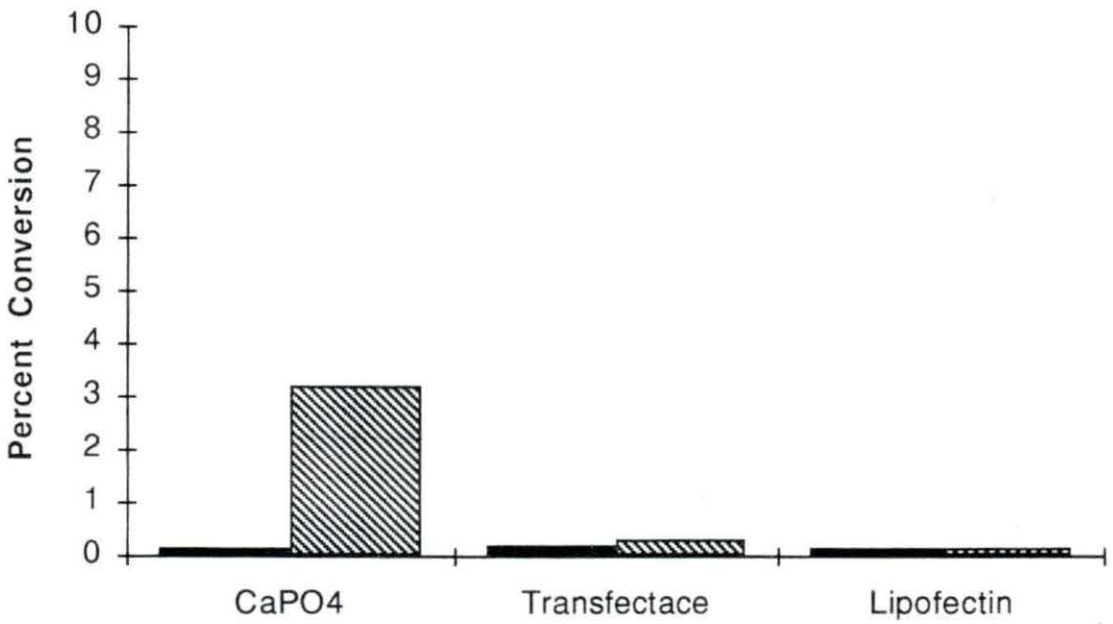


Figure 9. Comparison of different transfection methods in HMCs. Individual transfections were done with 10μg of pMA-1 LTR CAT and 5μg of pRS-Etat-M (black bars) or 30μg of pMA-1 LTR CAT and 10 μg of pRS-Etat-M (hatched bars). Cells were harvested 40-48 hours post transfection, and assayed for CAT activity. Results are expressed as the percent of ^{14}C-chloramphenicol converted to acetylated forms.

We observed 40 to 70% conversion of choramphenicol to mono- and in di-acetylated forms ED cells, and only 3% conversion in HMC.

Macrophage activation.

There are many reports in the literature describing retroviral LTR responsiveness to inducible or tissue specific transcription factors (14,24,56,72,91). To determine if the EIAV LTR was responsive to macrophage activation, transfected macrophages were stimulated with *E. coli* lipopolysacharride (5 μg/ml) and PMA (10 ng/ml) (Figure 10). Results of this experiment indicated that macrophages stimulated with PMA had significantly higher CAT activity than unstimulated and LPS-stimulated HMC. The increased CAT activity following PMA stimulation was highest in HMCs transfected with pTh-1.111 LTR CAT. In subsequent experiments, transfections were performed in non-stimulated and PMA stimulated macrophages to determine if inducible transcription factors may play a role in LTR function in macrophages.

CAT Activity of LTR CAT Constructs

CAT activity in ED cells

To test the promoter ability of the LTRs in ED cells, nearly confluent ED cells in 60 mm dishes were transfected with 10 μg pMA-1 LTR CAT, pTh-1.111 LTRCAT, pTh-1.11x302 LTR CAT or pTh-1.152x302 LTR CAT. Included in each transfection

