ABSTRACT

JIA, BIN. Role of the Cytoplasmic Tail of Equine Infectious Anemia Virus Transmembrane Glycoprotein in Acute Disease Induction. (Under the direction of Dr. Frederick J. Fuller.)

Equine infectious anemia virus (EIAV) is a macrophage-tropic lentivirus of horses. EIAV is unique among lentiviruses in that a further cleavage event occurs within the N-terminus of the cytoplasmic tail (CT) of transmembrane (TM) glycoprotein and yields a C-terminal non-glycosylated p20 protein. The p20 comprises more than two-third of the CT domain and contains both of the amphipathic α-helices.

To test the role of the EIAV CT domain in acute disease induction, we constructed a p20-truncated clone (p19/wenv17Δp20) on the background of a highly virulent EIAV infectious clone p19/wenv17 by introducing three termination codons into the N-terminal coding region of p20. The derived virus replicated at a delayed and lower level compared with that of parental virus in equine macrophages *in vitro*. *In vivo,* the p19/wenv17Δp20 virus showed attenuation and did not induce acute disease like the parental (p19/wenv17) virus. The viral load in ponies infected by p19/wenv17Δp20 virus was about 10-1000 fold lower than that of ponies infected by parental (p19/wenv17) virus. *In vitro* studies on the properties of the p20-truncated virus showed that truncation of the p20 did not impair the envelope glycoprotein incorporation into virions. There was also no severe defect in virus replication. The delayed and lower level replication of p20-truncated virus compared with parental virus was most probably due to small delays in several steps in the virus life cycle. In addition, p20 expressed in *trans* could not compensate for the absence of p20 in the p20-truncated virus.

ROLE OF THE CYTOPLASMIC TAIL OF EQUINE INFECTIOUS ANEMIA VIRUS TRANSMEMBRANE GLYCOPROTEIN IN ACUTE DISEASE INDUCTION

by

BIN JIA

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APPROVED BY:

Barbara Sherry, PhD	**Scott M. Laster, PhD**
Wayne A. Tompkins, PhD	**Frederick J. Fuller, PhD**
	Chair of Advisory Committee

BIOGRAPHY

I was born in Harbin, the northeastern part of China. I attended high school in Harbin from 1982 to 1988, and graduated from the Biotechnology Department of North-East Agriculture University in Harbin, China with a BS in 1992. From 1992 to 1997, I worked as a research assistant in Harbin Veterinary Research Institute, the Chinese Academy of Agriculture Sciences. From 1998 to 2000, I worked as a visiting researcher in the National Center for AIDS Prevention & Control of the Chinese Academy of Prevention Medicine in Beijing. In 2001, I began a graduate program of Comparative Biomedical Sciences at the College of Veterinary Medicine, North Carolina State University.

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LITERATURE REVIEW

A: EQUINE INFECTIOUS ANEMIA VIRUS (EIAV)

1. Overview of Equine Infectious Anemia (EIA)

Equine infectious anemia virus (EIAV) is a macrophage-tropic lentivirus of the retroviridae family and the causal agent of the disease named equine infectious anemia (EIA) (68, 91). This infectious disease was first described as early as 1843 and was the first animal disease proven to be caused by a filterable agent (10, 11, 55). EIAV only infects members of the equidae family and there is no evidence that it can replicate in other species. Instead of inducing a slow progressive disease as other lentiviruses, EIAV infection causes a rapid disease progression with a variable course including acute, chronic and asymptomatic (inapparent carriers) disease phases (39). After initial exposure to virulent virus strains, infected horses undergo an acute phase disease with a short duration between one week to a month represented by fever, thrombocytopenia and high-titer plasma viremia. Most infected horses can survive the acute disease phase and progress to the chronic or persistent phase, which is characterized by periodic episodes of clinical disease (39). Chronic EIA is considered a period of dynamic interaction between host and virus and recurrent episodes are correlated with emergence of antigenically variant viruses (66, 68). The chronic stage generally lasts for 6 to 12 months, during which time most infected horses progress to an inapparent carrier stage without clinical signs that is mediated by establishment of strict immunologic control over virus replication (figure 1) (74).

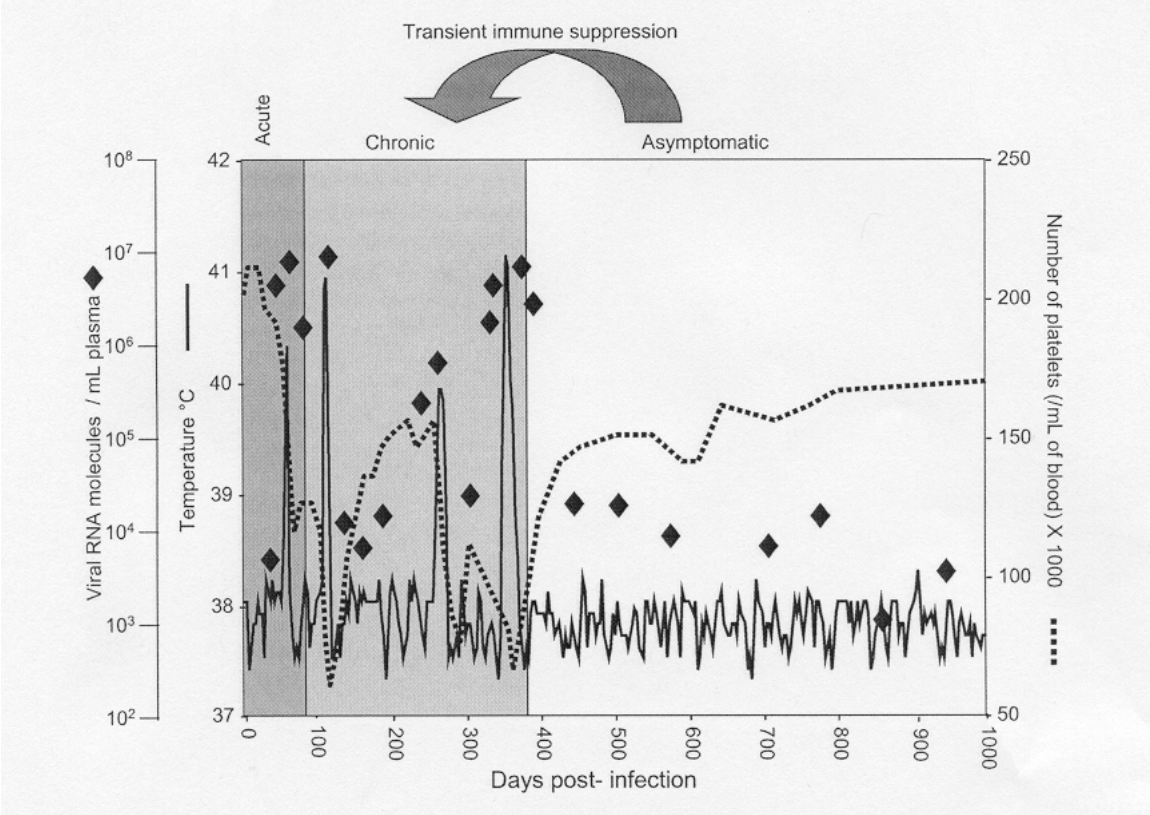


Figure 1. Clinical and virological profiles of ponies experimentally infected with EIAV. Febrile episodes are defined by a rectal temperature (—) above 39°C in conjunction with a reduction of the platelets (••••) below 100,000/µl of whole blood. Viral RNA load in the plasma is indicated by (♦). EIA may be divided into three phases: acute, chronic, and asymptomatic. As indicated by the grey arrow on top, asymptomatic animals may go back into chronic disease following naturally or experimentally-induced immune suppression. (Leroux, 2004)

Transmission of EIAV is mainly mediated through transfer of infected blood or body fluids to susceptible recipients mediated by insect vectors such as the stable fly or horsefly (40, 98). Virus can also be transmitted iatrogenically and *in utero* infection can occur. Thrombocytopenia is the earliest and most consistent laboratory abnormality observed in febrile horses (17). Anemia is primarily attributed to the reduced span of erythrocytes resulting from both intravascular and extravascular hemolysis due to immune mediated responses to viral infection (61). Besides this, EIAV is able to directly bind to red blood cells and activate complement-mediated lysis (88). Antibodies to EIAV are also able to incite complement binding to erythrocytes (78). Equine leukocytes are able to phagocytose red blood cells that are covered in complement and virus, which may contribute to anemia (89). In addition, bone marrow suppression can also induce anemia (61).

EIAV infection does not result in severe immune deficiency. Many of the observed clinical symptoms are a direct result of the immune response to viral infection (61). Febrile episodes are strictly correlated with a high level of viremia, which are a direct result of extensive replication of virus within the tissue macrophages (50, 68, 80). During the inapparent carrier phase, only a low level of viremia can be detected with no clinical signs (18, 31, 39).

2. Cellular and humoral immune response:

During the acute phase of EIAV infection, the initial CTL response contributes to the clearance of the primary viremia (31, 62, 73, 85). Epitopes most frequently recognized by CTLs are mainly located on the matrix protein (p15) and viral core protein

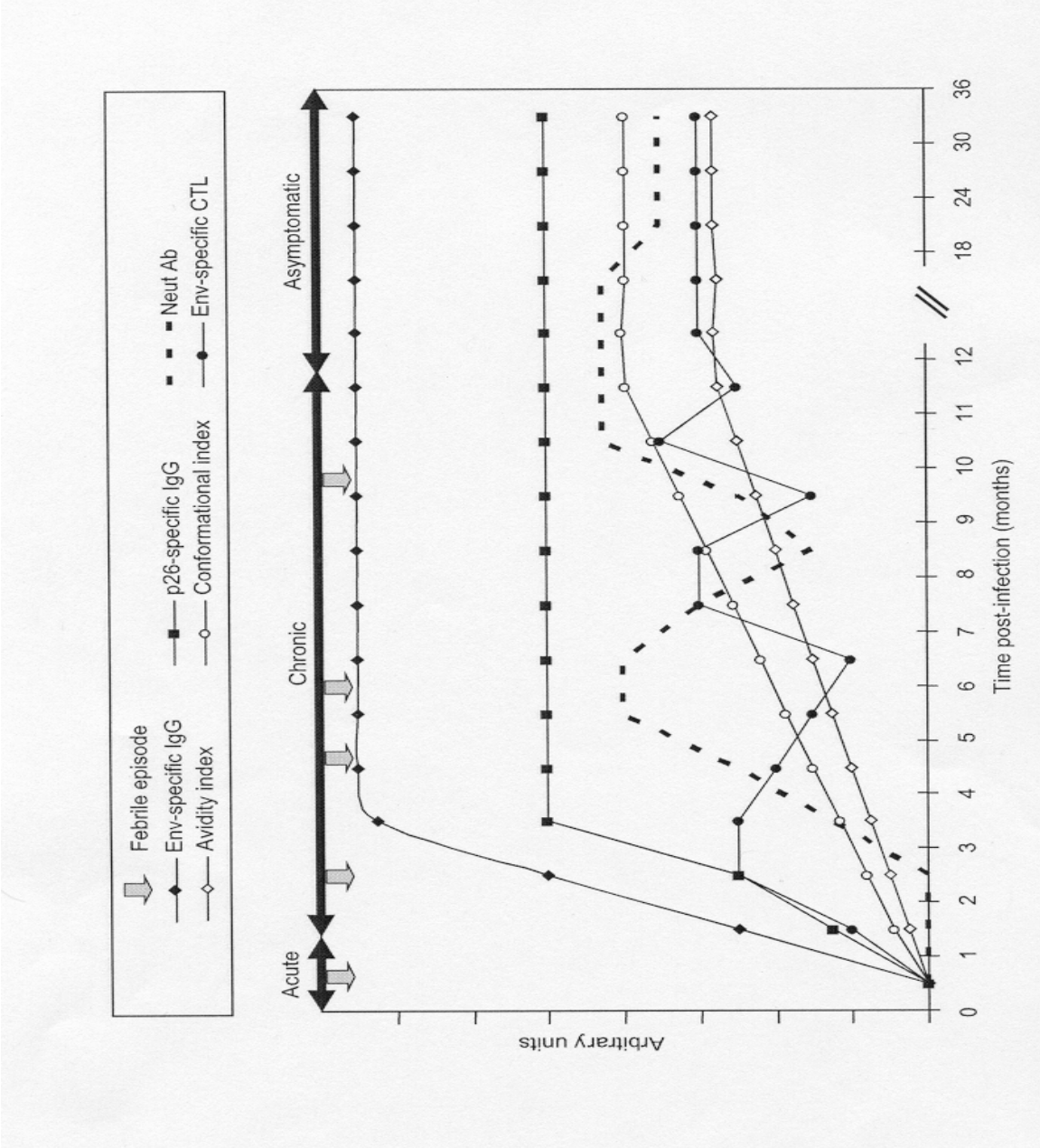


Figure 2: Maturation of the immune response in EIAV infected animals. The figure represents the evolution of EIAV-specific antibody (Env-specific IgG and p26 specific IgG and the EIAV-specific cellular response (Env-specific CTL).During the first ten months of infection, the antibody evolves gradually from a population characterized by low-avidity, non-neutralizing and predominately linear epitope specificity to an antibody population characterized by moderate to high-avidity, neutralizing and predominately conformational epitope specificity. Neutralizing antibodies appear only after 2 to 3 months of infection. Immune parameters are expressed in arbitrary units according to the time post infection. (Leroux, 2004).

(p26). Surface unit (SU) protein, the middle third of pol protein, transmembrane protein (TM) and S2 also contain identified CTL epitopes (63). Non-neutralizing, EIAV-specific antibodies can be detected at 7 to 10 days after infection with those to the envelope surface subunit (gp90) occurring first and followed by antibodies to core protein (p26). Antibodies to the TM protein are found at 3 to 4 weeks post infection (15, 67).

EIAV antibodies with neutralizing activity mainly specific for conformational epitopes, occur within 6 to 8 month after infection, which occurs well past the acute disease phase (30). Antibody-dependent cellular cytotoxicity (ADCC) is not present during the chronic disease phase (105).

In persistently infected ponies, high levels of CTL activity and neutralizing antibodies are present (31, 63). Suppression of the immune system of the ponies in inapparent disease phase with corticosteroid or cyclophosphamide may induce reoccurrence of clinical symptoms, which suggests the disease is under temporal immune control (47). After adoptive transfer of EIAV stimulated lymphocytes (derived from persistently infected half-sibling donors) to healthy severe combined immunodeficiency (SCID) Arabian foals, which are sensitive to EIAV infection with persistent high-level viremia, EIAV specific CTL and neutralizing antibodies can be induced after challenge. This provides further evidence that a specific immune response is required for termination of viremia in acute infection (64) (Figure 2).

3. EIAV structure, genomic organization and gene products

EIAV belongs to the family of retroviridae and the subgroup lentivirus. The virus particle includes a dense cone-shaped core and surface spike with a diameter of about 140 nm and shares the same ultrastructure as other lentivirus (26,106). The spike protein

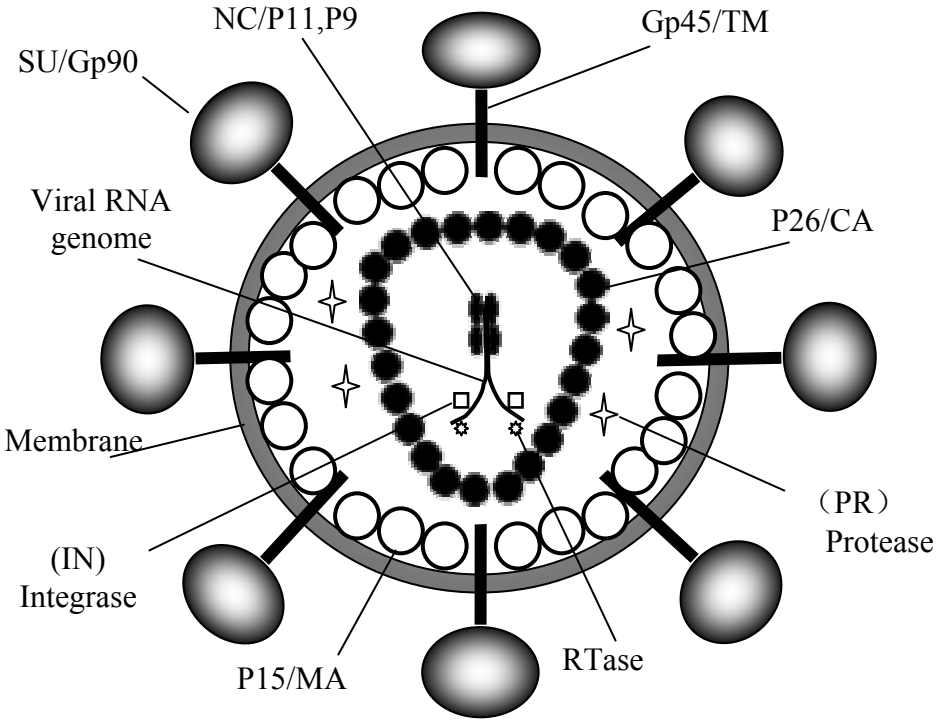


Figure 3: Schematic structure of the EIAV virion.

contains two glycoprotein subunits, the surface (SU) and transmembrane (TM) proteins (36). The viral core is formed by four non-glycosylated structure proteins including p15 (matrix), p16 (capsid), p11 (nucleocapid) and p9 (function in late assembly) (33, 80). The virion also contains four enzymes required for synthesis of viral DNA and integration of viral DNA into the host genome including protease, reverse transcriptase, integrase and dUTPase (7, 49). The genome contains two identical single-stranded RNA molecules that are closely associated with the nucleocapid p11 (13). Each RNA molecule has a 5' cap and 3' poly A tail. In addition, each virus particle also contains a few molecules of lys3 tRNA, which is used as primer for reverse transcriptase (49) (figure 3).

EIAV has the typical genomic organization of all lentiviruses (103). The full-length of the genome is about 8.2kb and it is flanked by 5' and 3' long terminal repeats (LTRs). From 5' to 3', it contains three structural genes *gag, pol* and *env*. There are also three shorter open reading frames in the genome named S1 (*tat*), S2, and S3 (*rev*), which encode regulatory proteins (1, 68) (figure 4). In this regard, the EIAV genome is simpler than HIV, which includes six regulatory or accessory proteins (Tat, Rev, Vif, Vpr, Vpu and Nef) (25).