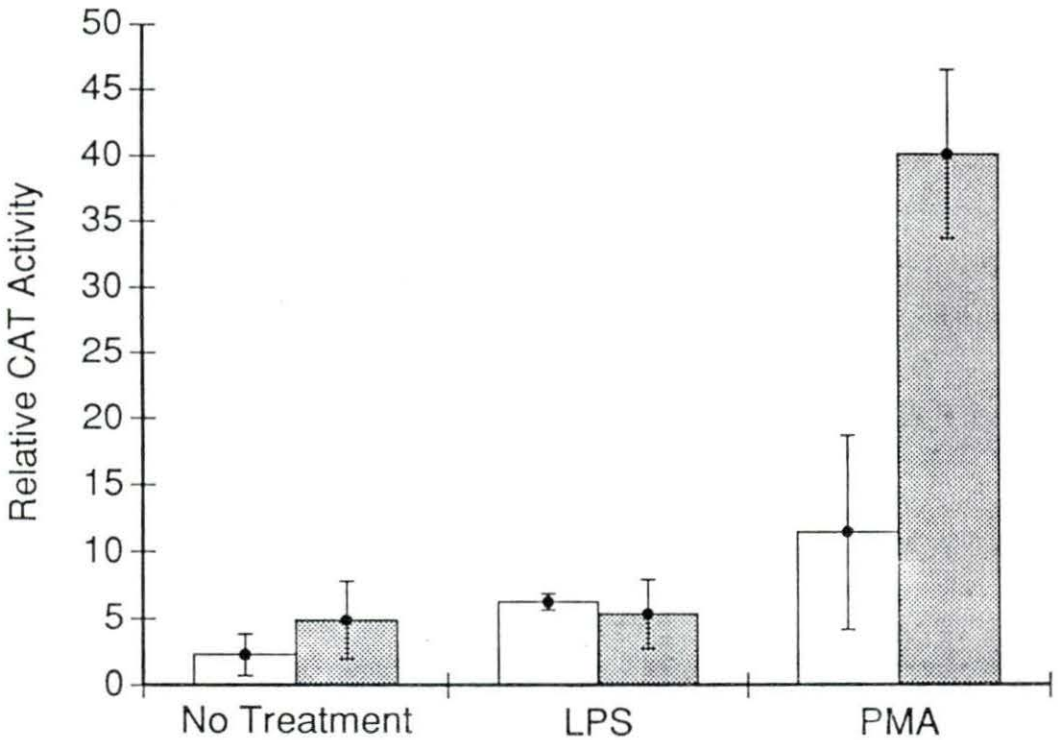


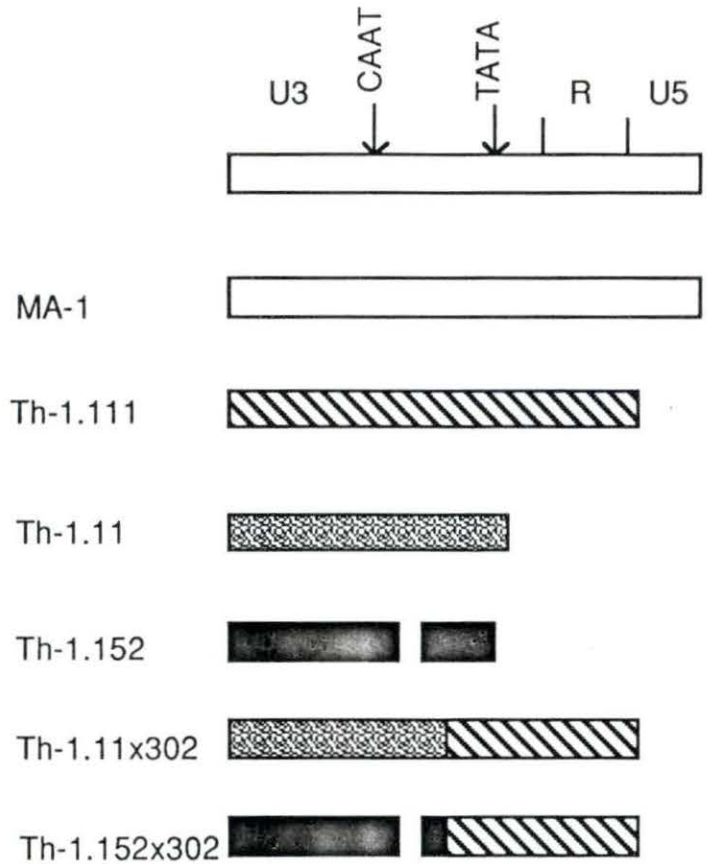
Figure 10. The effect of LPS and PMA on CAT activity in HMCs. Duplicate HMCs were co-transfected with 30μg pMA-1 LTR CAT (white bars) or pTh-1.111 LTR CAT (gray bars) and 15μg pRSEtat-M. Five hours post transfection HMC were stimulated with LPS (5μg/ml) or PMA (10ng/ml). Cells were harvested at 36 hours post stimulation and assayed for CAT activity as described in text.

was 1 ng of pRS-Etat-M and 5 μg of pCH110. Transfections were prepared in quadruplicate and lysates derived from each transfection were assayed twice, for a total of eight assays. CAT assay reactions were performed with 15 μl of lysate containing 40 to 65 ng of protein, incubated 1.5 hours at 37°C. Results were expressed as relative CAT activity compared to pCAT Control and statistically analyzed by analysis of variance.

Differences were observed between the LTRs in their ability to promote the CAT gene expression in ED cells (Figure 11). The LTRs could be separated into three statistically distinct groups, designated A, B, and C ($p<0.05$). Group A is the MA-1 LTR, group B is Th-1.111 and Th-1.11x302 LTRs, and group C is Th-1.152x302 LTR. The MA-1 LTR had the highest CAT activity in ED cells, Th-1.111 and Th-1.11x302 had intermediate activity, and Th1.152x302 had the lowest activity. The differences between group A and groups B and C cannot be entirely attributed to differences within the hypervariable region because the MA-1 LTR is 20 base pairs longer than the Th-1 LTRs. The difference between groups B and C can be attributed to the hypervariable region, in that the 14 base pair deletion within Th-1.152 significantly lowers CAT activity in ED cells.