4. EIAV individual gene products

a) Gag

The *gag* gene encodes proteins required for the generation of the virus particles and therefore plays a key role in virus assembly and budding. In the cytoplasm of the infected cells, Gag polyproteins are synthesized from the genome-length mRNA. The *gag* and *pol* genes are overlapped and it has been demonstrated that when ribosomes translate the

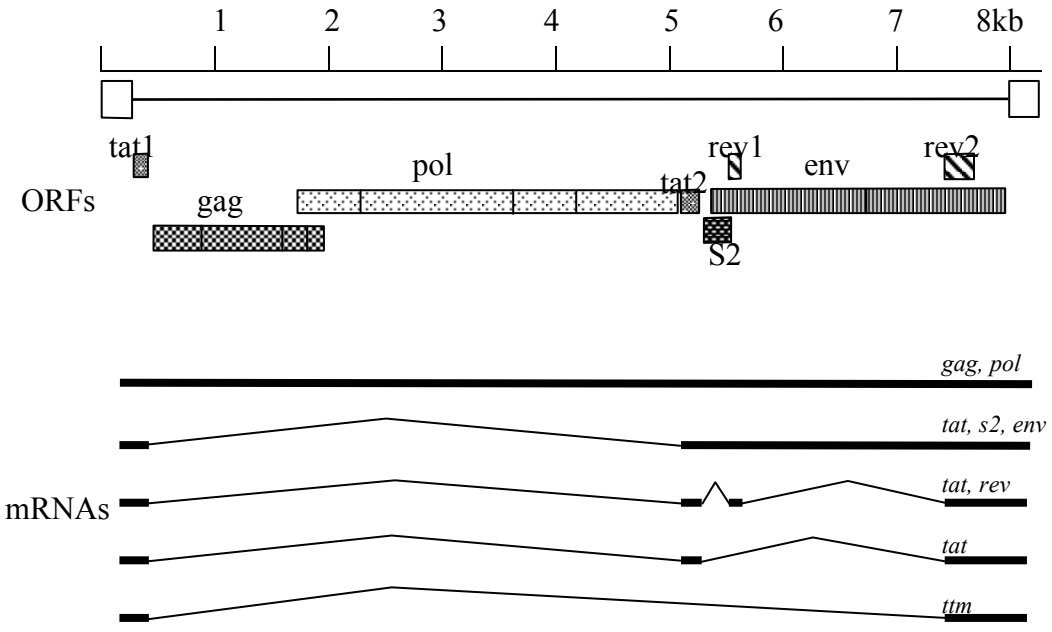


Figure 4: Schematic diagram of provirus genomic structure and transcription products of EIAV.

unspliced genomic RNA of retroviruses, 95% of translation yields Gag proteins while only about 5% of translation yields Gag-Pol proteins through –1 ribosome frame-shifting (102). For EIAV, it was reported recently that the frame-shifting efficiency and viral replication are dependent on a "slippery" sequence, a five-base-paired GC stretch and a pseudoknot structure (14). The Gag precursor protein will undergo further cleavage to form the above-mentioned four non-glycosylated structure proteins of EIAV. MA (matrix) or p15 localizes at the inner surface of the envelop lipid bilayer and is responsible for proper assembly of virus particles. The capsid protein (CA) p26 forms the virion core and is most often used as a diagnostic antigen (18). The NC (nucleocapsid) protein, p11 associates with the viral RNA genome as its name implies. The p9 protein is known now to facilitate release of the budding virus particle from the host cell plasma membrane through a YPDL motif as a late assembly domain (L domain) (80).

b) Pol

The *pol* gene encodes enzymes responsible for the synthesis of viral DNA and its further integration into the host cell genome (69). Protease (PR) mainly cleaves Gag and Gag-Pol precursors that produce mature Gag and Pol proteins during virion maturation (43, 99). Reverse transcriptase (RT) synthesizes proviral DNA from the viral RNA genome and RNase H degrades RNA from RNA/DNA hybrids formed during virus replication (44, 49). The integrase is responsible for the integration of proviral DNA into the host genome (39). The enzyme dUTPase is necessary for efficient viral replication in primary equine macrophage cultures by preventing uracil incorporation into the viral DNA (54, 76, 93, 97,104). The enzyme dUTPase is only present in EIAV, feline immunodeficiency virus (FIV), visna virus and caprine arthritis-encephalitis virus

(CAEV) and is not possessed by HIV, SIV and bovine immunodeficiency virus (BIV) (19,86,96).

c) Env

The *env* gene encodes the Env precursor protein of 135kd and is highly glycosylated (84). The precursor protein undergoes further cleavage mediated by cellular proteases and yields a surface unit protein (SU) gp90 and a transmembrane protein (TM) gp45. The two proteins are nonconvalently joined and most likely exist as a trimeric complex. Together they mediate viral budding and penetration into the host cells (35). The surface protein contains the putative receptor-binding domain and the major epitopes recognized by neutralizing antibodies (38). A transmembrane domain within the TM subunit anchors the nonconvalently associated SU/TM complex (oligomeric env) to the cell membrane, where incorporation into budding virions can occur. EIAV is also unique among all lentivirus in that a further cleavage event occurs at the carboxyl terminus of gp45 and yields a non-glycosylated 20kDa protein, designed p20, with about 175 amino acids. Amino acid sequencing of the N-terminal p20 shows that the cleavage occurs at a His/Leu site (87).

d) S1 (Tat), S2 and S3 (Rev)

Tat is required for virus replication because it controls gene expression via trans activation of the viral LTR (20, 70, 87). The Tat protein functions by binding the U3 region of LTR as well as the tat-response domain (TAR) located in the R region of the LTR (83, 94, 99).

The EIAV *rev* gene is essential for expression of structural protein genes and the production of new virus. The Rev protein contains a nuclear export signal (NES) that

works by mediating the nucleocytoplasmic transport of partially spliced viral mRNAs encoding viral structure proteins by binding with its cis-acting rev response element (RRE) (EIAV is thought to have 2 RREs) (3,32,65). In addition to regulating viral mRNA transport, EIAV Rev can also induce alternative splicing of EIAV mRNA *in vivo* (28, 65). The binding site for EIAV Rev and cellular splicing factors has been mapped to a 55-nucleotide purine rich exonic element (16). The function of S2 is unclear now and current knowledge has shown that it is not essential for virus replication *in vitro* but is required for disease expression *in vivo* (52, 53).

5. EIAV cell tropism

EIAV predominantly infects monocytes and macrophages (90). *In vivo*, endothelial cells are also infected during acute infection (71). Viral replication in experimentally infected ponies mainly occurs within the spleen and liver while in asymptomatic animals, viral DNA and RNA persist at low levels in the spleen, liver, lymph node, bone marrow, peripheral blood mononuclear cells (PBMC) and kidney (67,72,90,92). *In vitro*, different strains of EIAV exhibit different cell tropism. Besides equine macrophages, several laboratory adapted EIAV strains can infect and replicate in feline embryonic cells (FEA), canine osteosarcoma cells (D17), equine dermal fibroblasts (EDC), canine thymoma cells (CfT2h), equine endothelial cells, and canine macrophage cell lines (DH82) (4,34,45,46,59). On the other hand, replication of the Wyoming strain (Wy), a highly virulent wild type strain of EIAV, is restricted to equine macrophages in which it replicates to high titer without loss of virulence. LTR sequences of Wy strains have been showed to contribute to the restriction (75). The LTR regions affect both cellular tropism and virulence due to the hypervariable enhancer region (8, 56, 57, 58, 60, 77). In this

aspect, it is different from HIV, where the LTR does not appear to determine cell tropism (79).

6. EIAV replication

a) Virus entry into cells

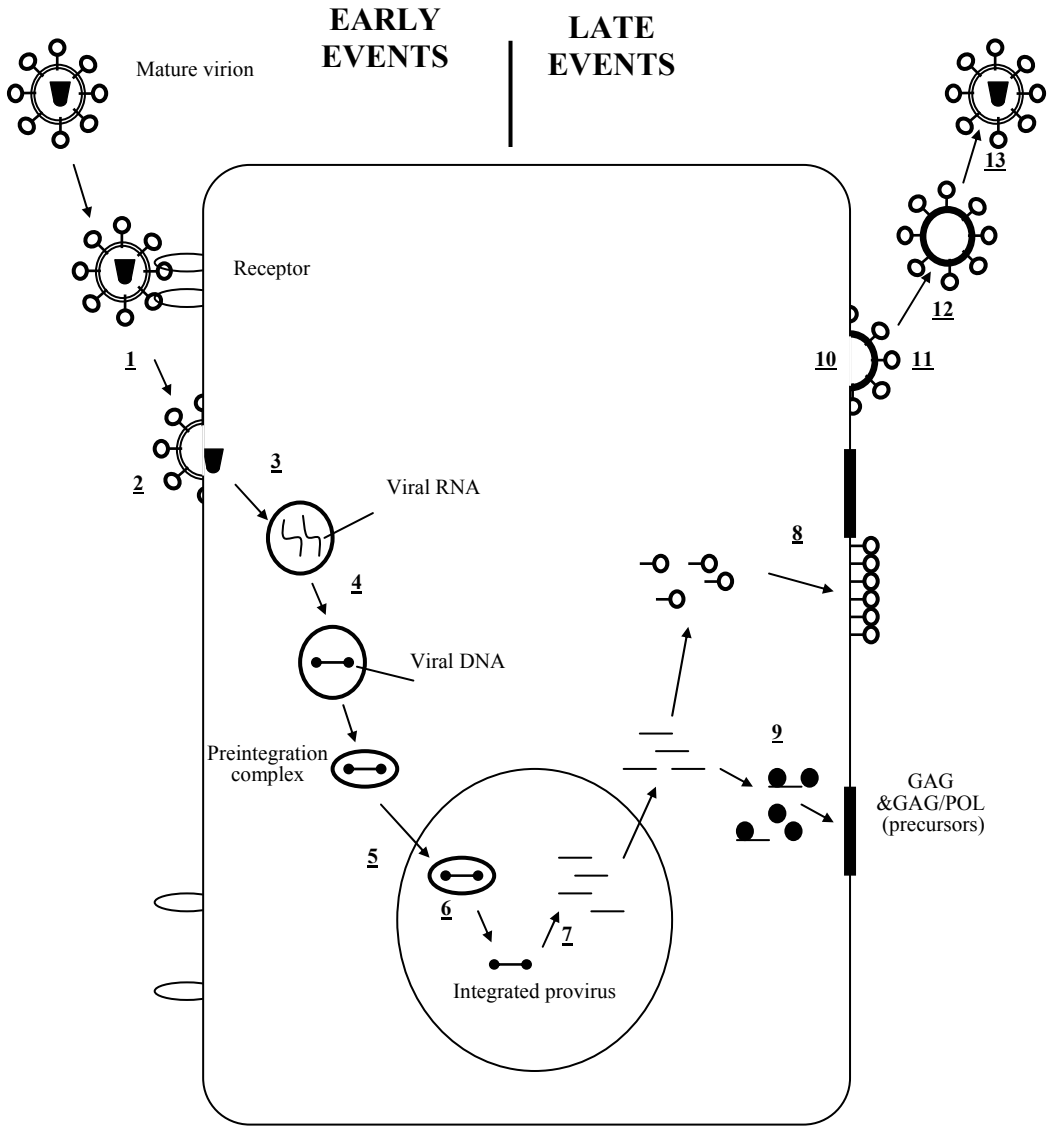
A cellular receptor for EIAV has not been identified. A putative receptor-binding sequence has been described in the variable domain of the surface glycoprotein gp90 (68). This region has a limited sequence homology, but an extensive structural similarity to the CD4 binding domain of the HIV-1 gp120 (48). EIAV does not induce syncytia formation in any of the known permissive cells and cellular entry is considered to utilize an endocytic entry mechanism to infect cells (29,107). Following entry, the viral genome is uncoated to prepare for the initiation of viral replication. The mechanism and factors involved in uncoating of EIAV are not currently known.

b) Reverse transcription and integration

After entering the cytoplasm, the following steps of EIAV replication are similar to all the other lentiviruses. Initiation of reverse transcription occurs in the cytoplasm and the final product, a double-stranded linear DNA proviral intermediate is then actively transported into the nucleus, where it is randomly integrated into the genome of host cells (21). The efficiency of integration among different lentiviruses varies and whether or not additional viral and cellular factors are required is still unknown (101).

c) Transcription and translation

Transcription of viral RNA is controlled by the enhancer and promoter elements located in the hypervariable U3 region of the LTR (54). The U3 region contains binding



1. BINDING
2. MEMBRANE FUSION
3. UNCOATING
4. REVERSE TRANSCRIPTION
5. NUCLEAR IMPORT
6. INTEGRATION
7. TRANSCRIPTION
8. ENV TRANSPORT
9. GAG TRANSPORT
10. MEMBRANE CURVE
11. ENV INCORPORATION
12. BUDDING /RELEASE
13. VIRON MATURATION

Figure 5: Schematic representation of the EIAV life cycle (Zhou, 2001).

sites for cellular transcription factors belong to the MDBP, PEA2, AP-1, and *ets* families (9). A full-length genomic RNA is first generated from the 5' LTR U3-R junction to the poly A site upstream of the 3'LTR (Figure 4). This full-length RNA is used as new viral genome and will be packaged into virions later. It also serves as the genomic mRNA for synthesizing the Gag and Pol polyproteins. A single cleavage event is required to separate the Gag-Pol precursor protein and the Env precursor (19). This cleavage is mediated by the virus-encoded protease, whereas subsequent envelope polyprotein cleavage is mediated by cellular proteases. In HIV, this cleavage occurs in the late Golgi compartment mediated by furin and is required for expression of envelope glycoproteins that will function in cell-to-cell or virus to cell interaction (5, 22). The enzyme responsible for the SU-TM cleavage in EIAV is still unknown.

At least three-splice donor and three-splice acceptor sites have been identified involved in mRNA splicing of EIAV in infected cells (50). The first donor site is located immediately upstream from the start of the gag gene. It is used for the generation of all the multiply spliced transcripts described so far and represents the end of the first exon of *tat*. The second exon of *tat* is located between *pol* and *env*. Tat may be expressed from at least three different transcripts, in which all require protein synthesis initiation from a non-AUG codon (20). The *rev* gene can be transcribed from two exons, both situated in the *env* gene on an alternative reading frame. The S2 protein can be expressed from a tricistronic (*tat*/S2/*env*) and /or biscistronic (S2/*env*) transcripts (Figure 4). Besides Tat, S2, and Rev, a 27kda protein of unknown function that is located in the Golgi and endoplasmic reticulum has been found *in vitro* named truncated transmembrane protein (Ttm). The *ttm* gene is composed part of the first exon of *tat* (N-terminal 29 amino acids)

and a portion of the 3' terminal region of the transmembrane protein (218 amino acids) (2). Immediately downstream of the transmembrane anchor domain is a splice acceptor site for *ttm* (2).

d) Assembly, budding and release

Assembly and budding of C-type retroviral particles are a complex process that requires the coordination of the processing of the required components through the intracellular transport pathway and targeting to the plasma membrane. The Gag protein drives particle assembly and both Gag-Pol and Env polyproteins are required for the formation of infectious particles (37, 41, 42,102). Both virus assembly and budding occur at the host plasma membrane and this is different from type B and D retroviruses that preassemble viral cores in the cytoplasm (102).

The detailed mechanism of EIAV assembly and release is still poorly understood. A recent finding is that p9 is known now to facilitate release of the budding virus particle from the host cell plasma membrane through a YPDL motif as a late assembly domain (L domain) (80). As a type C retrovirus, EIAV follows the general process as other lentiviruses. Two copies of single-stranded unspliced genomic RNA are packaged into the virion with the help of certain regions on Gag which can recognized the viral RNA and a package signal (*psi*), located between the 5'UTR and the *gag* gene, is thought to mediate this event (51). A zinc-finger nucleic acid-binding motif at the 3'end of the Gag protein has been shown to mediate the specific interaction between viral RNA and capsid protein (68). The Gag and Gag-Pol precursor proteins are synthesized on free ribosomes and transported through the cytoplasm, either individually or as small aggregates. In HIV-1, the targeting of the capsid precursors to the plasma membrane is mediated in part

by the myristylated N-terminal glycine residue of the MA protein (6, 23). For EIAV, MA does not contain myristic acid (33).

The precursor Env proteins are synthesized on membrane-bound ribosomes and are transported through the secretory pathway of the cell (37). They are then transported to the Golgi where the attached oligosaccharides are processed and the cleavage between gp90 (SU) and gp45 TM occurs. Following the processing, they will localize to the plasma membrane and eventually be incorporated into the plasma membrane for budding (37). Electron microscopy studies for primate lentiviruses show the newly budded particles have an immature morphology of a doughnut-shaped appearance containing Gag proteins located around the periphery of the particle just beneath the plasma membrane-derived lipid envelop (24). The viral protease then becomes activated and accomplishes the formation of a centrally located cone-shaped viral cone by cutting the peripheral proteins (24).

The N-terminal region of EIAV Gag p9 contains a late assembly domain (L) that is required for efficient budding of Gag (80). EIAV p9 contains a YPXL domain that is critical for late domain function, which is distinct from all other lentiviruses with a conserved PTAP sequence (12, 28, 35, 80). The EIAV YPXL domain of p9 binds to the medium chain (AP-50) of the plasma membrane localized AP-2 clathrin-associated adapter protein complex (81). This YPXL domain is not interchangeable with the PTAP as well as the PPPY (late assembly domain used by oncoretroviruses) because they do not bind to AP-50 (81). This may imply that EIAV is different from other lentiviruses and retroviruses by utilizing different components of cellular processing pathways to accomplish the common assembly step of particle formation.

Once the nucleocapid complex is formed, it interacts with the regions of the plasma membrane that have incorporated high concentrations of viral Env proteins and immature virus particles can be released by budding (37). Maturation of the virus particles, which is required for infectivity, is a process involving activation of the viral protease and cleavage activity of the Gag-Pol precursor proteins (97). Mature virions are recognized as typical conically shaped condensed cores by electron microscope (106).

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Function of the Cytoplasmic Tail of Lentivirus Transmembrane Protein

1. Organization and structure of lentivirus cytoplasmic tail

Lentiviruses belong to a subgroup of the Retroviridae family and currently have seven members including human immunodeficiency virus (HIV-1, HIV-2), simian immunodeficiency virus (SIV), maedi-visna virus (MVV), caprine arthritis-encephalitis virus (CAEV), feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV) (18). These viruses share similarities both in virion morphology and in genome organization (18). The genomes of replication-competent lentiviruses range in size from 8 to 12 kb and from the 5'- to 3'-end, encode *gag* (for group-specific antigen), *pol* (for polymerase), and *env* (for envelope glycoprotein) genes. In addition to the three structural genes, lentiviruses also encode regulatory/accessory proteins such as Tat which is critical for efficient transcription activation from viral LTR and Rev which influences viral mRNA splicing and/or transport. The number of regulatory/accessory genes varies among each lentivirus from the minimum of 3 genes in EIAV and maximum of 6 genes in HIV-1 (48). The Gag and Pol are initially synthesized together as a precursor protein and then separated by a cleavage event to form Gag $Pr55^{gag}$ precursor polyprotein. The synthesis of this Gag-Pol polyprotein requires ribosomes to shift translational reading frame once or twice in a –1 direction to read through the stop codon in the gag open reading frame. The Gag precursor proteins are required for the generation of the virus particles and therefore play a key role in virus assembly and budding. This precursor will be cleaved by the viral protease (PR) to form the matrix (MA), capsid (CA), nucleocapid (NC) and a small C terminal protein during virus maturation. The precursor of Pol will also be further cleaved by the viral protease (PR) to form individual enzymes as protease

(PR), reverse transcriptase (RT), and integrase (IN), which are responsible for the synthesis of viral DNA and its further integration into the host cell genome (96). For EIAV, FIV, CAEV and MVV, there is a dUTPase (DU) encoded between RT and IN (38, 104). The *env* gene encodes a precursor protein, which is highly glycosylated (121). Unlike the Gag and Pol precursors, which are cleaved by the viral PR, the Env precursor will be further processed by a cellular protease during Env trafficking to the cell surface. In HIV, this cleavage occurs in the late Golgi compartment mediated by furin and is required for expression of functional envelope glycoprotein that will function in cell-to-cell or virus to cell interaction. This processing results in the generation of the surface (SU) Env glycoprotein and the transmembrane (TM) glycoprotein (10, 41). The structure of TM proteins of lentiviruses is highly conserved (66). This protein contains an amino terminal hydrophobic fusion peptide, an extracellular domain, a hydrophobic membrane anchor domain, and a carboxy terminal intracytoplasmic domain (66, 88,102). This carboxy terminal intracytoplasmic domain is commonly called cytoplasmic tail (CT) (Figure 6). More recent studies revealed that a region of the C-terminal tail of the HIV-1 gp41 contains a neutralizing epitope. Based on the fact that antibody does not cross lipid membranes and infectious virus is by definition intact, the author predicted that part of the C-terminal tail of gp41 containing that epitope was looped back through the viral membrane to the exterior in free virions, or that it was normally hidden and was exposed intermittently (26).