CAT activity in equine macrophages

To test LTR promoter ability in equine macrophages, HMC were transfected with 30 μg pMA-1 LTR CAT, pTh-1.111 LTR CAT, pTh-1.11x302 LTR CAT and pTh-1.152x302 LTR CAT. Included in each transfection was 10 μg of pRS-Etat-M and 5 μg of pCH110. LTRs, pRS-Etat-M and pCH110 were transfected in triplicate



	Relative CAT Activity		
	ED Cells n=8	Macrophages No PMA n=9	Macrophages with PMA n=7
MA-1	8.19 ± 1.4^{a1}	3.02 ± 2.8^{2}	2.08 ± 2.5^{2}
Th-1.111	6.51 ± 1.9^{b1}	$1.35 \pm .52^{2}$	1.79 ± 1.3^{2}
Th-1.11	ND	ND	ND
Th-1.152	ND	ND	ND
Th-1.11x302	6.18 ± 1.2^{b1}	2.72 ± 2.7^{2}	4.49 ± 4.5
Th-1.152x302	4.46 ± 1.3^{c1}	2.98 ± 2.7	1.85 ± 1.1^{2}

Figure 11. Relative CAT activity of variant LTRs in ED cells, macrophages and macrophages stimulated with PMA. Letters indicate statistically different groups ($p < 0.05$) within a cell type. Numbers indicate statistical differences within a LTR across cell types.

in both PMA stimulated and non-stimulated HMCs and repeated three times, resulting in a total of nine transfections for each LTR CAT plasmid in both PMA stimulated and non-stimulated HMCs. CAT assays were performed with 85 μl of lysate containing 45 to 90 ng of protein. The reaction was incubated 4 hours and 1 μl of 80 mM acetyl Co A was added at the second and third hour of incubation. Because 85 μl of lysate was used for the CAT assay reaction, the lysates could only be analyzed once. Results in each experiment were expressed as relative CAT activity compared to pCAT Control and results of all experiments were analyzed statistically by analysis of variance.

When comparing LTRs within a group either in PMA stimulated or non-stimulated macrophages, no differences were seen in the ability of the LTRs to promote the CAT gene. When comparing differences between groups, i.e. PMA stimulated macrophages vs. non-stimulated macrophges, no differences were seen.

When comparing EDs as a group with either non-stimulated HMCs or PMA stimulated HMCs, the overall relative CAT activity was significantly higher in ED cells. Comparison of individual LTRs across cell types indicated that MA-1 and Th-1.111 had significantly higher CAT activity in ED cells than in either stimulated or non-stimulated HMCs. Th-1.11x302 had significantly higher CAT activity in ED cells than in non-stimulated HMCs, but not in stimulated HMCs. Th.152x302 had significantly higher CAT activity in ED cells than in stimulated HMCs, but not in

non-stimulated HMCs. On the whole, CAT activities were lower in macrophages than ED cells.

DISCUSSION

Equine infections anemia virus causes a variable disease in horses that may manifest itself in an acute, chronic or inapparent form. Molecular determinants of virulence or cell tropism have not been well characterized for EIAV, but studies with other retroviruses such as HIV and murine leukemia virus (14,16,27,29,54) suggest that the LTR may play a role. In the present study, LTR sequences from two closely related strains of EIAV were functionally characterized to examine the role of the LTR in EIAV pathogenesis. The Th-1 strain is a macrophage-trophic, virulent field isolate of EIAV (7). MA-1 is an avirulent isolate derived from Th-1 by multiple passage on ED cells. Comparison of MA-1 and Th-1 LTR sequences indicated that the majority of the differences were clustered in a hypervariable region between the CAAT and TATA boxes (9). The Th-1 and MA-1 LTRs were tested in transient expression assays to determine if these sequence differences played a role in the differences seen in the biological characteristics of the two virus strains. Results indicated that the differences in cell tropism between MA-1 and Th-1 does not map to the hypervariable region in the LTR.

Four LTRs were used, one from the MA-1 strain of virus and three from the Th-1 virus strain. The MA-1 LTR (322 bp) was excised from pUC 19 by *Hin*dIII digestion, isolated by electroelution and subcloned into the transient expression vector pCAT Basic. The Th-1 LTRs (Th-1.111, Th-1.11 and Th-1.152), were excised from pGEM by *Xba*I digestion and subcloned into pCAT Basic. The

structure of all LTR CAT plasmids was verified by restriction enzyme digestion, Southern blot hybridization, and DNA sequencing.

The Th-1 LTRs Th-1.11 and Th-1.152 have 3' deletions which resulted in the loss of the TATA box and the TAR region. Utilizing a unique *Af*III site within all of the LTRs, chimerics were constructed, thus isolating differences among Th-1 LTRs to those solely within the hypervariable region. These constructs were again verified by restriction enzyme digestion, Southern blot hybridization, and DNA sequencing.

The LTR CAT constructs were tested in ED cells and HMCs, and transfection conditions were optimized for both cell types. Three types of transfection methods were tested: calcium phosphate DNA precipitation, and the cationic lipid formations Transfectace™ and Lipofectin™. Results indicated that Transfectace™ gave the highest transfection efficiency in ED cells and calcium phosphate DNA precipitation gave the highest transfection efficiency in HMCs.