A major structure difference between the TM glycoproteins of lentiviruses and oncoviruses is in the length of the cytoplasmic tail (CT). Although presented in all

retroviruses, the CT is consistently longer in lentiviruses than in oncoviruses (47). With the exception of FIV, the cytoplasmic tail of all lentiviruses is over 120 amino acids in length. For primate lentiviruses including HIV-1, HIV-2 and SIV, it is about 150-200 residues long and for EIAV, it is 226 residues (75) (Figure 7). In sharp contrast, the cytoplasmic tail of the TM glycoprotein in the oncogenic retroviruses such as Mason-Pfizer monkey virus (MPMV), Rous sarcoma virus (RSV), feline leukemia virus (FeLV), and mouse mammary tumor virus (MMTV) is between 32 and 43 amino acids in length (47).

For primate lentiviruses and EIAV, the CT domain contains conserved amphipathic alpha helices near their carboxyl terminus (75,125) (figure 8). These helices are positively charged, arginine-rich and highly hydrophobic (125). They are often classified as surface-seeking or membrane-associate sequences, and in lentiviruses have been referred to as lentivirus lytic peptides (LLP) due to their ability to bind and disturb lipid bilayers (88,125). These structures are similar to antimicrobial peptides like cecropins (insect) and magainins (amphibians) but share no sequence homology (59). Peptides representing the amphipathic alpha helical regions of HIV have been found to alter membrane ionic permeability and induce cytolytic effects on both prokaryotic and eukaryotic cells (90,137). These helices in HIV-1 have also been shown to bind cellular calmodulin resulting in the inhibition of normal calmodulin-regulated signal transduction pathways (3). Interruption of this pathway may contribute to T-cell anergy in HIV patients (18). In addition, these helices have been confirmed to cause apoptosis and necrosis *in vitro* in a concentration-dependent manner (110). A region of the C-terminal tail of the HIV-1 gp41 has been found to contain a neutralizing epitope. This provides

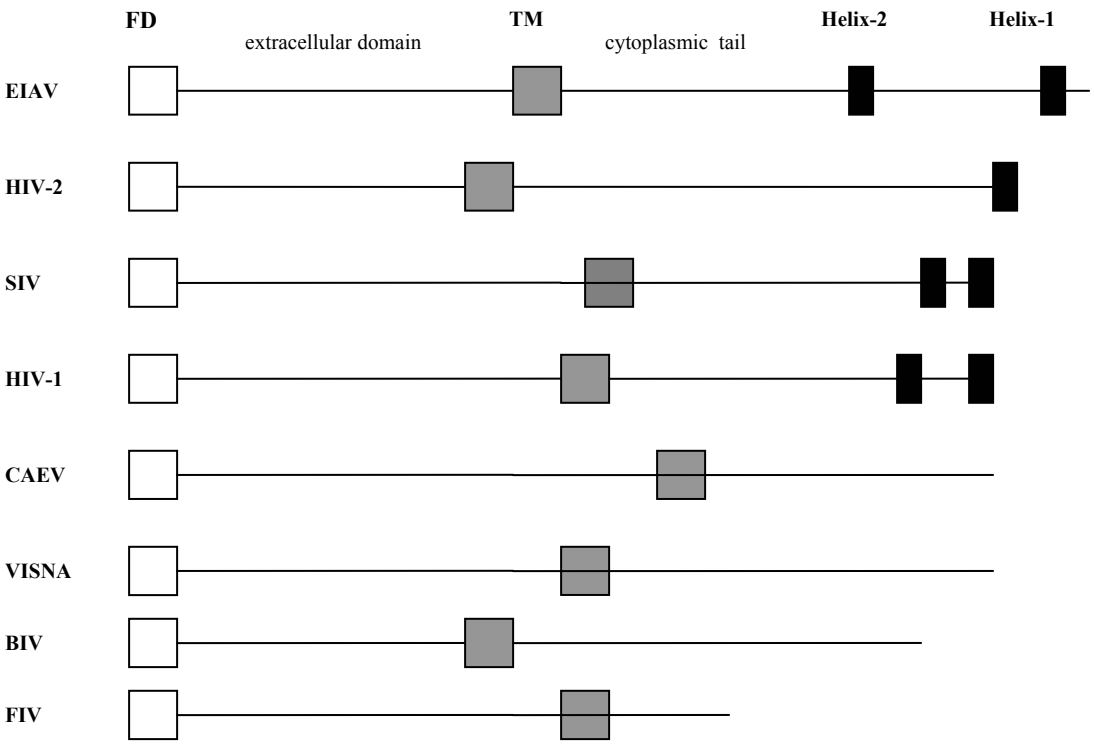


Figure 6: Linear diagram of the transmembrane proteins from eight lentiviruses. HIV-1: human immunodeficiency virus type 1, HIV-2: human immunodeficiency virus type 2, SIV: simian immunodeficiency virus, BIV: Bovine immunodeficiency virus, FIV: feline immunodeficiency virus, VISNA: maedi-visna virus. CAEV, caprine arthritis-encephalitis virus, EIAV: equine infectious anemia virus. FD: fusion domain (white box), TM: transmembrane region (grey box), Helix: alpha-helical domain (black box).

evidence of its membrane binding potential (26). Besides these helices, a leucine zipper motif connected with the N-terminus of HIV-1 LLP1 has also been shown to interact with lipid bilayers (72).

Among all lentivirus, EIAV is unique in that a further cleavage event occurs at the carboxyl terminus CT domain of gp45 to yield a non-glycosylated 20kDa protein, designed as p20, with about 175 amino acids. Amino acid sequencing of the N-terminal p20 protein indicates that the cleavage occurs at the His/Leu site, which is located within the N-terminal of CT domain (117). The p20 protein is presumably generated after maturation of virions probably mediated by viral protease due to the fact that it can only be detected in virions (117). The p20 protein contains both of the above-mentioned amphipathic alpha helical regions. Since the TM proteins will be efficiently cleaved during virion budding or release, the predominant type of TM proteins that exist in virions are N-terminal glycosylated gp32 and C-terminal p20 with only a trace amount of gp45 (117). The location of p20 within the virions has not been determined but it is predicted to be membrane-associated due to the presence of amphipathic alpha helices. This cleavage event within EIAV TM is similar to that of murine leukemia virus (MuLV) and MPMV, which are also subjected to a post-assembly cleavage resulting in a protein named the R peptide. This R peptide has already been confirmed to be generated within the virions by the viral protease (13, 51,147). The R peptide is 16 amino acids in length and contains 10 conserved amino acids (14). The R peptide acts as an inhibitor of fusion prior to cleavage and deletion of the R peptide results in increased cell fusion (25). The fusion inhibition of the precleaved R peptide is suppressed by mutations to the

upstream region of the R peptide (79). In regard to the function of R peptide, the cleavage of p20 is not essential for virus replication *in vitro* (58).

2. Role of lentivirus cytoplasmic tail

The length and structure differences of the cytoplasmic tails between lentiviruses and oncogenic retroviruses are striking and imply important differences in their respective roles in the virus life cycle. Current literatures on the role of cytoplasmic tail (CT) in lentiviruses can be summarized to the following six points.

a) Virus Replication

Although the CT region of lentiviruses is important for optimum virus infectivity, many reports have shown that the cytoplasmic tail of the HIV-1, HIV-2, SIV and EIAV is not essential for the replication of virus at least *in vitro* (18,31,44,54,57,64,117). Studies by various CT mutants confirm that the ability to replicate with a truncated cytoplasmic tail is cell-type dependent and truncation-length dependent (19, 63, 65, 73,135,144,148). It seems that natural selection favors a full-length cytoplasmic domain in certain situations and a shorter one in others. For example, viruses of HIV with truncated cytoplasmic tails produced from HeLa, A 3.01 and SupT1 cells showed a greatly reduced infectivity; those from Sw480 and MT-4 cells retained significant infectivity. This implies that a host cell factor is involved. The same experiment also suggests that if the CT truncation is 61 residues, the infectivity of progeny virus in permissive or non-permissive cells is at the lowest point. Either an increase or decrease in the size of the CT domain from 61 residues will increase the infectivity. The degree of increase also seems to be a function of the number of increased or decreased residues (63). Passage of SIV in

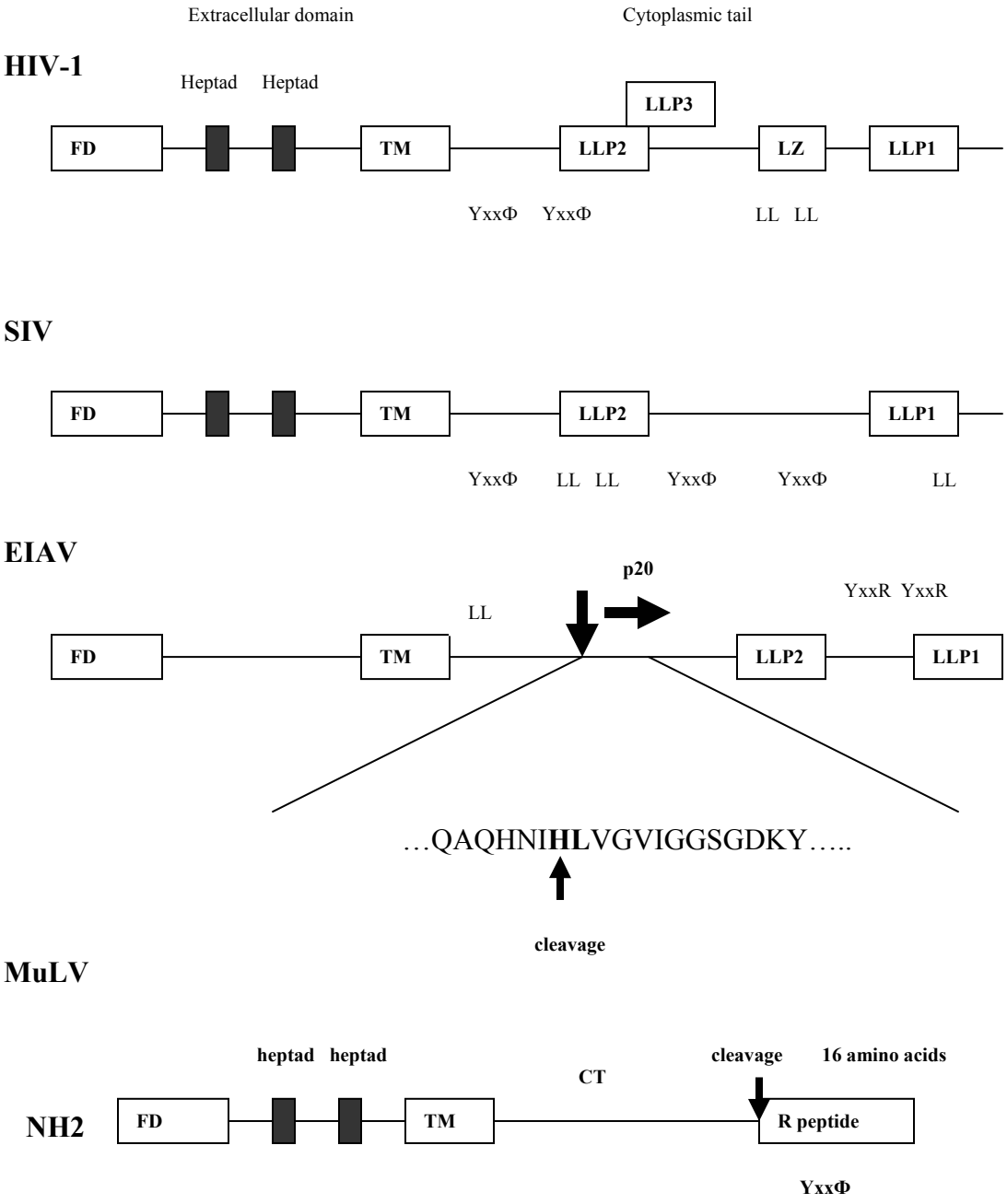


Figure 7: Schematic structure of lentivirus cytoplasmic tail and murine leukemia virus (MuLV) R peptide. FD: fusion domain. TM: transmembrane region. LZ: leucine zipper motif. LLP: lentivirus lytic peptide. YxxΦ: Tyrosine-based motif (Y: Tyrosine, x: any amino acid, Φ: amino acid with a bulky hydrophobic side chain). LL: dileucine motif. Heptad: α-helical domain.

human cell lines selected for virions with a truncated form of the cytoplasmic domain. When those viruses were passaged back in rhesus monkeys or in cultured macaque lymphocytes, reversion of the premature stop codon was observed, resulting in the production of virions with a full-length cytoplasmic tail (19, 65, 73). Therefore, it appears that the emergence of truncated cytoplasmic tails in virus isolates represents an adaptation of non-human primate lentiviruses to replicate in human cell lines. Similarly, EIAV containing a truncated TM domain preferentially replicates in canine cells (Cf2Th), whereas a full-length envelope is maintained upon passage of EIAV in equine cells (116). This observation has been further confirmed by demonstrating a cluster of termination codons in the gp45 of several PCR-amplified clones of EIAV (106). All these studies demonstrated that the requirement for the cytoplasmic domain of TM for replication is dependent on the host cell type. This may suggest that the cytoplasmic tail of lentiviruses has a specific role in the *in vivo* target cell and its truncation represents an artifact of *in vitro* virus replication. However, this phenomenon is not restrictive. It is reported that one of the HIV-1 reference strains with a 100 amino acid truncation on cytoplasmic tail can still productively infect peripheral blood mononuclear cells (PBMC) as well as Hut78 cells *in vitro* (135).

b) Glycoprotein incorporation

Virus infectivity depends on the functional and structural integrity of the envelope glycoproteins. The mechanism that CT truncation affecting virus replication is not well understood and has been naturally traced to the role of the cytoplasmic tail for Env incorporation into virions (33, 45,63,65,69, 84,129,149). For enveloped viruses, the incorporation of viral glycoproteins into budding virions is essential for the production of

infectious virus particles (43, 67). The Env proteins of retroviruses are synthesized on ribosomes in the endoplasmic reticulum (ER), undergo oligosaccharide processing and proteolytic cleavage (a cellular furin-type protease for HIV) in the Golgi complex, and then transported to the cell surface (70,85,107,113,122). For human immunodeficiency virus type 1, minor alterations in the conserved C-terminal region of the gp41 cytoplasmic tail can result in reductions in infectivity that correlate for most, but not all, constructs with a decrease in glycoprotein incorporation into virions (108).

Mapping of the functional region critical for envelope incorporation into virus particles within the cytoplasmic tail of SIV by Env mutants and short, in-frame deletions, suggests that domains in the C-terminal of TM protein are required for Env incorporation as well as Env-mediated entry (30, 84). However an interesting phenomenon is that although truncation including the critical domain will affect the Env incorporation, infectivity as well as entry of the virus, further deletion beyond the domain will at least partially rescue the lost functions (30, 63, 84). For SIV, deletion of 20 to 80 residues from the carboxyl terminus of the SIV CT eliminates the incorporation of the Env glycoprotein into particles and correspondingly impairs the infectivity of the virus. However, further truncation of the SIV TM protein by 100 to 140 amino acids restored the ability of the Env protein to associate with the Gag particles. More interestingly, Env mutants bearing 44 or 22 amino acids of CT could incorporate into virions at levels significantly higher than the wild type (84). It also shows that, if the truncation is from a specific site downstream of the C-terminal third of the cytoplasmic tail, virus infectivity will be drastically enhanced with respect to that conferred by the full-length Env protein (84,141).

Based on the structure of mature lentiviruses, the matrix protein (MA) is located in the inner side of the shell and is in close association with the lipid bilayer and the viral envelope. *In vitro,* intracellular interaction of simian immunodeficiency virus Gag and Env proteins has been reported (86). An Env incorporation block imposed by mutations in the HIV-1 and SIV matrix domain of the Gag polyprotein can be reversed by expression of an Env protein with a short TM cytoplasmic tail (42, 43, 49). This observation supports the idea that an interaction between the TM cytoplasmic tail and the matrix domain modulates Env incorporation into virions. The phenomenon that substitution of HIV Glu for Leu at residue 12 or 30 in the MA region will block Env incorporation into virions further implies that the long cytoplasmic tail of the trimeric Env has to fit the "cage hole" present in the lattice-like MA trimeric structure during virus assembly (62, 97). There is also genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and the α-helix 2 of gp41 cytoplasmic tail (141). Findings relating to the interaction of matrix domain and cytoplasmic tail are also consistent with the observation that domains in the C-terminal third of Env protein are required for Env incorporations as well as Env-mediated entry (30). Besides these, chimeras MuLV/ GaLV and HIV-1/MLV Gag also give indirect support for the critical function of cytoplasmic tail in Env incorporation (4, 68). Detailed mapping of the precise sequences or residues located in the cytoplasmic tail that directly interact with the MA or that modulate Env-gag interaction indicates that the C-terminal cytoplasmic tail has a critical role in Env-Gag interaction (29,30,42,43,84,141). A recent report investigating the effect of extension of the cytoplasmic domain of HIV-1 on virus replication have shown that HIV-1 Env can tolerate extension at the C-terminus to a certain degree

without loss of virus replication and Env incorporation into virions although further extension beyond 9 amino acids will impair virus infectivity by impairing env intracellular transport and incorporation into the virus. The same study also indicated that the immediate C-terminal portion of the cytoplasmic tail is not critical for Env-Gag interactions (20).

Sufficient cell surface expression of oligomeric lentivirus envelope protein that is correctly folded and glycosylated is the prerequisite for incorporation into virions. Deletion of the carboxy terminus of the cytoplasmic domain of HIV-1 gp41 may increase both protein transport and processing of gp160 but leads to decreased incorporation of glycoproteins into virions (42, 45, 55,149,151). Protein synthesis and processing of the TM protein in simian immunodeficiency virus (SIV) is also regulated by the cytoplasmic domain. Most SIVs isolated by culturing in human cells possess a premature stop codon that truncates the cytoplasmic tail of gp41 (19,140,143). The truncation of the cytoplasmic domain may increase surface expression, fusogenicity, infectivity (19,118,132,133,152). The similar phenomenon has also been observed in HIV-2, which is closely related to SIV (149). So an alteration of the cytoplasmic tail can result in reductions in infectivity that correlated for most but not all with a decrease in cell surface expression and virion incorporation of envelope glycoprotein. The post entry steps in virus life cycle are predicted to be affected and cellular factors are believed to regulate the process.

c) Fusion

The cytoplasmic tail of the TM proteins of retroviruses has been shown to play different roles in virus-to-cell mediated fusion. In the case of SIV, the length of the

cytoplasmic tail (CT) of TM has an effect on virus entry and a truncated CT enhances fusion activity of TM (118,152). It also appears that the cytoplasmic domain in certain cell types modulates cell fusion activity of HIV-2 TM (93).

In the case of oncoretroviruses such as MuLV and MPMV, cleavage of TM is an event occurring under normal conditions resulting in a small protein named the R peptide (14,115). This cleavage is required for the SU-TM complex to perform its essential function including fusion. Mutation in the cleavage site results in the blocking of normal processing of TM and reduction of viral infectivity (112,115). The mechanism of the uncleaved R peptide inhibition to fusion probably may come from its interaction with cellular factors such as actin, microfilaments and cell-surface integrins, which have been implicated in the fusion process by viral envelope proteins (1, 2, 16, 53, 61). When the R-peptide truncated envelope proteins are coexpressed with R-peptide containing envelope proteins, the high fusion activity of the R-peptide truncated proteins was unaffected, indicating the R peptide does not inhibit the cell fusion activity by a trans-dominant mechanism (147). Although the TM cleavage of MPMV is required for fusion activity and initiation of infection, it also appears that mutants completely lacking a cytoplasmic tail were nonfusogenic. This phenomenon implies that changes in the cytoplasmic tail can have a positive or negative effect on fusion. Further experiments also show that this nonfusogenic phenotype is not caused by a lower level of glycoprotein accumulation at the cell surface (14).

EIAV is the only lentivirus in which a cleavage event occurs during budding and maturation of virions to yield a C-terminal non-glycosylated p20 protein (117). But unlike the R peptide of oncoretroviruses, the cleavage of EIAV TM is not essential for

replication of virus *in vitro*. Neither truncation of p20 or mutation of the cleavage site to prevent cleavage will abrogate the virus replication (12, 58). So the significance of this TM cleavage may be different from that of the R peptide.