To further optimize CAT activity, plasmid pRS-Etat-M, containing the EIAV *tat* gene, was co-transfected with the LTR CAT plasmids. In ED cells, it was found that the strong *trans*-activating ability of Tat was masking subtle enhancer differences within the LTRs. Therefore, the amount of pRS Etat-M was reduced to 1 ng per transfection. This was not necessary in HMC due to their inherently lower CAT activity.

The MA-1 LTR, which was derived from an ED adapted strain of EIAV, had the highest CAT activity of all the LTRs in ED cells. Th-1.111 and Th-1.11x302 had

intermediate CAT activity, and Th-1.152x302 had the lowest CAT activity. The Th-1 LTRs were all derived from a macrophage trophic field strain of EIAV, and these results may indicate that cell tropism maps to the EIAV LTR. However, MA-1 LTR is 20 bp longer than the Th-1 LTRs, and it is not possible to ascribe the differences in CAT activity solely to differences within the hypervariable region. Furthermore Th-1.11x302 and MA-1 are identical in the hypervariable region and differ only in the extra 20 base pairs at the 3' end of MA-1. Therefore, the differences in CAT activity seen between these two LTRs indicate that the 20 base pairs at the 3' end of the MA-1 LTR has an effect on LTR activation in ED cells. In addition, there was no difference in CAT activity between Th-1.111 and Th-1.11x302, indicating that the 4 base pair substitution in the hypervariable region did not alter promoter activity in ED cells. Together, these results suggested that the hypervariable region did not play a role in ED cell tropism.

The hypervariable region was found to affect the overall promoter activity of the EIAV LTR in ED cells. When comparing group B to group C, one can ascribe the lower CAT activity to differences in the hypervariable region, in as much as a deletion in this region significantly lowered CAT activity in ED cells. Further experiments in ED cells would include the construction of chimeric LTRs between the 3' end of the MA-1 LTR and the 5' ends of Th-1.11 and Th-1.152 in order to limit variation to the hypervariable region.

No statistically significant differences were observed among LTRs in horse macrophage cultures. Furthermore, no differences were observed between PMA

stimulated and non-stimulated cells. Stimulation of macrophages with PMA or LPS did not increase LTR driven promoter activity. Either PMA did not induce transcription factors in HMCs or the LTRs did not have the binding sites for the induced transcription factors. In all cases, CAT activity in HMCs was significantly lower than CAT activity in ED cells. The lower level of CAT activity in macrophages may indicate that these cells are poor initiators of EIAV LTR driven transcription. It is also possible that transfection efficiency in macrophages was much lower than in ED cells. However, we were unable to determine comparative transfection efficiency because levels of β galactosidase in each cell type were below the sensitivity of the assay.

There are a number of different ways to interpret the results in HMCs. There are many technical difficulties encountered in working with a primary cell culture. Each time an experiment was run, macrophages were collected from a different horse, and a detailed medical history was not available. The presence of subclinical infections or undue stress which may have had an effect on the results are factors that cannot be ruled out. In addition, a number of different horses were used throughout the experiments, and it was observed that the macrophages from different horses had varying levels of activity with the majority having low activity. Perhaps this parallels what is seen in natural EIA infections where the majority of infected animals are subclinical carriers. This suggests that there may be genetic predisposition for disease. Another possible variable is the presence of mixed populations of macrophages within a culture. Cells within a single culture were

observed to be morphologically heterogeneous, and different populations of macrophages may vary in responsiveness to stimulation with PMA or permissiveness for viral replication.

Steps that were taken to control for variation included standardization of cell lysates with respect to protein concentration. Suggestions for additional experiments would include quantitation of tumor necrosis factor and interleukin 1 in HMC supernatants in order to assess levels of macrophage activation. Collection of cells from a single horse may control for horse to horse variability. Another possibility would be to use a continuous macrophage cell line, but unfortunately there is no equine macrophage cell line available at this time. Although there are human and murine macrophage cell lines available, this adds another level of separation from the actual *in vivo* system.

Our results indicated that the LTR is not a determinate of cell tropism for EIAV. Although studies on murine leukemia virus and Friend leukemia virus have shown that determinants of cell tropism reside within the LTR (14,16,27,29,54), studies on HIV indicate that the envelope gene contains the determinants of cell tropism (23,59). This may reflect the differences between lentiviruses and oncoviruses. In another study performed in our laboratory, it was shown that the Th-1 isolate of EIAV consists a heterogeneous mixture of genotypes which differ in the S3 and envelope sequence (1). This seems to parallel occurrences in HIV, but the function of the EIAV heterogeneity in these regions are unknown at this time.

Genomic and antigenic variation have long been recognized characteristics of EIAV. Variation occurs from febrile cycle to febrile cycle (45,51,73,78,79), and recently, genetic variation has been shown to be present at a single time point (1,9). The biological function of variant genotypes is unknown. One study showed that a number of isolates of EIAV were Rev defective suggesting that defective genotypes may be an important mechanism of viral latency (1). It is possible that variation also results in dysfunctional, or poorly functional LTRs also as a mechanism of viral latency. Perhaps only a very small population of virus genotypes exists in which LTRs are strong activators of the initiation of transcription. If so, the LTRs examined in the present study may not wholly reflect the genotypes present *in vivo*.

In summary the hypervariable region does affect transcriptional activity in ED cells, but does not appear to play a major role in determining EIAV cell tropism *in vitro*. However, additional studies of LTR transcriptional activity in HMC are needed to minimize variability and optimize transfection efficiency. Further characterization of variant LTRs may shed light on the role of the LTR in regulation of viral gene expression and persistence.

LITERATURE CITED

1. Alexandersen, S. and S. Carpenter. 1991. Characterization of variable regions in the envelope and S3 open reading frame of equine infectious anemia virus. J. Virol. **65**:4255-4262.

2. Anand, R., F. Siegal, C. Reed, R. Cheng, S. Floilenza, and J. Moore. 1987. Non-cytocidal natural variants of human immunodeficiency virus isolated from AIDS patients with neurological disorders. Lancet **ii**:234-238.

3. Anginiard, 1859. Observations de contagion par infection de l'anhemie idiopathique du cheval. Rapport de la commission. Rec. Med. Vet. **36**:639.

4. Archer, B.G., T. B. Crawford, T. C. McGuire, and M. E. Frazier. 1977. RNA-dependent DNA polymerase associated with equine infectious anemia virus. J. Virol. **22**:16-22.

5. Arya, S.K., C. Guo, S. F. Josephs, and F. Wong-Staal. 1985. *trans*-Activator gene of human T-lymphotropic virus type III (HTLV-III). Science **229**:69-73.

6. Benjamin, L. 1990. Genes IV. Oxford University Press, Oxford.

7. Benn, S., R. Rutledge, T. Folks, J. Gold, L. Baker, J. McCormick, P. Feorino, P. Piot, T. Quinn, and M. Martin. 1985. Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire. Science **230**:949-951.

8. Bothe, K., A. Aguzzi, H. Lassmann, A. Rethwilm, and I. Horak. 1991. Progressive encephalopathy and myopathy in transgenic mice expressing human foamy virus genes. Science **253**:255-257.

9. Carpenter, S., S. Alexandersen, M. J. Long, S. Perryman, and B. Chesebro. 1991. Identification of a hypervariable region in the long terminal repeat of equine infectious anemia virus. J. Virol. **65**:1605-1610.

10. Carpenter, S. and B. Chesebro. 1989. Change in host cell tropism associated with in vitro replication of equine infectious anemia virus. J. Virol. **63**:2492-2496.

11. Carpenter, S.L. 1985. A quantitative study of the development of neutralizing antibodies to two isolates of equine infectious anemia virus. Deptartment of Veterinary and Animal Science. University of Massachusetts., Amerst, Mass.

12. Carre, H. and H. Vallee. 1904. Sur l'anemie infectieuse du cheval. Compt. Rend. Acad. Sci. **139**:1239.

13. Carvalho, M. and D. Derse. 1991. Mutational analysis of the equine infectious anemia virus *tat*-responsive element. J. Virol. **65**:3468-3474.

14. Celander, D. and W. A. Haseltine. 1984. Tissue-specific transcription preference as a determinant of cell tropism and leukaemogenic potential of murine retroviruses. Nature **312**:159-162.

15. Chang, D.D. and P. A. Sharp. 1990. Messenger RNA transport and HIV *rev* regulation. Science **249**:614-615.

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

17. Cheevers, W.P., B. G. Archer, and T. B. Crawford. 1977. Characterization of RNA from equine infectious anemia virus. J. Virol. **24**:489-497.

18. Cheevers, W.P. and T. C. McGuire. 1985. Equine infectious anemia virus: immunopathogenesis and persistence. Rev. Infect. Dis. **7**:83-88.

19. Chiodi, F., A. Valentin, B. Keys, S. Schwartz, B. Åsjö, S. Gartner, M. Popovic, J. Albert, V. -A. Sundqvist, and E. -M. Fenyö. 1989. Biological characterization of paired human immunodeficiency virus type 1 isolates from blood and cerebrospinal fluid. Virology **173**:178-187.

20. Clements, J.E. 1985. Hypothesis on the molecular basis of nononcogenic retroviral diseases. Rev. Infect. Dis. **79**:68-74.

21. Coffin, J.M. 1990. Retroviridae and Their Replication, p.1437-1500. In B.M. Fields and D.M. Knipe (eds.), Fields Virology. Raven Press, New York.

22. Coffin, J.M. 1991. Retroviridae and their replication, p.645-708. In B.N. Fields and D.N. Knipe (eds.), Fundamental Virology. Raven Press, New York.

23. Collman, R., B. Godfrey, J. Cutilli, A. Rhodes, N. F. Hassan, R. Sweet, S. D. Douglas, H. Friedman, N. Nathanson, and F. Gonzalez-Scarano. 1990. Macrophage-tropic strains of human immunodeficiency virus type 1 utilize the CD4 receptor. J. Virol. **64**:4468-4476.

24. Cullen, B.R. and W. C. Greene. 1989. Regulatory pathways governing HIV-1 replication. Cell **58**:423-426.

25. Cullen, B.R. and W. C. Greene. 1990. Functions of the auxiliary gene products of the human immunodeficiency virus type 1. Virology **178**:1-5.

26. Dahlberg, J.E. 1988. An overview of retrovirus replication and classification. Adv. Vet. Sci. Comp. Med. **32**:1-35.