The mechanism by which the cytoplasmic tail of the retroviral TM affects the fusion activity is not clear. It might relate to: (1) transmembrane alterations in the conformation of the external domain, including fusion peptide; (2) cytoskeletal association of glycoproteins in the plasma membrane (1,2,16,53,61); (3) glycoprotein transport to the plasma membrane or stability on the cell surface. Recent reports suggest a mechanism that binding of the gp41 cytoplasmic tail to gag within immature HIV-1 particles inhibits Env conformational changes on the surface of the virions that are required for membrane fusion. This "inside-out" regulation of HIV-1 fusion could play an important role in the virus life cycle by preventing the entry of immature, non infectious particles (145). To further support this, protease (PR)-mediated gag processing has been found to regulate the interaction between HIV-1 gp41 cytoplasmic tail and matrix (MA) protein. The protease inactive (PR-) virions bearing wild-type envelope displayed defects in cell-cell fusion and this defect could be reverted by truncating the gp41 cytoplasmic tail (94). The results also suggest that interactions between unprocessed Gag and gp41 cytoplasmic tail suppress fusion.

d) Cytopathic effect (CPE)

Unlike oncogenic retroviruses, which normally establish a resistant infection in target cells, lentiviruses usually establish a productive infection resulting in death of the infected cells though they are also able to persistently infect cells (95). The mechanisms

for lentivirus cytopathicity are still not clearly known but it is predicted that a multifactorial process including both viral and host cell factors may be involved.

A high level of unintegrated proviral DNA possibly resulting from cell superinfection (56,60,95), virus-specific mRNAs (83,111) and direct action of *env* gene products (74), are probably the main factors thought to correlate with lentiviral cytopathicity. From the point of direct action of env products in lentivirus cytopathology, most studies focused on the roles of Env protein in damaging the membrane of infected cells. Sequence analysis of various strains of HIV demonstrated that truncation of the CT domain of TM could affect the cytopathogenic potential of the virus (40,46,130,151). Two possible TM mediated cytopathic mechanisms are fusion-induced syncytia formation (80), or direct disruption of the cell membrane integrity resulting in intracellular calcium influx and cell death, either by necrosis or via apoptosis (27,35). It is now known that HIV-1 CPE is caused by either syncytium formation or single cell lysis, both processes are considered to be involved in the transmembrane glycoprotein gp41. Syncytium formation is induced by the binding of the fusion domain of gp41 with cellular receptor and this binding facilitates the fusion of the viral membrane with host cell plasma membranes (130). This process can be inhibited in the presence of soluble CD4 or neutralizing antibodies (130). Enfuvirtide, a HIV-1 membrane fusion inhibitor and also the first entry inhibitor approved for the treatment of HIV-1 infected patients in USA, targets this fusion process. The function of the cytoplasmic tail in this fusion process has been discussed in the above "fusion" section.

Besides syncytium formation and membrane fusion, reports also suggest that single cell lysis may be more relevant than syncytium formation *in vitro* and *in vivo* in inducing

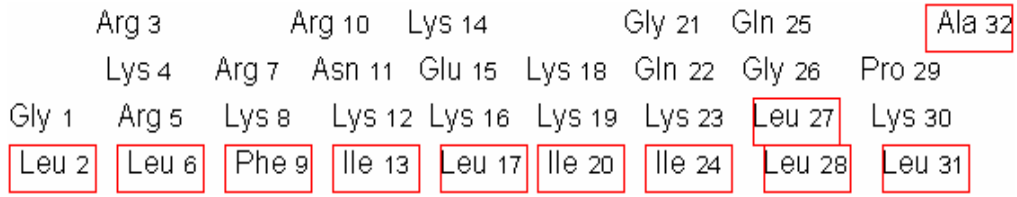
cytopathic effect (17,131). It has been shown that non-syncytium-inducing HIV-1 strains that are macrophage-tropic are cytopathic for CD4+ T lymphocytes and this finding suggest that HIV-1 CPE could be independent of syncytia-forming ability. This single cell lysis is also not inhibited by soluble CD4 and should be mediated either by necrosis or apoptosis (17,131). To support this, HIV-1 infected lymphoblasts have been shown to undergo apoptosis *in vitro* via activation of a Ca2+-dependent endonuclease (28, 87,138). However, necrosis is probably more prevalent than apoptosis in HIV-1 infected cell cultures and depend on the cell lines used (5,17).

The CT domain of the EIAV and HIV has been implicated in the process of single cell lysis through cell membrane destabilization based on secondary and tertiary structure prediction. Indeed, the cytoplasmic tail of the HIV-1, HIV-2, SIV and EIAV TM protein contains two or three highly hydrophobic domains, with the potential to form an amphipathic alpha helix (82,142). These segments are positively charged and rich in arginine residues. In contrast to this, sequence comparisons show that oncogenic retroviruses such as HTLV-1, RSV and MuLV lack such characterization (11). An amphipathic helix can be defined as α-helix with opposing polar and nonpolar faces oriented along the long axis of the helix (125) (Figure 8). Such structures are commonly found in a variety of lipid-associating proteins, such as apolipoproteins and in many cases involved in protein-protein interactions. Based on the physicochemical and structural characteristics, naturally occurring amphipathic alpha helices can be divided into seven categories: Class A, for apolipoproteins, H for polypeptide hormones, L for lytic peptide, G for globular proteins, K for calmodulin-regulated protein kinases, C for coiled-coil proteins and M for transmembrane proteins. Further studies on the CT domain of HIV-1

have shown that the arginine-rich segment is structurally similar to peptides of the class L. The cytolytic peptides, which could form multimers, have a high affinity for lipids and are generally treated as part of the antibacterial defense mechanism of a variety of organisms. Their binding to lipid can induce membrane disruption, resulting in osmotic lysis and death of bacterial cells (88). Though all these peptides may vary considerably in their amino acid sequence, they all retain the characteristic positively charged amphipathic alpha helical structure.

The mechanism that allows the amphipathic helical structures to disrupt membranes is not completely clear and computer modeling has shown that the two-amphipathic alpha helices in the cytoplasmic tail of HIV-1 gp41 could fold back on each other and form a tertiary structure stabilized by salt bridges and hydrogen bonds (142). A similar tertiary structure could be observed by examining the CT domain of the EIAV TM protein (92). It appears that this structure may aggregate to form channels or pores through the cell membrane and then destabilize it to induce cytopathic effect. Synthesized peptides corresponding to the conserved alpha helices segments of HIV-1, which are designed as lentivirus lytic peptides (LLPs) could mediate killing of prokaryotic (Staphylococcus) and eukaryotic cells (HUT-78 cells) when added exogenously and at relatively high concentration (88). Additional studies show that the toxic prosperities of the LLPs are not restricted to a specific cell type (90). ^{51}Cr-release assay and ion flux experiments have revealed that the LLPs are really able to aggregate and form pores of a defined size through cellular membranes. Though the LLPs may mainly contribute to the cytopathicity of lentiviruses, they are also predicted to have other roles in the virus life cycle. To support this, it has been observed that the LLPs have a remarkable similarity in sequence

A:



B:

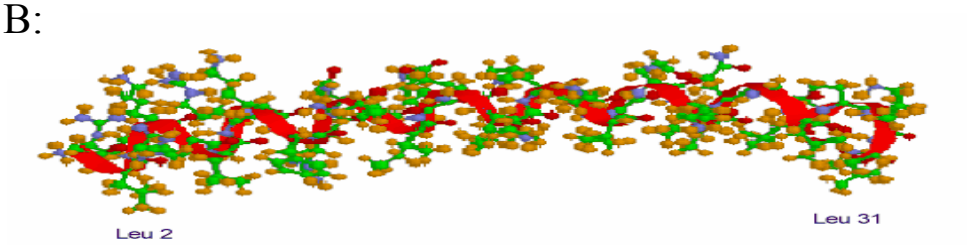


Figure 8: Schematic structure of amphipathic *α*-helix of lentivirus cytoplasmic tail. (A). In the amphipathic *α*-helix ,one side of the helix contains mainly hydrophilic amino acids such as Arg and Gly and the other side contains mainly hydrophobic amino acids such as Phe and Leu. The amino acid sequence of amphipathic α helix alternates between hydrophilic and hydrophobic residues every 3 to 4 residues, since the α helix makes a turn for every 3.6 residues. (B). the three-dimension (3D) structure of the amphipathic *α*-helix determined by nuclear magnetic resonance (NMR).

and structure with the S4 motif of monovalent cation channels, which are highly conserved between Na+ and K+ channels (139). For influenza virus A, its M2 protein also has a channel activity and is believed to play a critical role in mediating fusion of the virus envelope with cellular lipids during uncoating (34,109). The walls of ion channels are formed directly by the peptide amino acids (123). The α-helix domain of HIV-1 CT can interact strongly with lipid bilayers and cause the formation of pores (25). Some of the characteristics of these pores, including low ionic selectivity, are similar to those created by magainin and cecropin, two examples of cytolytic peptides found in amphibians and insects respectively that function as antibiotics (11).

HIV-1 contains three highly conserved lentivirus lytic peptide designed LLP-1, LLP-2 and LLP-3 in the C-terminal region of the cytoplasmic tail and they are thought to be associated with the inner surface of viral and cellular membranes (89, 142). The multimerization potential and membrane binding ability contributed by the LLP of the cytoplasmic tail may play an important role in virus replication. The N-terminal segment of LLP-1 contains a structural determinant critical for modulating Env stability (23, 24, 25, 77, 78). Two-conserved cysteine residues, with one located within the LLP-1 region and another close to the N-terminal of LLP-2/3 have been shown to be the targets for palmitoylation and are required for envelope association with lipid rafts and assembly on budding virions (119). Envelope proteins lacking the two cysteines are excluded from lipid rafts. Substitution of the cysteines with alanines or serines will eliminate raft association and severely reduce envelope incorporation and infectivity (7). In addition, HIV-1 LLP could also interact with calmodulin with its positively charged amino acids

(91,134,136). The sequestration of calmodulin by LLP is hypothesized to interfere with calmodulin-dependent signal transduction pathway in cellular dysfunction.

e) Surface envelope glycoprotein internalization

Viral envelope proteins expressed at the cell surface are available for cellular and humoral immune surveillance by the host. It is of critical importance for viruses to minimize the surface expression of their envelope proteins at the surface of infected cells.

Rapid endocytosis by clathrin-coated vesicles is a common method used by cells for surface protein transportation and recycling. This kind of transport is carried out in a constitutive manner (21) (Figure 9). Endocytosis is also a mechanism used by some viruses to keep the number of exposed envelope proteins in check and retrograde those that are not packaged into virions. Virus envelope glycoproteins subjected to endocytosis often contain specific motifs in their cytoplasmic domain that will be recognized during their interaction with clathrin adaptor proteins and formation of a clathrin-coated pit (8, 9,15). Both retrovirus and lentivirus cytoplasmic tails also contain the corresponding endocytic motif, even though the length of cytoplasmic tail for oncoretroviruses is much shorter than that of lentiviruses. Lentivirus envelope proteins have been shown to undergo rapid endocytosis but this is under strict control in order to have enough expression of surface unit protein at the cell surface and effective incorporation into virions. When HIV-1 Gag is coexpressed with Env, a down regulation of Env endocytosis will ensue (37). It is presumed that the competition for Env binding between Gag and Ap-2 is the cause of this down regulation (37).

The amino acid motif YXXΦ (Y,Tyr; X, any amino acid; Φ, amino acid with a bulky hydrophobic side chain) and dileucine motif are two kinds of endocytic signals that have

been identified on the cytoplasmic tails of retroviral transmembrane proteins (15).In the case of HIV-1, SIV, HTLV-1, MuLV, MPMV, RSV and BLV, the cytoplasmic domain of TM has been demonstrated to bind, via two highly-conserved tyrosine motifs (YXXΦ), to the μ2 domain of the clathrin adapter protein AP-2 at the plasma membrane and μ1 domain of AP-1 at the trans-golgi network (9,99).

The TM of HIV-1 is known to contain at least two tyrosine signals involved in endocytosis. The first signal, RQGYSPL, interacts with the μ2 (medium) chain of AP-2 and the entire AP-2 complex (8). It can also interact with μ1 and μ3A components of AP-1 and AP-3 respectively (99). A glycine residue (Gly) adjacent to the membrane-proximal tyrosine endocytic signal has been shown to affect the binding to the μ2 and Ap-2 complex (98). Another endocytosis signal (LFSYHRL) can also interact with μ1, μ2 and μ3A as well as the entire AP-2 complex but this signal is not completely required for endocytosis probably due to its distal location of the fusion domain (99). Further studies on the internalization/cellular trafficking of the endocytosis signals in the cytoplasmic tail of HIV-1, SIV and HTLV-1 has revealed that all three of the TM cytoplasmic tails interact with μ1 and μ2 complexes of AP-1 and AP-2 as well as the whole AP complex and their C-terminal region interacts with β2 adaptin subunit (6).

For HTLV-1, the membrane proximal tyrosine motif is the major determinant for interaction with the host cell and recruitment of AP complexes (6). The cytoplasmic tail of HTLV-1 TM is only 24 amino acids in length compared with more than 150 amino acids for primate lentiviruses suggesting that these endocytosis signals are widely used for retroviruses. However, RSV (Rous sarcoma virus) envelope glycoproteins, which contain a tyrosine endocytic motif and also undergo endocytosis from the cell surface as a

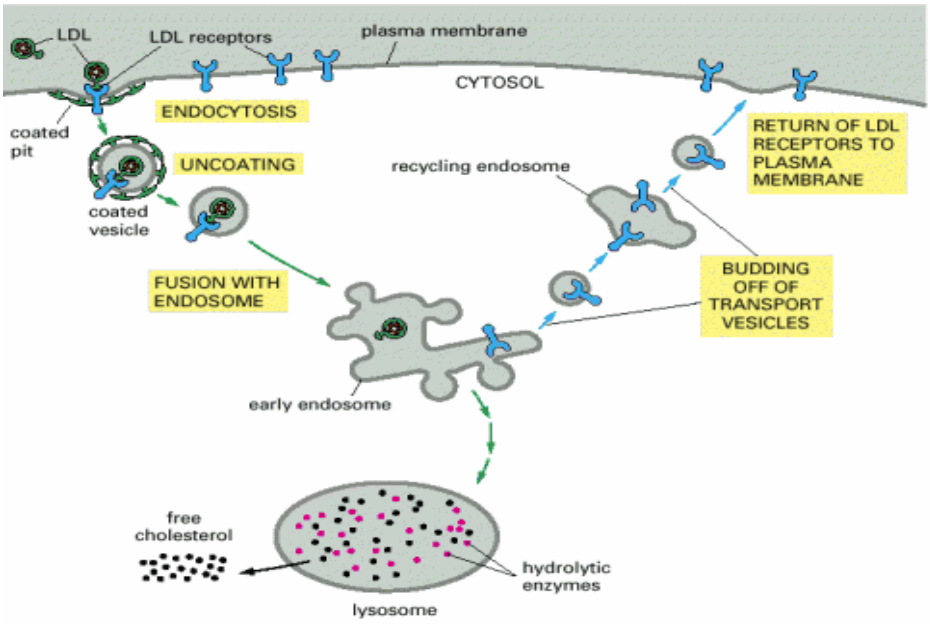


Figure 9: Schematic representation of low density lipoprotein (LDL) particles through receptor-mediated endocytosis. (1). At the plasma membrane, LDL receptors bind LDL particles and collect in clathrin-coated pits. (2).The clathrin-coated pits pinch off and form clathrin-coated vesicles. The vesicles loss clathrin coats and fuse with early endosome. (4). Empty receptor returns to cell surface. (5). Vesicles from early endosome fuse with late endosome and mature into lysosome. (6). LDL particles get digested, cholesterol released, used for membrane synthesis. (Molecular Biology of the Cell. 4th ed., Alberts *et al*, 2002)

means of regulating envelope protein exposure, have lower level of envelope endocytosis than the predicted rate (15). The reason for this altered internalization phenotype is that regions within the membrane-spanning domain have been shown to silence the endocytic internalization signal (98). Another method to control envelope surface expression is utilized by MLV and MPMV. Though these oncoretroviruses also contain a conserved internalization motif like lentiviruses, however, the envelope proteins are preferentially retained in the Golgi instead of trafficking to the cell surface (50, 98).

A second known endocytosis internalization signal of lentivirus is the dileucine motif. This dileucine motif has been found in several receptors, coreceptors and proteins including HIV-1 TM cytoplasmic domain, HIV-1 nef and the chemokine receptors (CXCR4 and CXCR2) and serves to mediate interaction with AP-1 and AP-2 (146,52). This dileucine motif has been found to be crucial in assisting the membrane-proximal tyrosine internalization domain of HIV-1 Env in binding to AP-1 (52). This result suggests that the dileucine motif is the commonly motif used to regulate lentivirus envelope protein expression on the cell surface as well as modulate some other cellular factors which are involved in virus replication.

f) Immunogenesis and pathogenesis

It is reasonable to predict that the cytoplasmic tail of lentiviruses has the capacity to alter immune responses to virus protein *in vivo* and pathogenesis of the virus. A molecular clone SIVmac239 containing a premature stop codon in the cytoplasmic tail replicates more efficiently than wild-type SIV in the human T-cell line HUT-78 by increasing Env incorporation into particles as well as higher fusogenicity and infectivity (126,152). Though this CT mutant virus replicates with a profile identical to parental

virus in the human T/B hybrid cell line CEMx174, it replicates less efficiently in primary rhesus PBMC (126,127). *In vivo* experiment shows that the CT mutant virus is unable to cause disease in juvenile macaques. Though this CT mutant virus still causes a high level viremia in the acute phase for neonatal rhesus macaques, a relatively lower viral load in the chronic phase of infection has been observed. In addition, no clinical signs of disease are found up to the first year of infection (127). These results demonstrate that the cytoplasmic tail of transmembrane Env is a locus for attenuation of SIV in rhesus macaques. When juveniles or neonate macaques are infected with the virus from SIVmac239 molecular clone with the cytoplasmic tail truncation, it can provide partial protection from mucosal challenge with pathogenic virus (128). Recent studies on mapping of the pathogenic epitopes on EIAV Env suggest that compared with surface unit protein, the transmembrane region does not contribute a lot to the pathogenesis (105). Mutation of the tyrosine-dependent endocytosis signal in the cytoplasmic tail of SIV TM has also been shown to cause attenuation *in vivo* (103). A leucine zipper motif in the cytoplasmic tail of HIV TM, which is predicted to function for membrane binding, is also required for replication *in vitro* and pathogenesis *in vivo* (71). We predicted that truncation of the SIV cytoplasmic tail containing the putative endocytosis signal may increase the envelope expression on host cell surfaces and incorporation into virions. This phenotype changes may make the virus more sensitive to immunological control and increase immune-mediated clearance of the virus. Another possibility is that truncation of the SIV cytoplasmic domain may cause conformational changes in the surface protein leading to exposure of epitopes that are sensitive to neutralization. To support this, it has been observed that truncation of the cytoplasmic domain of HIV-1 induces exposure of

conserved regions in the ectodomain and results in the marked neutralization sensitivity of envelope protein (36).

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Truncation of the Cytoplasmic Tail of Equine Infectious Anemia Virus Transmembrane Protein Attenuates the Virus *in Vivo*

Introduction

A major difference between the transmembrane proteins of lentiviruses and oncoviruses is in the intracytoplasmic extension from the transmembrane segment. Although present in all retroviruses, this extension is consistently longer in lentiviruses than in oncoviruses (13, 26). With the exception of FIV, the cytoplasmic tail of all lentiviruses is over 120 amino acids in length. For primate lentiviruses including HIV-1, HIV-2 and SIV, it is about 150-200 residues long and for equine infectious anemia virus (EIAV), it is 226 residues (26). In sharp contrast, the intracytoplasmic domain of the TM glycoprotein in the oncogenic retroviruses such as Mason-Pfizer monkey virus (MPMV), Rous sarcoma virus (RSV), feline leukemia virus (FeLV), and mouse mammary tumor virus (MMTV) is between 32 and 43 amino acids in length (13). The cytoplasmic domain of lentiviruses has been shown to affect a variety of virus functions including virus replication (20), fusion activity (69), virus-induced cytopathogenicity (52), envelope incorporation into virions (36), surface envelope glycoprotein internalization (28), pathogenicity (59) and immunogenicity (10).

Among all lentiviruses, EIAV is unique in that a further cleavage event occurs at the carboxyl terminus (CT) domain of gp45 and yields a nonglycosylated 20kD protein, named p20, with about 175 amino acids, which comprise more than two thirds of the C-terminal cytoplasmic domain. Amino acid sequencing of the N-terminal p20 shows that the cleavage occurs at His/Leu site for a specific strain (56). The p20 is presumably generated after maturation of virions mediated by viral protease due to the observation

that it can only be detected in virions (56). The p20 protein also contains both of the amphipathic alpha helical regions, which are analogous to lentivirus lytic peptides (LLPs) of primate lentivirus HIV-1, HIV-2, and SIV (34, 63). Since the TM proteins will be efficiently cleaved during virion budding or release, the predominant type of TM proteins that exist in virions are N-terminal glycosylated gp32 and C-terminal p20 with only a trace amount of gp45 (56). The location of p20 within the virions has not been determined but it is predicted to be membrane-associated due to the presence of the amphipathic alpha helices. The cleavage event of EIAV within the cytoplasmic domain is similar to that of murine leukemia virus (MuLV), Mason-Pfizer monkey virus (MPMV), and the gibbon ape leukemia virus (GaLV), which are also subjected to a post-assembly cleavage resulting in a protein named the R peptide (4, 9,15). This peptide has already been confirmed to be generated within the virions by the viral protease and both R peptides for MuLV and MPMV are 16 amino acids in length and contain 10 conserved amino acids (4, 15, 66). The cleavage within the cytoplasmic tail of MuLV at, or shortly after virus budding, activates fusion activity (5). The precleaved R peptide inhibits fusion and infectivity and this inhibition can be suppressed by introducing mutations to the upstream region of the R peptide (31, 55). It has been suggested that the virus has adopted this strategy to regulating Env fusogenicity in order to limit the expression of a potentially cytotoxic molecule on the cell surface (54, 55). In contrast to the function of the R peptide, the cleavage of EIAV transmembrane protein is not required for virus replication *in vitro* and mutation of the cleavage site to a non-cleavable type did not eliminate virus replication (17). Studies in our laboratory have also shown that expression

of p20 is not essential for virus replication. Virus with truncated p20 still replicates, although its replication is delayed compared with parental virus (3).

EIAV infection in horses results in a rapid disease progression instead of inducing a slow progressive time course as other lentiviruses. After initial exposure to a virulent virus strain, infected horses undergo an acute phase disease with a short duration between one week to a month represented by fever, thrombocytopenia and high-titer plasma viremia (23). The rapid disease occurrence makes it a useful model to study the acute pathogenesis of lentiviruses. Virus derived from the molecular clone SIVmac 239 is normally highly pathogenic in juvenile macaques but virus derived from a SIVmac239 clone containing a premature stop codon in the envelope cytoplasmic tail (CT) is attenuated. Though the CT mutant still causes a high level viremia in the acute phase for neonatal rhesus macaques, a relatively lower viral load in the chronic phase of infection with no clinical signs of disease for 1 year has been observed compared with wild type infection (59). These results demonstrate that the intracytoplasmic domain of the transmembrane Env is required for disease expression of SIV in rhesus macaques. In this study, we test the *in vivo* function of cytoplasmic domain of EIAV transmembrane protein by introducing premature stop codons into the N-terminal coding region of p20 on the background of a highly virulent infectious clone p19/wenv17 (47). To characterize the phenotype of the p20-truncated virus, we also compare the level of the envelope incorporation into virions and the relative specific infectivity between p20-truncated and parental virus.

Materials and Methods

Cells and virus.

Feline embryonic adenocarcinoma (FEA) cells were obtained from Dr. Susan Payne at Texas A&M University. The cells were cultured in Dulbecco modified medium (DMEM) containing 5% fetal bovine serum (FBS).

Equine monocyte-derived macrophages (eMDMs) were made from the peripheral blood of Shetland ponies by centrifugation from Ficoll (Histopaque-1077; Sigma, MO). The cells were washed with phosphate-buffered saline (PBS with Mg^{2+} and Ca^{2+}) two times and plated in $25cm^2$ flasks in RPMI-1640 (Cellgro) with 10% autologous horse serum. After 24 hours incubation in 37°C with 7% CO2, the medium was changed and the attached macrophages were washed twice with cold PBS and used for virus infection.

The EIAV pSPeiav19 full-length infectious clone is a prototype derived from the cell culture adapted Malmquist strain. Virus particles derived from pSPeiav19 are avirulent, replicate both in FEA cell with no cytopathic effect (CPE) and equine monocyte-derived macrophage cultures with CPE. The EIAV p19/wenv17 infectious clone is highly virulent and virus derived from this clone is routinely fatal when administrated in high dose (47). It is derived by substituting most of the envelope of pSPeiav19 with highly virulent Wyoming strain and at the same time, replacing the pSPeiav19's LTR with Wyoming LTR. Differences between pSPeiav19 and p19/wenv17 also existed in the *rev* gene due to the substitution of envelope sequences (47).

Construction of EIAV p20 truncated proviral clone

Three termination codons were introduced into the 8th, 22nd and 26th amino acid of the N-terminal p20-coding region without affecting the coding sequence of Rev (Figure

10A and B, page 116). To avoid the interference of restriction enzyme sites on the vector (pLG338) for the infectious clone, the SphI and EcoRI fragment which includes most of the envelope gene and full-length of 3'LTR was subcloned into the corresponding site of pGEM-Teasy vector (Promega) (15). The first stop codon (8th amino acid of p20) was generated by general PCR. The upstream primer p20Mu1-F (5'-GGAATTCTGACCGGTGGA***TAA***GGGGAC-3') with Age I site located in the N-terminal of p20 and the stop codon TAA replacing TCA were paired with the downstream primer p20Mu1-R (5'GGAATTCGCTTCGAATCTCCAGGTC-3') with the SfuI site within the p20 coding region. The amplification reaction was carried out using the Expand High Fidelity PCR system (Roche) with 50ng of plasmid DNA as template. The concentration of Mg^{2+} is 1.5mM. Cycling parameters were 94°C 15 seconds, 60°C 30 seconds and 72°C 30 seconds (30cycles). PCR product was purified by QIAquick Gel Extraction Kit (Qiagen) and ligated with pGEM-Teasy T-A clone vector (Promega). Ligation mixture was transformed to DH5α competent cells and after identification by restriction enzyme cut; one of the positive clones with insert was used for further mutation. Introduction of another two termination codons (22nd and 26th amino acid) was done through QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). The complement prime pairs include 250ng p20Mu-F2 (5'-CTCCAGGAACGACTAGAATGGAGAATAAGAGGAGTACAAC-3') (positive strand primer) and 250ng p20Mu-R2 (5'-GTTGTAGTCCTCTTATTCTCCATTCTAGTCGTTCCTGGAG-3') (negative strand primer). 250ng of plasmid DNA was used as template. The cycling parameters were 95°C 50 seconds, 60°C 50 seconds and 68°C 8 minutes for 18 cycles. Following the cycling,

the PCR product was placed on ice for 2 minutes and then treated with restriction endonuclease Dpn I supplied with the kit at 37°C for 4 hours. One microliter of the Dpn I treated PCR product was used to transform XL10-Gold ultracompetent cells supplied with the kit according to the manual. Clones from the transformation were purified by QIAprep Spin Miniprep Kit and sequenced to confirm the introduction of the three termination codons. One positive clone containing the derived mutations was digested by Age I and Sfu I and the insert was used to replace the corresponding fragment in the Sph I and EcoR I pGEM-Teasy subclone. Hinf I was used to identify the substitution because the second termination codon (the 22nd amino acid of p20) abrogates the Hinf I site present on the parental coding region. Finally, the SphI and EcoR I fragment containing the three termination codons within the N-terminal p20 region was cloned back in the pLG338 vector containing the other region of the virus genome and a new proviral clone with a truncated p20 was constructed. Since all of the three sites used for introducing stop codons are the same for pSPeiav19 and p19/wenv17, this enabled us to establish the p20-truncated proviral clone for both full-length clones with the same strategy (Figure 10C, page 116).The new clones are named pSPeiav19Δp20 and p19/wenv17Δp20 respectively.

Transfection and preparation of virus stock.

Approximately 10 μg of purified plasmid DNA were transfected into FEA cells respectively using FuGene 6 transfection reagent (Roche). Cells were grown in 25cm^2 flask for 24 hours with about 50%-70% confluency before transfection. For each transfection, 20μl of FuGene reagent was first added to 0.8ml DMEM media (Cellgro) with no other additions for 5 minutes. Then the media containing transfection reagent was added drop by drop to the tube containing DNA. After 15 minutes incubation, the mixture

was added drop by drop to the medium in 25cm^2 flasks. Medium was changed at 24 hours after transfection after washing 2-3 times with cold PBS. At 4 or 5 days after transfection, 1ml supernatant fluid was used to test reverse transcriptase activity and the remaining supernatant fluid was stored at -80°C for further infection.

For p19/wenv17Δp20, culture supernatant of transfected FEA cells containing 30,000cpm virus were used to infect macrophages established for 24 hours in 25mm^2 flasks. Prior to infection, the macrophages were washed twice with RPMI-1640 (Cellgrow) and the FEA culture supernatant was inoculated into the flasks. After one hour incubation at 37°C, the supernatant fluid was removed and macrophage growth media (RPMI+10% autologous horse serum) was added. Reverse transcriptase (RT) activity was detectable from 7 days after infection and culture supernatant with high RT levels were collected and stored at -80°C for further use.

Virus derived from p19/wenv17 clone was prepared by infecting established macrophages with supernatant fluid of transfected FEA cells. The infection and harvest procedure was the same as p19/wenv17Δp20. For pSPeiav19and pSPeiav19Δp20, FEA culture supernatant collected after transfection was used to reinfect FEA cells and derived virus was harvested 7 days after infection.

Reverse transcriptase (RT) assay

One ml of cell-free supernatant was centrifuged at 13,000g for 1 hour at 4°C. The supernatant was removed and the pellet was resuspended in 10µl lysis buffer (50mM Tris-HCl pH 8.3, 20mM dithiothreitol, 0.25% Triton X-100). After an incubation of 5 min at room temperature, the sample was mixed with 20µl of RT cocktail containing 50mM Tris-HCl pH7.9, 50mM KCl, 5mM dithiothreitol, 0.05% Nonidet P-40, 10mM

EGTA, 0.33 A_{260} unit of poly (dA)-poly (dT) $_{12\text{-}18}$ per ml, and 0.5uM (^{3}H) TTP at 62Ci/mmol. The reaction mixtures were then incubated at 37°C for 90min and subsequently spotted onto Whatman DE81 filters. Filters were air-dried, washed 3 times in 0.35M Na_2HPO4 for 15min each time, briefly rinsed in dH_2O, washed for 5min in 95% ethanol and then air dried. Finally they were placed into 5ml of scintillation fluid (Bio-Safe II, Research Products International Corp.) and counted with an automated beta counter (LKB1219, Wallac). Positive, negative and blank controls were included in the assay. The positive control consisted of a known virus stock containing 300,000cpm/ml. The negative control is uninfected cells and the blank control is DE81 filters with no sample applied.

Animal studies.

Virus derived from p19/wenv17Δp20 was intravenously (IV) injected into two EIAV negative Shetland ponies ($1.5x10^5$cpm/per pony). After infection, rectal temperatures were tested daily and disease episodes were defined as periods of significant increased body temperature. Blood serum and plasma samples were collected to detect the development of antibody, quantity the viral load and monitor the stability of termination codons introduced by viral genome recovery. Enzyme-linked immunosorbent assays (ELISA) were used to detect antibodies to a synthetic envelope peptide and a recombinant core antigen of EIAV using a commercial available kit (obtained from Centaur, Inc.). The infected ponies were observed for more than 50 days. As a control, p19/wenv17 virus was used to infect another two ponies ($1.0x10^5$cpm/per pony) by IV injection. These two ponies were monitored as those for p19/wenv17Δp20 infection and euthanized at 15 days post infection due to untreatable sustained fever.

RealTime RT-PCR for viral load detection from plasma samples

Viral RNA was extracted from 200µl plasma sample by High Pure Viral Nucleic Acid Kit (Roche) according to the instruction manual and diluted in 50µl elution buffer. A 10µl RNA sample was added to each of the reverse transcription mixes containing 2.5µl 10xBuffer without Mg^{2+}, 2.5µl 25mM Mg^{2+}, 7µl 2.5mM dNTP, 1µl RNA inhibitor, 1µl random hexamers and 1µl MuLV reverse transcriptase (50U/µl) from the GeneAmp RNA PCR kit (Applied Biosystems). After 5 minutes incubation at room temperature, the mixer was put at 42°C for 15 minutes and then 99°C for 5 minutes to inactivate the enzyme. A 12.5µl cDNA sample was directly used for a Taqman RealTime PCR assay performed on a Bio-Rad Icycler machine. Besides the 12.5µl cDNA template, each 50µl reaction also contained 3.75µl 10X buffer with 15mM Mg^{2+} (Applied Biosystems), 7µl 25mM Mg^{2+}, 1µl 10mM dNTP, 1µl 10mM upstream primer (5'-TTCCCATGACAGCAAGGTTT-3'), 1µl 10mM downstream primer (5'-TCCATTGTCTATATGTCTGCCTAAA-3'), 0.25µl 10mM Taqman probe (5;-FAM-CAAAAGCAGGCTCCATCTGTCTTTCTCTAGGACT-TAMRA-3') and 0.25µl AmpliTag Gold (5U/µl) (Applied Biosystem). Cycling parameters were 95°C for 30s and 60°C for 1 min (45cycles). DNA standards consisted of p19/wenv17 parental plasmid at 10^1-10^7 copies per reaction. A 50µl reaction for standard plasmid contained 5µl 10X buffer with 15mM Mg^{2+} (Applied Biosystems) and 8µl 25mM Mg^{2+} with the same components as the cDNA reaction. Real time data were analyzed by Bio-Rad Icycler software.

Western Blot

Supernatant fluids containing virus were collected and virions were pelleted through a 10% sucrose layer in TNE buffer (10mM Tris, 1mM EDTA,0.15M NaCl) by ultracentrifugation (1 hour, 100,000g, 4 °C). The pellet was resuspended in 1X NuPage LDS sample buffer (Invitrogen), heated at 70°C for 10min and electrophoresed on a 4-12% NuPage Bis-Tris Gel. Proteins were transferred onto a polyvinylidene difluoride (PVDF) plus membrane (Omnicron) and the membrane was blocked overnight at room temperature on a shaking platform with TBST (0.13M NaCl, 0.015M Tris-HCI, 0.005M Tris-base, 0.1% Tween-20) containing 10% powdered milk. The membrane was next incubated with horse anti-EIAV antibody (1:100) in TBST+10% powder milk for 90 min. After several washes in TBST, the membrane was subsequently incubated with 1:5000 horseradish peroxidase (HRP)-conjugated goat anti-horse IgG (H+L) (obtained from KPL) for 60min. Finally, the antigen-antibody complexes were detected by ECL Western Blotting Analysis System (Amersham Life Science) and visualized through Kodak X-OMAT X-ray films.

Relative specific infectivity assay

Diluted virus was used to infect healthy FEA cells cultured in 24 well plates. Seven days post infection; 1ml supernatant fluid for each dilution was collected and detected by reverse transcriptase (RT) assay.

Viral genome recovery and reversion identification

Viral RNA was extracted from 200µl culture supernatant or plasma samples by High Pure Viral Nucleic Acid Kit (Roche) according to the instruction manual and diluted in 50µl of elution buffer. The reverse transcriptase assay was performed as previously

described. For RT-PCR, five-microliters of cDNA and 10ng of p19 plasmid were used as template in a 50µl reaction to amplify a 250bp fragment flanked by primer pair p20Mu1-F and p20Mu1-R, which contained the second (22nd amino acid) and third (26th amino acid) termination codon introduced into the N-terminal region of p20 by Expand High Fidelity PCR system (Roche). The concentration of Mg^{2+} and each primer was 1.5mM and 0.02 µM respectively. Thermal cycling parameters were 94°C 15 seconds, 60°C 30 seconds and 72°C 30 seconds (30cycles). PCR product was purified by QIAquick Gel Extraction Kit (Qiagen) and treated with Hinf I (NEB) at 37°C for 1 h. The digestion mix was analyzed on a 2% agarose gel. DNA samples undigested by Hinf I confirmed the maintenance of the second termination site within the p20 coding region.

PCR analysis of EIAV proviral DNA in nucleus.

The method used to extract nuclear DNA is as described by Bukrinsky and Panganiban (7, 45) with a small modification. Briefly, the infected cells were washed twice with ice-cold phosphate-buffered saline (PBS, pH7.2) and lysed by the ice-cold lysis buffer (0.1M NaCI, 10mM Tris pH 7.9, 0.5% NP40 and 1.5mM $MgCI_2$) at $1x10^7$ cells per ml. After 10 minutes at room temperature, nuclei were pelleted at 6000cpm for 10 minutes at 4°C. The pelleted nuclei were washed twice with cold PBS and deproteinated with proteinase K in the presence of 1.2% SDS at 70°C for 15 minutes. Five-fold volume of the DNA binding buffer (Buffer PB, Qiagen) was added to the SDS supernatant and well mixed. The mixer was passed the QIAquick® Spin-column. After washing once by the washing buffer (Buffer PE, Qiagen), the DNA was eluted from the column with 50µl elution buffer (10mM Tris, pH 8.0). For amplification of the proviral DNA, 100ng of nuclear DNA was used as template. The primer pairs (upstream, 5'-TTCCCATGACAGCAAGGTTT-3';

downstream, 5'-TCCATTGTCTATATGTCTGCCTAAA-3') amplifies a 100bp gag fragment. The cycling parameters were 95°C for 20 seconds, 60°C for 20 seconds, 72°C 20 seconds for 30 cycles. For SYBR RealTime PCR, virus was used to infect cells and nuclear DNA was extracted at 4 hours after infection. Besides the 100ng nuclear DNA as template, each 50µl reaction also contained 5µl 10X buffer with no Mg^{2+} (Applied Biosystems), 11µl 25mM Mg^{2+}, 1µl 10mM dNTP, 1µl 10mM upstream primer (5'-TTCCCATGACAGCAAGGTTT-3'), 4.5µl 10mM downstream primer (5'-TCCATTGTCTATATGTCTGCCTAAA-3'), 10µl 1:10000 diluted SYBR (Sigma) and 0.25µl AmpliTag Gold (5U/µl) (Applied Biosystem). Cycling parameters were 95°C for 30s and 58°C for 1 min (35cycles). DNA standards consisted of pSPeiav19 parental plasmid at 10^1-10^7 copies per reaction. Real time data were analyzed by Bio-Rad Icycler software.

Virus binding assay.

About 50,000-10,000 cpm viruses in 0.15ml culture supernatant were used to cover equal number of FEA cells in 24-well plates. Empty wells without cells were also covered at the same time as a control. The plates were incubated at 37°C for 1 hour and reverse transcriptase (RT) activities left in the culture supernatant of each well were detected. For calculation of binding efficiency, the RT activity obtained from each well containing cells was divided by the average RT activity of wells without cells and the percentage obtained was treated as the proportion of virus not binding on the cells. The data were analyzed by *student t-test.*

Results

Construction of p20 truncated proviral clone.