27. Dai, H.Y., M. Etzerodt, A. J. Baekgaard, S. Lovmand, P. Jorgensen, N. O. Kjeldgaard, and F. S. Pedersen. 1990. Multiple sequence elements in the U3 region of the leukemogenic murine retrovirus SL3-2 contribute to cell-dependent gene expression. Virology **175**:581-585.

28. Derse, D., P. L. Dorn, L. Levy, R. M. Stephens, N. R. Rice, and J. W. Casey. 1987. Characterization of equine infectious anemia long terminal repeat. J. Virol. **61**:743-747.

29. DesGroseillers, L. and P. Jolicoeur. 1984. The tandem direct repeats within the long terminal repeat of murine leukemia viruses are the primary determinant of their leukemogenic potential. J. Virol. **52**:945-952.

30. Dingwall, C., I. Ernberg, M. J. Gait, S. M. Green, S. Heaphy, J. Karn, A. D. Lowe, M. Singh, M. A. Skinner, and R. Valerio. 1989. Human

immunodeficiency virus 1 *tat* protein binds *trans*-activation responsive region (TAR) RNA *in vitro*. Proc. Natl. Acad. Sci. USA **86**:6925-6929.

31. Dorn, P., L. DaSilva, L. Martarano, and D. Derse. 1990. Equine infectious anemia virus *tat*: Insights into the structure, function, and evolution of lentivirus *trans*-activator proteins. J. Virol. **64**:1616-1624.

32. Dorn, P.L. and D. Derse. 1988. cis- and trans-acting regulation of gene expression of equine infectious anemia virus. J. Virol. **62**:3522-3626.

33. Dreguss, M.N. and L.S. Lombard. 1954. Experimental studies in equine infectious anemia. University of Pennsylvania Press, Philadelphia.

34. Dynan, W.S. 1989. Modularity in promoters and enhancers. Cell **58**:1-4.

35. Fisher, A.G., B. Ensoli, D. Looney, A. Rose, R. C. Gallo, M. S. Saag, G. M. Shaw, B. H. Hahn, and F. Wong-Staal. 1988. Biologically diverse molecular variants within a single HIV-1 isolate. Nature **334**:444-447.

36. Garrett, E.D., L. S. Tiley, and B. R. Cullen. 1991. Rev activates expression of the human immunodeficiency virus type 1 *vif* and *vpr* gene products. J. Virol. **65**:1653-1657.

37. Gonda, M.A., H. P. Charman, J. L. Walker, and L. Coggins. 1978. Scanning and transmission electron microscopic study of equine infectious anemia virus. Am. J. Vet. Res. **39**:731-740.

38. Gorman, C.M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard. 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated tranfection. Proc. Natl. Acad. Sci. USA **79**:6777-6781.

39. Guy, B., M. P. Kieny, Y. Riviere, C. LePeuch, K. Dott, M. Girard, L. Montagnier, and J. P. Lecocq. 1987. HIV F/3' orf encodes a phosphorylated GTP-binding protein resembling an oncogene product. Nature **330**:266-269.

40. Haase, A.T. 1986. Pathogenesis of lentivirus infections. Nature **322**:130-136.

41. Hanahan, D. 1983. Studies on transformation of *E.coli* with plasmids. J. Mol. Biol. **166**:557-580.

42. Hanly, S.M., L. T. Rimsky, M. H. Malim, J. H. Kim, J. Hauber, M. D. Dodon, S. -Y. Le, J. V. Maizel, B. R. Cullen, and W. C. Greene. 1989. Comparative

analysis of the HTLV-I Rex and HIV-1 Rev *trans*-regulatory proteins and their RNA response elements. Genes Dev. **3**:1534-1544.

43. Hess, J. L., J. A. Small, and J. E. Clements. 1989. Sequences in the visna virus long terminal repeat that control transcriptional activity and respond to viral *trans*-activation: Involvement of AP-1 sites in basal activity and *trans*-activation. J. Virol. **63**:3001-3015.

44. Ho, D.D., T. R. Rota, and R. T. Schooley. 1985. Isolation of HTLV III from cerebrospinal fluid and neural tissues of patients with neurologic syndromes related to the acquired immunodeficiency syndrome. N. Engl. J. Med. **313**:1493-1497.

45. Hussain, K.A., C. J. Issel, K. L. Schnorr, P. M. Rwambo, and R. C. Montelaro. 1987. Antigenic analysis of equine infectious anemia virus (EIAV) variants by using monoclonal antibodies: epitopes of glycoprotein gp90 of EIAV stimulate neutralizing antibodies. J. Virol. **61**:2956-2961.

46. Issel, C.J. and L. Coggins. 1979. Equine infectious anemia: current knowledge. J. Am. Vet. Med. Assoc. **174**:727-733.

47. Issel, C.J. and L. D. Foil. 1984. Studies on equine infectious anemia virus transmission by insects. JAVMA **184**:293-297.

48. Jacks, T. and H. E. Varmus. 1985. Expression of the Rous sarcoma virus pol gene by ribosomal frameshifting. Science **230**:1237-1242.

49. Jones, K.A., J. R. Kadonaga, P. A. Luciw, and R. Tjian. 1986. Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. Science **232**:755-758.