EIAV has the longest cytoplasmic tail (226 amino acids) of the transmembrane protein among the lentiviruses (1). It is also unique in that a further cleavage event occurs within the transmembrane protein during virus maturation and yields an N-terminal glycosylated gp32 and a C-terminal nonglycosylated p20 with about 175 amino acids (56). Previous studies in our laboratory have shown that expression of the p20 is not essential for virus replication *in vitro* even though replication of the p20-truncated viruses is delayed compared with wild-type viruses (3). To test the role of the cytoplasmic tail of the EIAV transmembrane protein in disease expression, we introduced three termination codons within the N-terminal coding region of p20 on the background of the EIAV p19/wenv17 infectious molecular clone (47) (Figure 10B, page 116). As the p20 N-terminal coding region overlaps the *rev* gene coding region in an alternative open reading frame, the introduction of the three termination codons does not affect the amino acid sequence of rev (Figure 10A and B, page 116). In addition, the three new codons of rev are widely used in the virus genome and they should not down-regulate the expression of Rev.

Replication of p20 truncated virus *in vitro*

Virus derived from p19/wenv17 clone can only replicate in equine monocyte-derived macrophages (eMDMs), which is determined by the transcription activation region within the long terminal repeat (LTR) (72). Because equine monocyte-derived macrophages (eMDMs) are very sensitive and do not allow optimized transfection, we transfected p19/wenv17Δp20 plasmid DNA to FEA cells first and tested the presence of reverse

transcriptase (RT) activity for the culture supernatant at 72 and 96 hour after transfection, as we have previously described (47,48). One-milliliter aliquots of the FEA supernatant with ~30,000 cpm virus were used to infect eMDMs to generate virus stocks. Previous studies in our laboratory have demonstrated that the replication of p20-truncated virus on the background of an avirulent infectious clone pER, which can replicate either in eMDMs or in equine dermis cells as well as other cell lines is significantly delayed and lower in both equine dermis cells and eMDMs compared with parental virus (3). To study the kinetics of viral replication of p19/wenv17Δp20 virus, about 20,000-30,000 cpm of p19/wenv17 or p19/wenv17Δp20 virus was used to infect eMDMs. At 10 days after infection, the replication of the p19/wenv17Δp20 virus is still at a lower level. The RT activity can reach about 40,000 cpm/ml supernatant at 12 days post infection (Figure 11, page 117). In contrast, for p19/wenv17 parental virus, the RT activity exceeds more than 10,000 cpm/ml supernatant fluid at 7 days post infection and reaches about 60,000-70,000 cpm/ml supernatant fluid at 10 days post infection (Figure 11, page 117). We concluded that the replication of the p19/wenv17Δp20 virus is delayed and lower than that of parental p19/wenv17 virus. In detecting the relative specific infectivity of pSPeiav19 and pSPeiav19Δp20, we also observed the same phenomenon that replication of pSPeiav19Δp20 virus was about 2-10 fold lower than that of its parental virus when serially diluted virus was used to infect roughly the same number of FEA cells for 7 days. The replication of p19/wenv17Δp20 virus in equine macrophages is also similar to that of the SIVmac239 mutant containing a premature stop codon in the cytoplasmic tail of transmembrane protein in primary rhesus PBMC. Although the replication for the SIVmac239 mutant is more efficient or identical compared with wild-type SIV in the

human T-cell line HUT-78 and human T/B hybrid cell line CEMx174 respectively (58), it is less efficiently than wild-type SIVmac239 in primary rhesus PBMC (59).

Infection of Shetland ponies by p19/wenv17 and p19/wenv17Δp20 virus

In the case of SIV, virus derived from the molecular clone SIVmac239 with a truncated cytoplasmic tail was attenuated and did not induce disease in juvenile macaques (59). Though this mutant virus causes a high level viremia in the acute phase for neonatal rhesus macaques, compared with wild type infection, a relatively lower viral load in the chronic phase of infection has been observed with no clinical signs of disease for 1 year (59). These results demonstrate that the cytoplasmic domain of the transmembrane protein is required for disease expression in juvenile macaques. When we studied the influence of long terminal repeat (LTR) and envelope (Env) on the virulence phenotype of EIAV by substituting the corresponding region of virulent p19/wenv17 clone with avirulent clone pSPeiav19, we found both Env and LTR is required for disease expression (47,48). We were interested in determining if the cytoplasmic tail domain of the EIAV transmembrane protein was required for acute disease expression. $1.5x10^5$ cpm p19/wenv17Δp20 virus was used to infect two EIAV-negative Shetland ponies by intravenous injection. Another two EIAV-negative Shetland ponies were infected with $1.0x10^5$ cpm parental p19/wenv17 virus as a control. Acute disease was observed from the ninth day post infection (dpi) for both p19/wenv17 virus infected ponies and the high fever did not subside at 15 dpi when the ponies were killed. In contrast, for ponies infected with p19/wenv17Δp20 virus, no temperature increase was induced for the two ponies during the observation period of more than seven weeks (Figure 12, page 118). Virus specific antibodies were assessed with a commercial ELISA assay utilizing

synthesized peptides for transmembrane and capsid protein and quantified by the OD value at 480nm wavelength. All animals exhibited detectable antibody responses by the second week after infection. The OD value for ponies infected with p19/wenv17Δp20 virus peaked at 13 dpi. After that time, the antibody level was maintained for the total observation period (Figure 13, page 119). Viral loads in infected ponies were detected from plasma samples by an established Taqman RealTime PCR protocol, which amplified a 100bp gag fragment. The threshold sensitivity of the assay is approximately 10 copies. The level of viral load remained stable between 10^2-10^5copies/ml plasma for both p19/wenv17Δp20 virus infected ponies (Figure 14A, page 120). In contrast, for ponies infected with p19/wenv17B virus, the viral load started from 10^4-10^5 copies/plasma samples before the disease period and reached 10^6-10^8 copies/ml plasma during febrile episodes, which was about 10^1-10^3 fold higher than that of p19/wenv17Δp20 infected ponies (Figure 14B, page 120). This reflected again that the higher level of viremia was correlated with disease expression.

Envelope incorporation into virions of p20-truncated virus

Lentivirus infectivity depends on the functional and structural integrity of the envelope glycoproteins. For enveloped viruses, the incorporation of viral glycoproteins into budding virions is essential for the production of infectious virus particles (12, 22). We were interested in determining if our cytoplasmic tail mutants of EIAV displayed a difference in the relative incorporation of envelope into virions by comparing the ratio of surface unit (SU) protein gp90 to capsid protein p26. Virions were pelleted from supernatants of FEA cells infected with pSPeiav19or pSPeiav19Δp20 virus through a 10% sucrose layer by ultracentrifugation and virus particles were subjected to SDS-

PAGE and Western-Blot analysis with horse anti-EIAV serum as primary antibody. The concentration of surface unit (SU) gp90 and capsid protein p26 was determined by visualizing the density of specific bands. When a certain amount of virions for each virus was loaded, we observed a higher ratio of gp90 to p26 for p20-truncated virus compared with that for parental virus (Figure 15A, page 121). This result indicated that there was a higher amount of envelope incorporation into per virus particle for p20-truncated virus than that for parental virus. To further estimate the level of SU incorporation for p20-truncated virus, pSPeiav19 parental virions were diluted 2- and 4-fold. The highest dilution (4-fold) provided an equal amount of p26 proteins in the virion with that of the loaded pSPeiav19Δp20 virus. The undiluted parental virus had an approximately equal density for gp90 band with that of the loaded pSPeiav19Δp20 virus. The results indicate that at least a 4-fold increase of SU incorporation into virions for pSPeiav19Δp20 virus was found compared with that for parental pSPeiav19 virions (Figure 15B, page 121). In repeating the experiment, the increase for the envelope incorporation into virions of pSPeiav19Δp20 is around 5-fold compared with that of parental pSPeiav19 (data not shown). We were unable to compare the ratio of SU to CA proteins for p19/wenv17 and p19/wenv17Δp20 virus because it is hard to obtain enough p20-truncated virus and interference of the media (RPMI 1640+autologous horse serum) for macrophages with the primary antibody for Western-blot analysis. FEA cells have not been found to select virus with a truncation of the cytoplasmic domain and in fact, we have observed reversion of the pERΔp20 termination codon during passage *in vitro* before (3). This suggests that FEA cells may favor the growth of virus with a full-length cytoplasmic tail.

Relative specific infectivity of p20-truncated virus

The entry of lentivirus particles into target cells is mediated by interaction of the viral Env proteins on the virion surface with a cellular receptor or receptors. The cellular receptor for EIAV is still unknown though we have found that the virus can attach and enter a variety of cell types including human, avian, fish and amphibian by detecting the presence of proviral DNA within these cells upon infection (our unpublished data). The higher-level incorporation of envelope proteins into virions for p20-truncated virus compared with parental virus could provide the virus with more chances to bind cellular receptors and correspondingly increase the infectivity as observed in the case of SIVmac with truncated cytoplasmic tail in a human T-cell line (69). However, what we observed is a delayed and lower level replication for p20-truncated virus in FEA cells and equine macrophages as well as lower viral loads *in vivo*. This observation led us to test the possibility that p20-truncated EIAV may be immature, defective or less efficient for infection, possibility due to an altered conformation of the envelope proteins. To test if the p20-truncated EIAV virus is defective in infection, we measured the relative specific infectivity of this mutant virus by infecting FEA cells in 24-well plates with serially diluted pSPeiav19Δp20 or parental pSPeiav19 virus. Reverse transcriptase (RT) activity of supernatant for each dilution was tested at 7 days post infection. The reason we use pSPeiav19Δp20 instead of p19/wenv17Δp20 is still because of the difficulty to control the quality of equine MDMs. FEA cells favor the full-length cytoplasmic tail and a single premature stop codon used to truncate p20 could be shown to revert in FEA cells (3). In the experiment, the diluted virus used was from 5000 cpm to 156 cpm with a 2-fold serial dilution. As observed for p19/wenv17Δp20 virus, the replication of pSPeiav19Δp20 was

lower (about 2-10 fold) than that for parental virus. Based on the RT activity of uninfected FEA cells in 10 separate experiments, the threshold for positive samples is determined as 484 cpm by the average of the ten samples plus (a two-fold) standard deviation. The results show that the relative specific infectivity is 8 $TCID_{50}$/5000 cpm for pSPeiav19Δp20 virus and 16 $TCID_{50}$/5000 cpm for parental pSPeiav19 (Figure 16, page 122). This indicated that the relative specific infectivity for p20-truncated virus is only two-fold lower than that for parental virus. This 2-fold difference is really not extraordinarily high especially after considering the delayed replication for p20-truncated virus as observed for p19/wenv17Δp20 virus. This suggests that p20-truncated EIAV virus does not have a severe defect in replication and the observed delayed and lower replication could be the result of inefficiency in one or more steps in the virus life cycle.

Efficiency of p20-truncated virus in the early and late phase of the life cycle

Since the delayed and lower level of replication for p20-truncated virus compared with the parental virus is not caused by impaired envelope glycoprotein incorporation into virions and a severe defect in replication, it is suggested that the delayed and lower level replication may be a result of inefficiency or delay occurring in several steps of the virus life cycle. Under this hypothesis, we designed an experiment to determine if the delay occurs before or after virus genome integration and transcription. Our previous experience with the p19/wenv17 clone indicated that even though transient transfection to FEA cells could produce a certain amount of virus, these virions (or p19/wenv17 virus) didn't replicate after reentering and integrating into the host genome due to lack of the appropriate transcription factors for the p19/wenv17 LTR in non-macrophage cell types (37). We transfected FEA cells with the same amount of p19/wenv17 and

p19/wenv17Δp20 plasmid DNA on 24-well plates respectively and monitored the reverse transcriptase (RT) activities in each well from the supernatant fluid at 3, 4, 5 days post transfection. The RT activity for p19/wenv17Δp20 transfection was about 2-fold lower than that for p19/wenv17 transfection, which indicates the delay of virus replication for p20-truncated virus occurred after virus genome transcription including translation, assembly and maturation (Figure 17A, page 123). In another experiment, 2000 cpm p19/wenv17 and p19/wenv17Δp20 virus were treated with DNase I and then used to infect equal number of FEA cells in 24-well plates. At 4 and 8 hours after infection, nuclear DNA was extracted from each well and 100 ng of nuclear DNA was used as template to amplify 100 bp of the EIAV *gag* gene under identical condition. The viral genome can be detected in the nucleus at four hours after infection for both p19/wenv17 and p19/wenv17Δp20 viruses. This indicates that there is no delay occurred for p20-truncated virus in the early phase of the life cycle (Figure 17B, page 123). To quantify the viral DNA entering the nucleus, 10,000 cpm pSPeiav19 and pSPeiav19Δp20 virus were treated with DNase I and then used to infect equal number of FEA cells in 35x10 mm flasks (FALCON 1008) for 4 hours. Copies of proviral DNA in 100ng nuclear DNA were compared by a SYBR RealTime PCR assay. The result also indicates that there is no significant difference (p=0.09) for the number of proviral DNA in the nucleus between the two kinds of viruses (Figure 18, page 124).

Binding efficiency of p20-truncated virus.

The cytoplasmic tails of primate lentivirus transmembrane protein have been shown to change fusion activity and neutralization sensitivity (10, 69). We were interested to know if truncation of cytoplasmic tail of EIAV would affect the virus binding to the cellular

receptor. To test this question, an established virus binding assay was used. Based on the comparison of RT activity change between the wells containing cells and wells without cells in 24-well plates after virus inoculation, it was indicated that this assay could detect the virus binding on the cells ($p<0.05$) (Figure 19A, page 125). We also observed that incubation for 1 hour after inoculation with 0.15ml culture supernatant containing ~50,000-100,000 cpm virus was a suitable time for detecting virus binding (data not shown). When the binding efficiency of pSPeiav19Δp20 virus was compared with that of parental pSPeiav19 virus, there is no significant difference ($p>0.05$) between the two viruses for binding on the cells (Figure 19B, page 125).

Discussion:

We demonstrated in this report that the expression of EIAV p20 is not essential for virus replication. This observation is consistent with our previous observations (3). We tested the role of p20 *in vivo* by introducing three termination codons into the N-terminal coding region of p20 on the background of the virulent p19/wenv17 EIAV infectious molecular clone. The virus derived from the p20-truncated mutant replicates in a delayed and lower level *in vitro* compared with parental virus as we have observed from previous studies (3). Shetland ponies were asymptomatic when infected with a high dose of p19/wenv17Δp20 virus for more than seven weeks. These results indicate that p20 is essential for acute disease expression.

The replication of SIV and HIV-1 mutants with truncated cytoplasmic tails is cell-type dependent (8, 20, 21, 25, 61, 64, 67). *In vitro,* selection favors a full-length cytoplasmic domain in certain situations and a shorter one in others. For example, passage of SIVmac in human T-cell lines selects for variants with premature stop codons that truncate the

cytoplasmic tail (25) while the truncated cytoplasmic tail will rapidly revert to full-length expression during replication in infected macaques (21,25,33). A single stop codon located within the cytoplasmic tail of SIV transmembrane protein would be expected to revert rapidly *in vivo* in rhesus macaques (21, 24, 25). These results indicate a selective advantage to SIV replication conferred by the full-length cytoplasmic tail *in vivo*. For EIAV, some cells producing a tissue-culture-adapted strain have been found to synthesize a truncated envelope precursor polyprotein and the point of truncation is just downstream from the membrane-spanning domain, close to the cleavage site of p20 (56). Since SIV and HIV replication may favor the full-length cytoplasmic tail in the natural target cells *in vivo*, in the design of truncation of p20, we introduced three termination codons in order to increase the difficulty for reversion. Previous studies in our laboratory have indicated that the introduction of a single premature stop codon within the N-terminal coding region of p20 results in reversion upon multiple passages *in vitro* (our unpublished results). In the case of SIVmac239, if only one premature stop codon is used to truncate the cytoplasmic tail of transmembrane protein, the time for reversion in juvenile macaques is about 8 weeks post inoculation (59). If three stop codons plus a +1 frame-shift along with mutation of three highly conserved, charged residues in the conserved C-terminal alpha-helix (LLP-1) are introduced at the same time, the mutants can stand from 25 to 66 weeks with an average of about 40 weeks in juvenile macaques and more than 24 weeks in neonate macaques before reversion (59). Because virus derived from the parental p19/wenv17 EIAV clone is highly virulent and can cause death within about two weeks post infection when administered at high dose (47), we presume that the three termination codons used to truncate p20 should be sufficient for testing the

role of p20 in acute pathogenesis (less than 50 days post-infection) based on the reversion time obtained from SIVmac239 in juvenile or neonate macaques. During the course of animal experiment, we have not found reversion of the three termination codons.

The cytoplasmic tail of the HIV-1, HIV-2, SIV and EIAV transmembrane protein contains two or three highly hydrophobic domains with the potential to form an amphipathic alpha helix (34, 63). These segments are positively charged and rich in arginine residues. They have the capacity to cause cell lysis through cell membrane destabilization (34,63). Synthesized peptides corresponding to the conserved α-helix forming peptide segments of HIV-1, which are designated as lentivirus lytic peptides (LLP) could mediate killing of both prokaryotic (Staphylococcus) and eukaryotic cells (HUT-78 cells) when added exogenously and at relatively high concentration (38). The toxic properties of the LLPs are not restricted to a specific cell type and are considered to contribute to the cytopathicity and necrosis of cells infected with HIV-1 (39). However, results from our previous studies showed that virus with a truncated-p20 could still induce a cytopathic effect (CPE) in eMDMs (3). This phenomenon was also observed from the p19/wenv17Δp20 virus in this study (data not shown). So it is suggested that the amphipathic α-helices of EIAV CT are not required for cytopathic effect in EIAV infection. High levels of unintegrated proviral DNA, possibly resulting from cell superinfection (16, 18, 43), virus-specific mRNAs (35, 53) and direct action of *env* gene products (27), correlate with EIAV cytopathicity.

The cytoplasmic domain of HIV-1 and SIV transmembrane protein was confirmed to modulate the envelope incorporation into cell membrane and virions (12, 14). Minor alterations in the conserved C-terminal region of the gp41 cytoplasmic tail of human

immunodeficiency virus type 1 (HIV-1) affected the infectivity that correlates for most but not all constructs with a decrease in glycoprotein incorporation (51). This modulation was thought to correlate with the interaction between the cytoplasmic tail of the transmembrane domain and the matrix domain of Gag. The evidence of this interaction is the observation that the Env incorporation block imposed by mutations in the HIV-1 and SIV matrix domain of the Gag polyprotein can be reversed by expression of an Env protein with a short TM cytoplasmic tail (11, 12, 14). The phenomenon of higher level envelope incorporation into virus particles for pSPeiav19Δp20 virus compared with parental virus was itself not surprising. Enhanced envelope density on particles was observed previously for an infectious molecular clone from SIVmac with a premature stop codon within the cytoplasmic domain in certain cloned human T-cells, which selected truncated CT virus *in vitro* (25). Accompanying the higher-level envelope incorporation into virions, it has also been observed that the entry, fusogenicity and infectivity of the CT truncated virus increased in HUT.78 T cells (69). However, what we observed was a delayed and lower level replication of p19/wenv17Δp20 virus and higher levels of envelope incorporation into virions. The less efficient replication was also observed for the SIVmac virus with a truncated cytoplasmic tail of transmembrane protein in primary rhesus PBMCs though virion envelope protein incorporation was not measured (59). It is reasonable to predict that a higher level of incorporation of envelope into virions will increase the binding with cellular receptors and entry into cells. This seemingly contradictory result (lower levels of virus replication) also implies that the reason for delayed and low level replication of p20-truncated virus occurs during the post-entry step of the virus life cycle. Of course, since it has been observed that

truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain and also results in increased neutralization sensitivity of envelope protein (10), we cannot exclude the possibility that truncation of the cytoplasmic domain may change the conformation of surface unit protein which may affect its entry into cells. However, based on the detection of binding efficiency, there is no significant difference between the pSPeiav19Δp20 virus and parental virus for binding on the cells. This result suggests that truncation of the cytoplasmic tail of EIAV transmembrane protein does not affect the envelope regions that are critical for the cellular receptor (coreceptor) binding.

Lentivirus envelope proteins expressed at the cell surface will be monitored by the cellular and humoral immune responses and infected cells will be killed. So it is advantageous for viruses to minimize the surface expression of their envelope proteins in infected cells. Endocytosis is a mechanism used to keep the number of exposed envelope proteins in check and retrograde those that are not packaged into virions. Glycoproteins subjected to endocytosis often contain conserved specific motifs on their cytoplasmic domain that will be recognized during their interaction with clathrin adaptor proteins and formation of clathrin-coated pits (1, 2). The amino acid motifs YXXΦ (Y,Tyr; X, any amino acid; Φ, amino acid with a bulky hydrophobic side chain) and dileucine motifs are two kind of endocytosis signals that have been identified on the cytoplasmic tails of retroviral transmembrane proteins (6). In the case of HIV-1, SIV, HTLV-1 (human T-cell leukemia virus), MuLV, MPMV, RSV and BLV (bovine leukemia virus), the cytoplasmic domain of TM has been demonstrated to bind, via two highly-conserved tyrosine motifs (YXXΦ), to the μ2 of the clathrin adapter protein AP-2 at the plasma membrane and μ1 of AP-1 at the trans-Golgi network (2,44). This YXXΦ motif cannot

be found on the cytoplasmic tail of EIAV but at the C-terminal of p20, there are two YXXR motifs, which are conserved for all the known EIAV strains. This YXXR motif can be used as an alternative internalization signal by influenza virus hemagglutinin and membrane-type 1 matrix metalloproteinase (MT1-MMP) (29, 62). The increased (~5-fold) envelope protein incorporation for p20-truncated virus compared with parental virus implies that there may be internalization signals, possibly the YXXR motifs, existing within the cytoplasmic domain of EIAV because the direct effect of loss of the endocytosis signal is a higher level expression of envelope protein on the cell surface(68). When we only truncated the C-terminal 56 amino acids of p20 by physically deleting this region from the virus genome, which contained the two YXXR motifs, we also observed a higher ratio of surface unit (SU) proteins gp90 to capsid protein p26 compared with parental virus (data not shown). In a recent publication, one of the CT mutants, SIV_{239} E767stop, has been shown to increase the surface unit (SU) protein incorporation into virions for 20- to 50-fold (68). We do not find this high level increase of envelope incorporation for p20 truncated virus possibly because other potential endocytosis signals may exist within the region upstream of p20 within the cytoplasmic tail. Sequence alignment has indicated that there is a conserved dileucine motif proximal to the membrane anchor region of EIAV. Disruption of the tyrosine-dependent endocytosis signal in the cytoplasmic tail of SIV TM could cause attenuation of the virus *in vivo* (46). It is suggested that truncation of the cytoplasmic domain containing the endocytosis signals may increase the envelope expression on the infected host cell surface and render the infected cells more sensitive to immunological control. Another possibility related to the attenuation is that truncation of the cytoplasmic domain may cause conformational

changes in the surface unit protein exposing conserved epitopes which are sensitive to neutralization. It has been observed that truncation of the cytoplasmic domain of HIV-1 induces exposure of conserved regions in the ectodomain and results in increased neutralization sensitivity of envelope protein (10). Even though for EIAV, it has been determined that the clearance of the primary plasma viremia correlates with the emergence of EIAV-specific CD8 cytotoxic T lymphocytes (CTLs) and non-neutralizing EIAV-specific antibodies (41), it is possible that p20-deleted virus could be more efficiently controlled by neutralizing antibodies.

The viral load detected by Taqman RealTime PCR for p19/wenv17Δp20 infected ponies was about 10^1-10^3 fold lower than those infected by parental virulent p19/wenv17 parental virus during disease episode. This difference was compatible with that obtained from ponies infected by S2 deleted virulent EIAV (EIAVΔS2), which also did not induce disease in ponies (30). The viral load difference in juvenile rhesus macaques between viremia and nonviremic stages and in neonatal rhesus macaques between acute phase and chronic phases infected by SIVmac with truncated cytoplasmic tail of transmembrane protein (SIVmac-M4) also showed the same pattern (59). Since horses infected with the EIAVΔS2 attenuated virus were protected from intravenous challenge with virulent virus (32) and SIVmac-M4 could also provide partial protection from mucosal challenge with pathogenic SIVmac 251 (60), it will be of interest to evaluate the potential of the p19/wenv17Δp20 as a candidate for an attenuated vaccine.

In the case of HIV, although it has generally been assumed that immature HIV particles are competent for entry into target cells, HIV-1 may have evolved a mechanism to prevent premature entry into target cells (42, 65). Maturation is also essential for virus

replication and the inhibition of proteases by mutation or drugs results in the production of immature virions, which are noninfectious (40, 49). Protease inhibitor acts during a late stage of virus replication (maturation) and the progeny virions are impaired for early post-entry steps of infection (40, 49). Recent studies on the role of HIV gp41 cytoplasmic tail on fusion activity show that the interaction between unprocessed Gag and the gp41 cytoplasmic tail suppresses fusion and truncation of the gp41 cytoplasmic tail reversed the fusion defect (42). The significance of this suppression has been suggested by another report that binding of the gp41 cytoplasmic tail to Gag within immature HIV-1 particles inhibits envelope protein conformational changes on the surface of the virion that are required for membrane fusion (40). This implies that the cytoplasmic tail of HIV gp41 could play a key role in the virus life cycle by inhibiting the production and the following entry of immature, noninfectious particles (65). Based on the detection of relative specific infectivity, efficiency of nuclear entry of proviral DNA and binding efficiency, for pSPeiav19Δp20 virus, there is no severe defect in virus replication and no delay occurred in the early phase of the life cycle. In contrast, a reduction in virus yield has been found in the late phase of the life cycle. This suggests that there is delay occurred in the virus particles morphogenesis for pSPeiav19Δp20 virus. Based on the observation from primate lentiviruses that interaction existed between matrix protein and C-terminal of cytoplasmic tail (11, 12, 14), our further experiments would test the hypothesis if the assembly of viral core with envelope protein for pSPeiav19Δp20 virus is defective and inefficient.

Virus derived from p19/wenv17Δp20 replicates at about 2-fold lower than that of parental p19/wenv17 virus *in vitro*. While *in vivo*, this difference has been increased to

about 10-1000 fold. Since there was only a small amount of virus used to initialize infection, it was predicted that the 2-fold difference *in vitro* could be expanded after multiple round of virus replication *in vivo*. On the other hand, the relatively lower level replication of p19/wenv17Δp20 virus could stimulate the host to establish immunological control over virus replication. In this regard, it is reasonable to consider testing p19/wenv17Δp20-derived virus as a potentially useful vaccine candidate.

After we have found that p19/wenv17Δp20 virus was attenuated *in vivo*, a number of interesting questions have emerged. It is unclear which step or steps in the virus life cycle is/are affected due to absence of the p20 region. Our results indicate that endocytosis signals exist within the cytoplasmic tail based on the observation of an increased level of envelope incorporation into virions for p20-truncated virus. Further studies are needed to determine if the non-standard YXXR motifs function as endocytosis signals. It would be interesting to determine the immune response and protection induced by infection with the p19/wenv17Δp20 virus against virulent virus challenge. It would also be useful to determine if the loss of cytoplasmic tail causes conformational changes on the surface unit (SU) protein.

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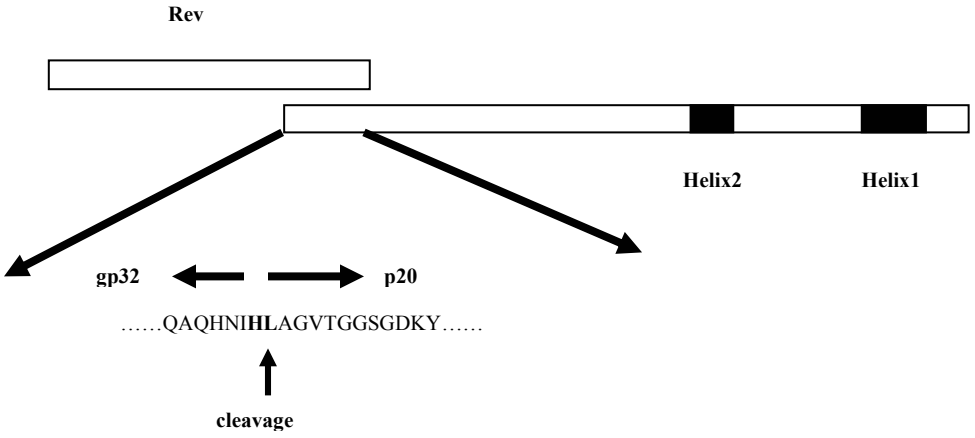
(A)



(B)

```
1     cta gca ggc gtg acc ggt gga tca ggg gac aaa tac tac aag cag   45
1      L   A   G   V   T   G   G   S   G   D   K   Y   Y   K   Q    15
                                  #1
46    aag tac tcc agg aac gac tgg aat gga gaa tca gag gag tac aac   90
16     K   Y   S   R   N   D   W   N   G   E   S   E   E   Y   N    30
                              #2              #3
```

Termination 1: tca-taa (8th amino acid), atc (Ile) to ata (Ile) for rev

Termination 2: tgg-tag (22th amino acid), ctg (leu) to cta (leu) for rev

Termination 3: tca-taa (26th amino acid), atc (Ile) to ata (Ile) for rev

(C) pSPeiavp19: HLAGVTGGSGDKYYKQKYSRNDWNGESEE

p19/wenv17: ----------R ----------

Figure 10: Truncation of p20 by introducing three termination codons to the N-terminal coding region. (a) Schematic structure of p20. (b) The p20 N-terminal coding region. "#" indicates the site for mutation. (c) Amino acid alignment of p20 N-terminal between pSPeiav19 and p19/wenv17 clones. The dash (--) indicates the identical region and underlined amino acids indicate the sites for introducing termination codons.

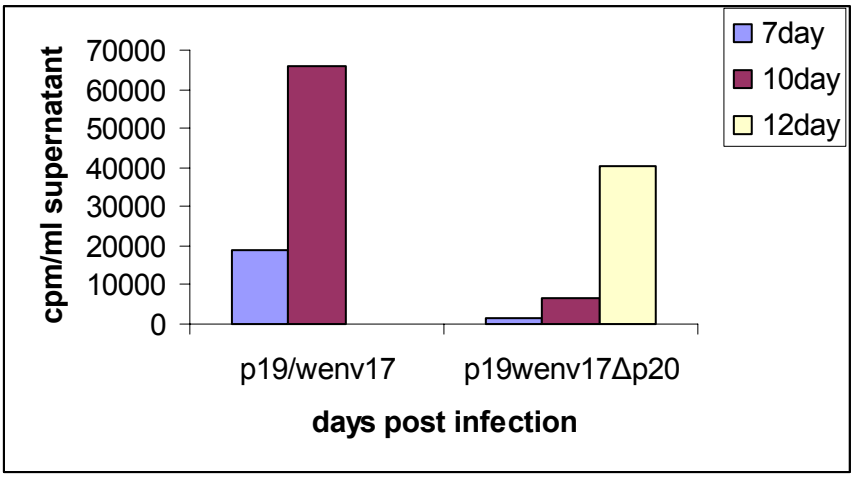


Figure 11: Replication of p19/wenv17Δ20 virus in equine macrophage cultures. About 20,000-30,000cpm p19/wenv17Δ20 or p19/wenv17 virus was used to infect equine macrophages. RT activity (RT) activity for 1ml culture supernatant was detected at 7, 10, 12 days post infection.

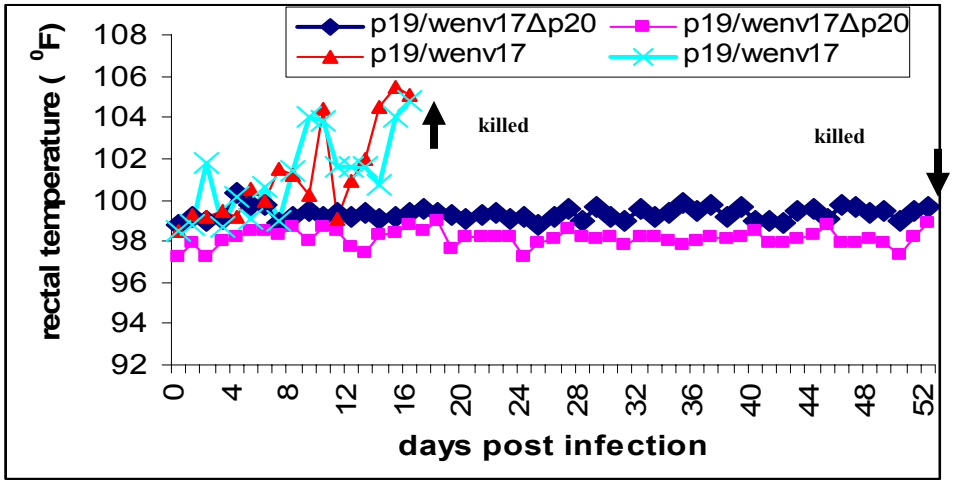


Figure 12: Clinical profiles of infected ponies. Two EIAV negative ponies (**X**,▲) were infected with $1.0x10^5$cpm p19/wenv17 and another two EIAV negative ponies (■,♦) were infected by $1.5x10^5$cpm p19/wenv17Δp20 virus. All infection was through intravenously injection. Rectal temperatures of infected animals were measured daily and disease episodes were defined as periods of significant increased body temperature. Arrows indicated the time that the infected ponies were killed.

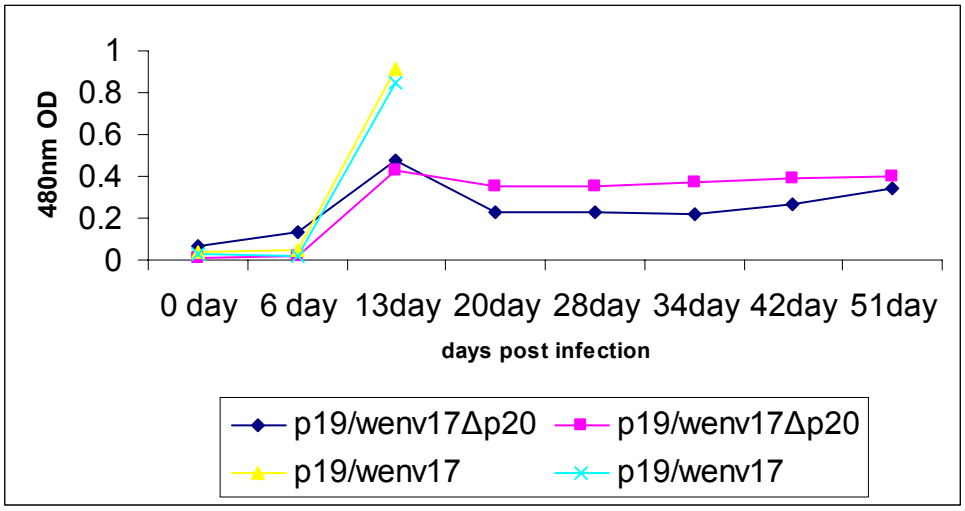
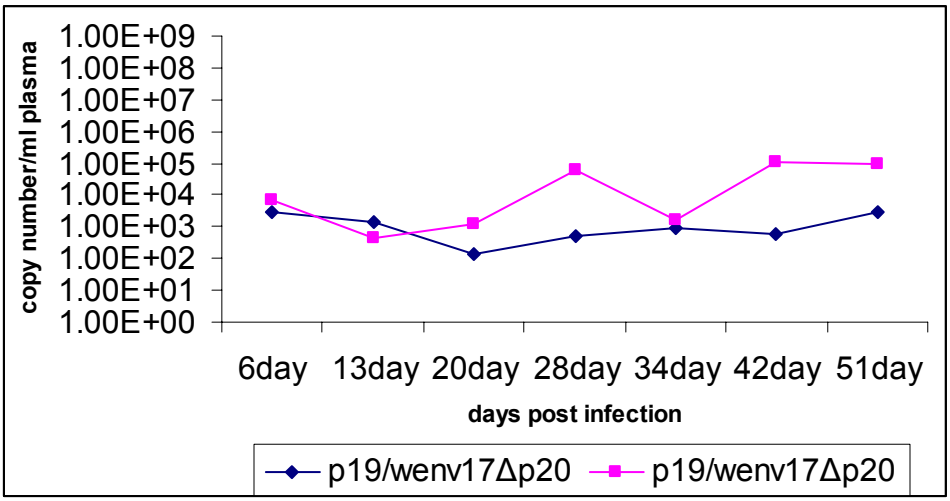


Figure 13: Antibody responses of infected ponies. Virus specific antibody responses of infected ponies were detected by commercial ELISA kit using peptides of transmembrane and capsid protein. Fifty-microliter undiluted serum samples were used to detect virus specific antibody response at indicated time points for each p19/wenv17 infected (X,▲) and p19/wenvΔp20 infected (■,♦) pony. OD values at 480nm wavelength represented the level of antibody response.

A:



B:

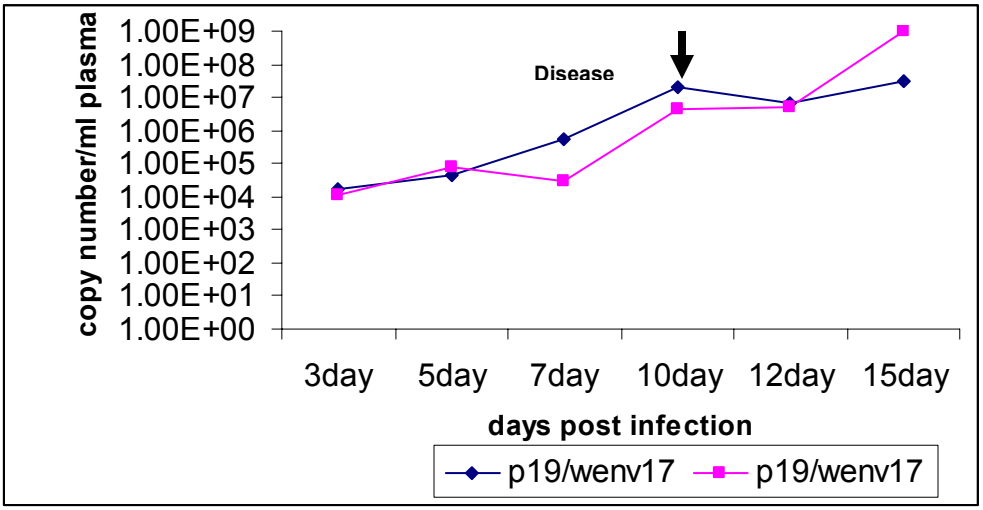


Figure 14: Viral load for infected ponies. 200 microliters plasma was used to extract viral RNA at indicated time points for infected ponies. Viral RNA corresponding to 40 microliters plasma was reverse-transcribed and cDNA corresponding to 20 microliters plasma were used for Taqman RealTime PCR. The viral copy number obtained from quantitative PCR was multiplied by 50 and presented as copy number in 1 ml plasma sample for p19/wenv17 (A) or p19/wenv17Δp20 (B) infected ponies.