50. Kono, Y. 1972. Recurrences of equine infectious anemia. Proc. 3rd Conf. Equine Infect. Dis. 175-186.

51. Kono, Y., K. Kobayashi, and Y. Fukunaga. 1973. Antigenic drift of equine infectious anemia virus in chronically infected horses. Arch. Ges. Virus. Forsch. **41**:1-10.

52. Koyanaoi, Y., S. Miles, R. T. Mitsuyasu, J. E. Merric, H. V. Vinters, and I. S. Y. Chen. 1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science **236**:819-822.

53. LaThangue, N.B. and Peter W.J. Rigby. 1988. Trans-acting protein factors and the regulation of eukaryotic transcription, p.1-32. In B.D. Hames and D.M. Glover (eds.), Transcription and Splicing. IRL Press, Washington D.C.

54. Lenz, J., D. Celander, R. L. Crowther, R. Patarca, D. W. Perkins, and W. A. Haseltine. 1984. Determination of the leukaemogenicity of a murine retrovirus by sequences within the long terminal repeat. Nature **308**:467-470.

55. Levy, J.A., J. Shimabukuro, H. Hollander, J. Mills, and L. Kaminsky. 1991. Isolation of AIDS-associated retroviruses from cerebrospinal fluid and brain of patients with neurological symptoms. Lancet **85**:586-588.

56. Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins. 1987. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. J. Virol. **61**:693-700.

57. Lieider, J.M., P. Palese, and F. I. Smith. 1988. Determination of the mutation ratio of a retrovirus. J. Virol. **62**:3084-3091.

58. Lignee, M. 1843. Memoire et observation sur une maladie de sang, connue sous le nom d'anhemie hydrohemie, cachexie aqueuse du cheval. Rec. Med. Vet. **20**:30.

59. Liu, Z., C. Wood, J. A. Levy, and C. Cheng-Mayer. 1990. The viral envelope gene is involved in macrophage tropism of human immunodeficiency virus type 1 strain isolated from brain tissue. J. Virol. **64**:6148-6153.

60. Lowy, D.R. 1986. Transformation and oncogenesis: retroviruses, p.235-263. In B.N. Fields and D.M. Knipe (eds.), Fundamental Virology. Raven Press, New York.

61. Malim, M.H., R. Fenrick, D. W. Ballard, J. Hauber, E. Böhnlein, and B. R. Cullen. 1989. Functional characterization of a complex protein-DNA-binding domain located within the human immunodeficiency virus type 1 long terminal repeat leader region. J. Virol. **63**:3213-3219.

62. Malim, M.H., J. Hauber, R. Fenrick, and B. R. Cullen. 1988. Immunodeficiency virus rev trans-activator modulates the expression of the viral regulatory genes. Nature **335**:181-183.

63. Malim, M.H., J. Hauber, S. Le, J. V. Maizel, and B. R. Cullen. 1989. The HIV-1 *rev trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature **338**:254-257.

64. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor.

65. Maniatis, T., S. Goodbourn, and J. A. Fischer. 1987. Regulation of inducible and tissue-specific gene expression. Science **236**:1237-1244.

66. Marriott, S.J. and J. N. Brady. 1989. Enhancer function in viral and cellular gene regulation. Biochim. Biophys. Acta **989**:97-110.

67. McGuire, T.C., T. B. Crawford, and J. B. Henson. 1972. Equine infectious anemia: Detection of infectious virus-antibody complexes in the serum. Immunol. Commun. **1**:545-551.

68. McGuire, T.C. and J. B. Henson. 1973. Equine infectious anemia - pathogenesis of persistant viral infection. Perspectives in Virology **8**:229-247.

69. McGuire, T.C., K. O'Rourke, and W. P. Cheevers. 1987. A review of antigenic variation by equine infectious anemia virus. Contrib. Microbiol. Immunol. **8**:77-89.

70. Meyerhans, A., R. Cheynier, J. Albert, M. Seth, J. Kwok, L. Sninsky, L. Morfeldt-Manson, B. Asjö, and S. Wain-Hobson. 1989. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolation. Cell **58**:901-910.

71. Murphy, J.J., M. Tracz, and J. D. Norton. 1990. Patterns of nuclear proto-oncogene expression during induced differentiation and proliferation of human B chronic lymphocytic leukaemia cells. Immunology **69**:490-493.

72. Nabel, G. and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T-cells. Nature **326**:711-713.

73. Narayan, O. and J. E. Clements. 1989. Biology and pathogenesis of lentiviruses. J. Gen. Virol. **70**:1617-1639.

74. Noiman, S., A. Yaniv, L. Sherman, S. R. Tronick, and A. Gazit. 1990. Pattern of transcription of the genome of equine infectious anemia virus. J. Virol. **64**:1839-1843.

75. Noiman, S., A. Yaniv, T. Tsach, T. Miki, S. R. Tronick, and A. Gazit. 1991. The Tat protein of equine infectious anemia virus is encoded by at least three types of transcripts. Virology **184**:521-530.

76. Parrott, C., T. Seidner, E. Duh, J. Leonard, T. S. Theodore, A. Buckler-White, M. A. Martin, and A. B. Rabson. 1991. Variable role of the long terminal repeat Sp1-binding sites in human immunodeficiency virus replication in T lymphocytes. J. Virol. **65**:1414-1419.

77. Payne, S., B. Parekh, R. C. Montelaro, and C. J. Issel. 1984. Genomic alterations associated with persistent infections by equine infectious anemia virus, a retrovirus. J. Gen. Virol. **65**:1395-1399.

78. Payne, S.L., F. -D. Fang, C. -P. Liu, B. R. Dhruva, P. Rwambo, C. J. Issel, and R. C. Montelaro. 1987. Antigenic variation and lentivirus persistence: variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. Virology **161**:321-331.

79. Payne, S.L., K. Rushlow, B. R. Dhruva, C. J. Issel, and R. C. Montelaro. 1989. Localization of conserved and variable antigenic domains of equine infectious anemia virus envelope glycoproteins using recombinant

env-encoded protein fragments produced in *Escherichia coli*. Virology **172**:609-615.

80. Pepinsky, R.B. and V. M. Vogt. 1984. Fine-structure analysis of lipid-protein protein-protein interactions of *gag* protein p19 of the avian sarcoma and leukemia viruses by cyanogen bromide mapping. J. Virol. **52**:145-153.

81. Rasty, S., B. R. Dhruva, R. L. Schiltz, D. S. Shih, C. J. Issel, and R. C. Montelaro. 1990. Proviral DNA integration and transcriptional patterns of equine infectious anemia virus during persistent and cytopathic infections. J. Virol. **64**:86-95.

82. Rice, N.R., L. E. Henderson, R. C. Swoder, T. D. Copeland, S. Oroszlan, and J. F. Edwards. 1990. Synthesis and processing of the transmembrane envelope protein of equine infectious anemia virus. J. Virol. **64**:3770-3778.

83. Roberts, J.D., K. Bebenek, and T. A. Kunkel. 1988. The accuracy of reverse transcriptase from HIV-1. Science **242**:1171-1173.

84. Rohdewohld.H, H. Weiher, W. Reik, R. Jaenisch, and M. Breindl. 1987. Retrovirus integration and chromatin structure: Moloney murine leukemia

proviral intergration sites map near DNase I-hypersensitive sites. J. Virol. **61**:336-343.

85. Rushlow, K., K. Olsen, G. Steigler, S. L. Payne, R. C. Montelaro, and C. J. Issel. 1986. Lentivirus genome organization: the complete nucleotide sequence of the env gene region of equine infectious anemia virus. Virology **155**:309-321.

86. Sakai, K., S. Dewhurst, X. Ma, and D. J. Volsky. 1988. Differences in cytopathogenicity and host cell range among infectious molecular clones of human immunodeficiency virus type 1 simultaneously isolated from an individual. J. Virol. **62**:4078-4085.

87. Salinovich, O., S. L. Payne, R. C. Montelaro, K. A. Hussain, C. J. Issel, and K. L. Schnorr. 1986. Rapid emergence of novel antigenic and genetic variants of equine infectious anemia virus during persistent infection. J. Virol. **57**:71-80.

88. Sawadogo, M. and A. Sentenac. 1990. RNA polymerase B (II) and general transcription factors. Annu. Rev. Biochem. **59**:711-754.

89. Shen, D. T., J. R. Gorham, J. B. Henson, T. C. McGuire, and T. B. Crawford. 1972. The natural history of equine infectious anemia. Western Vet. **1**:35-44.

90. Sherman, L., A. Yaniv, H. Lichtman-Pleban, S. R. Tronick, and A. Gazit. 1989. Analysis of regulatory elements of the equine infectious anemia virus and caprine arthritis-encephalitis virus long terminal repeats. J. Virol. **63**:4925-4931.

91. Short, M. K., S. A. Okenquist, and J. Lenz. 1987. Correlation of leukemogenic potential of murine retroviruses with transcriptional tissue preference of the viral long terminal repeat. J. Virol. **61**:1067-1072.

92. Sodroski, J., C. A. Rosen, F. Wong-Staal, S. Z. Salahuddin, M. Popovic, S. Arya, R. C. Gallo, and W. A. Haseltine. 1985. Transacting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. Science **227**:171-173.

93. Stephens, R. M., J. W. Casey, and N. R. Rice. 1986. Equine infectious anemia virus *gag* and *pol* genes: relatedness to visna and AIDS virus. Science **231**:589-594.

94. Stephens, R. M., D. Derse, and N. R. Rice. 1990. Cloning and characterization of cDNAs encoding equine infectious anemia *tat* and putative *rev* proteins. J. Virol. **64**:3716-3725.

95. Struhl, K. 1991. Mechanisms for diversity in gene expression patterns. Neuron **7**:177-181.

96. Temin, H. M. 1981. Structure, variation and synthesis of retrovirus long terminal repeat. Cell **27**:1-3.

97. Varmus, H. E. 1982. Form and function of retroviral proviruses. Science **216**:812-820.

98. Vijaya, S., D. L. Steffen, C. Kozak, and H.L. Robinson. 1987. Dsi-I, a region with frequent proviral insertions in Moloney murine leukemia virus induced rat thymomas. J. Virol. **61**:1164-1170.

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

100. Zhang, J., K. Logan, X. -B. Yu, E. A. Davis, M. Posner, and S. Ackerman. 1990. Early events of RNA polymerase II transcription initiation. Life Sci. **46**:729-735.

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