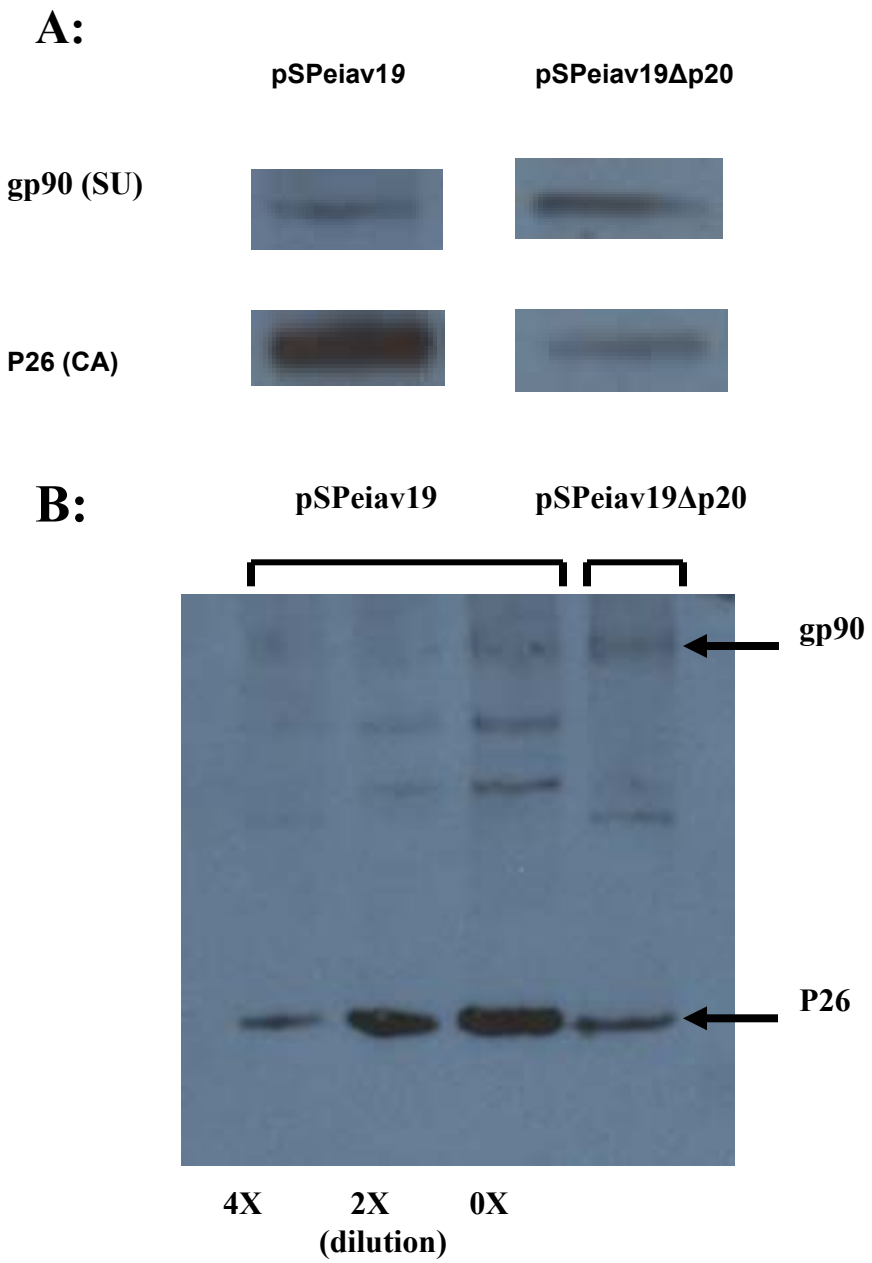


Figure 15: Analysis of envelope incorporation into virions for p20-truncated virus. Virions were pelleted from supernatants of FEA cells infected with pSPeiav19 or pSPeiav19Δp20 virus through a 10% sucrose layer by ultracentrifugation, subjected to SDS-PAGE analysis and transferred to PVDF membranes for Western-Blot analysis with horse anti-EIAV serum as primary antibody. The amount of surface unit (SU) gp90 and capsid protein p26 was determined by visualizing the density of specific bands. (A) Relative SU incorporation between pSPeiav19 or pSPeiav19Δp20 virions. (B) Estimation of the level of SU incorporation for pSPeiav19Δp20 virions. Parental pSPeiav19 virus was loaded as undiluted (0X), 2-fold diluted (2X) and 4-fold diluted (4X).

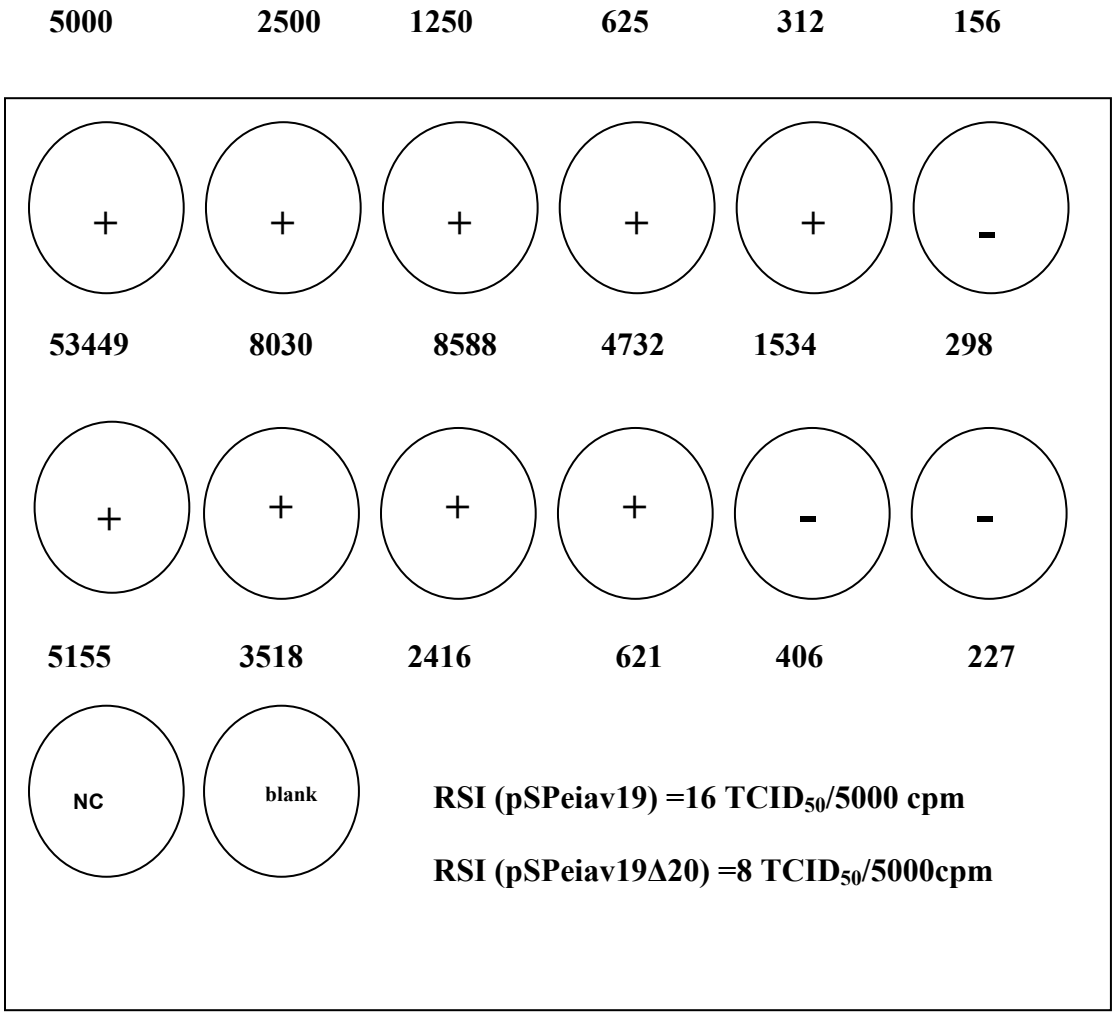
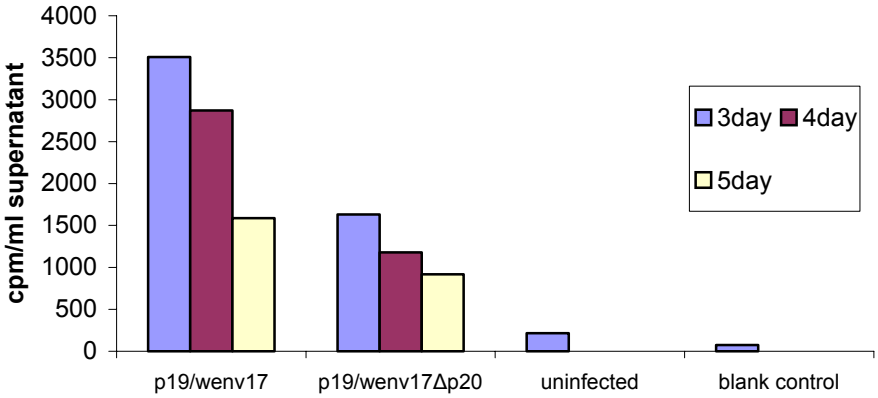


Figure 16: Detection of relative specific infectivity of pSPeiav19Δ20 virus. The pSPeiav19 or pSPeiav19Δ20 viruses were serially diluted in two-fold serials from 5000cpm to 156cpm and used to infect FEA cells on 24-well plates. At seven days post infection, reverse transcriptase (RT) activities for 1ml culture supernatant fluid from each well were detected. Unit of the number: cpm. NC: uninfected cells, "+"higher than positive threshold, "-" lower than positive threshold. Positive threshold (484cpm) was determined by the average of RT activity of 10 uninfected FEA cells plus a two-fold standard deviation.

A:



B:

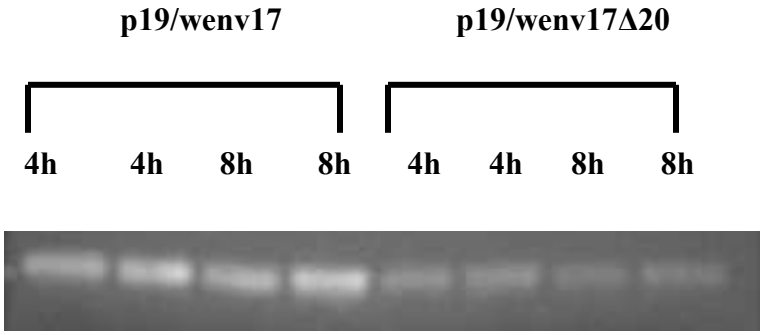


Figure 17: Efficiency of early phase and late phase of life cycle for p19/wenv17Δ20 virus. (A) 0.5ug p19/wenv17 (p19/wenv17) or p19/wenv17Δ20 (p19/wenv17) plasmid DNA was used to transfect roughly equal numbers of FEA cells on 24-well plate respectively. Reverse transcriptase (RT) activity for 1ml supernatant fluid from each well was tested at 3, 4, 5 days post transfection. Results were based on the average of two parallel samples at each time. (B) 2,000cpm p19/wenv17 (p19/wenv17) or p19/wenv17Δ20 (p19/wenv17Δ20) viruses were treated with 200U DNase for 10min at room temperature and used to infect roughly equal numbers of FEA cells on 24-well plate. At 4 hours and 8 hours after infection, nuclear DNA was extracted from the cells of each well. 100ng of nuclear DNA was used as template for amplification of a 100bp product located in viral *gag* gene.

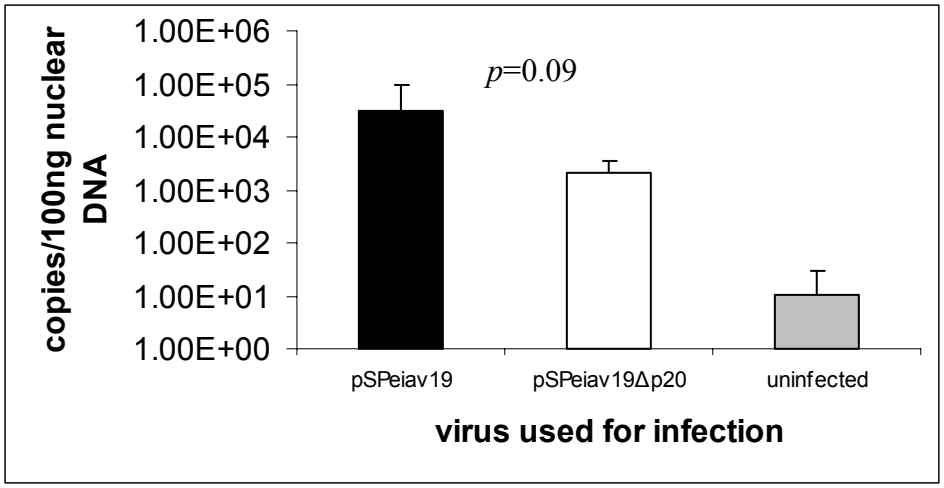
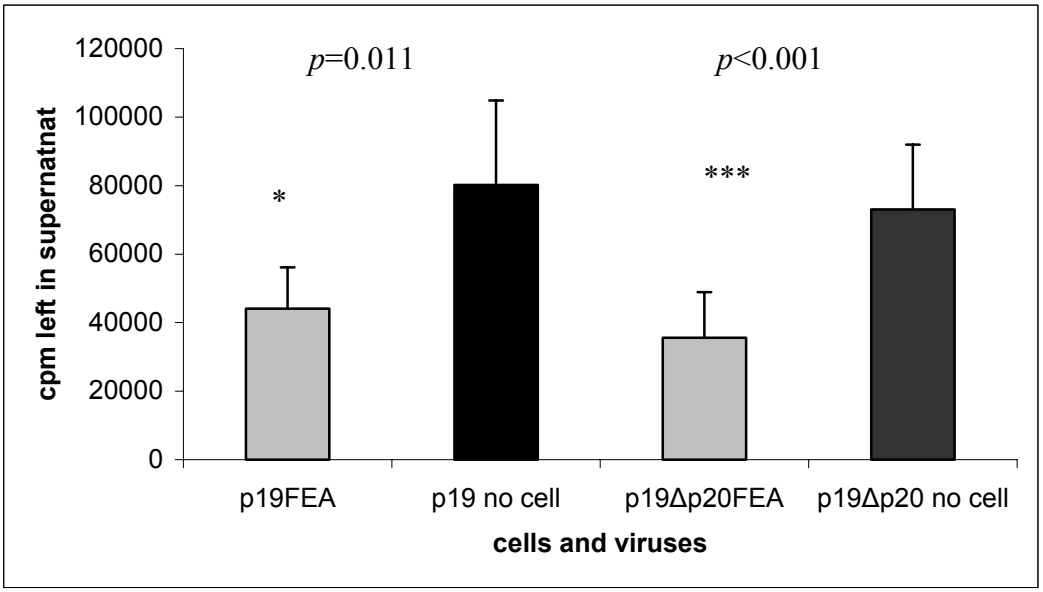


Figure 18: Quantitative comparison for nuclear entry of proviral DNA. 10,000 cpm pSPeiav19 and pSPeiav19Δp20 viruses were treated with DNase I and then used to infect equal number of FEA cells in 35x10 mm flasks (FALCON 1008). Nuclear DNA was extracted from the infected cells at 4 hours after infection. Copies of proviral DNA in 100ng nuclear DNA were compared by a SYBR RealTime PCR assay. Data were analyzed by *student t-test.*

A:



B:

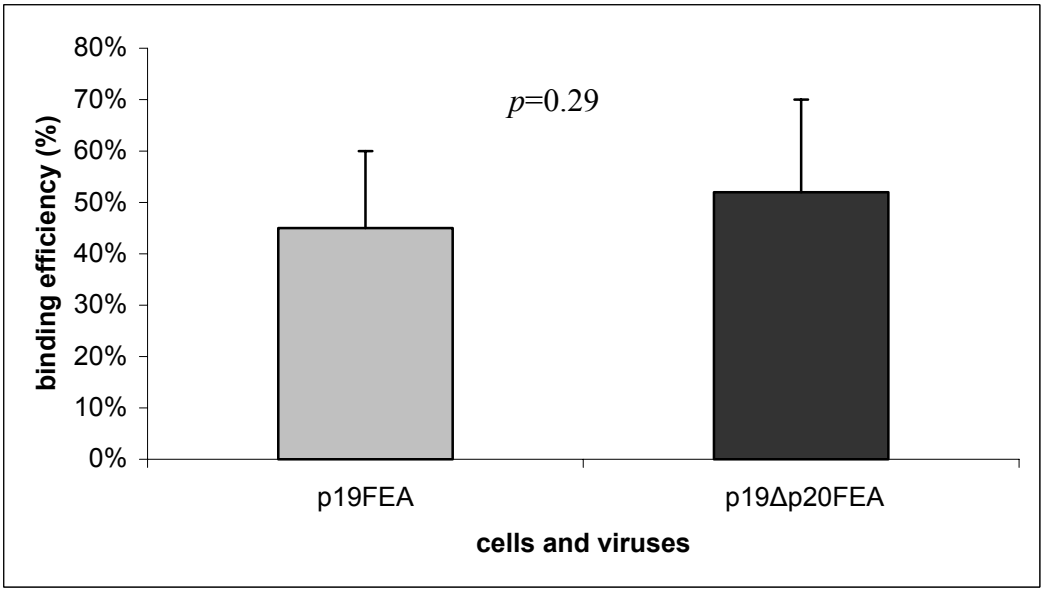


Figure 19: Binding efficiency of pSPeiav19Δp20 viruses. Parental pSPeiav19 (abbreviated as p19) and pSPeiav19Δp20 (abbreviated as 19Δp20) viruses in 0.15ml culture supernatant were used to cover equal number of FEA cells in 24-well plates. Empty wells without cells were also covered at the same time as a control. The plates were incubated at 37°C for 1 hour and reverse transcriptase (RT) activities left in the culture supernatant of each well were detected. Data were analyzed by *student t-test.* (A).Comparison of RT activities between wells containing cells and wells without cells. (B).Comparison of binding efficiency between pSPeiav19 and pSPeiav19Δp20 viruses. "*": significant, "***": strongly significant.

Role of the Cytoplasmic Tail of Equine Infectious Anemia Virus (EIAV) Transmembrane Protein Expressed in *Trans* in Replication of the Virus with Truncated Cytoplasmic Tail

Introduction

With the exception of feline immunodeficiency virus (FIV), the cytoplasmic tail (CT) of transmembrane protein (TM) for other lentiviruses is over 120 amino acids in length compared with 20 to 40 amino acids for most other retroviruses (39). The CT of lentiviruses has been shown to affect a variety of virus functions including virus replication (32), fusion activity (81), virus-induced cytopathogenicity (56), envelope incorporation into virions (49), surface envelope glycoprotein internalization (40), pathogenicity (64) and immunogenicity (20).

Although the CT domain of lentiviruses is important for optimum virus infectivity in target cells, it has been documented that it is not essential for the replication of HIV-1, HIV-2, SIV and EIAV because many isolated or cell adapted strains with partially truncated CTs can still replicate (11, 17, 24, 28, 29, 33, 59, 79). Studies on the CT mutants have shown that the ability to replicate with a truncated CT was cell-type dependent and truncation length dependent (12, 32, 34, 38, 68, 73, 76). It seems that selective pressures favor a full-length CT in certain situations and a shorter one in others.

For primate lentiviruses and EIAV, the CT domain contains conserved amphipathic *α-helices* designated as lentiviral lytic peptides (LLPs) (47, 72). The three LLPs located in the C-terminal of the CT domain of HIV-1 are thought to be associated with the inner surfaces of viral and cellular membranes (51, 72). The multimerization potential and membrane binding ability of CT for HIV-1 has been shown to play a crucial role in virus

replication (14,15,16,41) and that the N-terminal segment of LLP-1 contains structural determinants critical for modulating Env stability (42). Synthetic peptides corresponding to the conserved LLP segment could mediate killing of prokaryotic (Staphylococcus) and eukaryotic cells (HUT-78 cells) when added exogenously and at relatively high concentrations (50). Besides the contribution to the cytopathic effect of lentiviruses, the LLPs are also predicted to play other roles in the virus life cycle. Two-conserved cysteine residues, with one located within the LLP-1 region and another close to the N-terminal of LLP-2/3 of HIV-1 have been shown to be the targets for palmitoylation and reported to be required for envelope association with lipid rafts and assembly of budding virions (60). Envelope proteins lacking the two cysteines are excluded from lipid rafts. Substitution of the cysteines with alanines or serines will eliminate raft association and severely reduce envelope incorporation and infectivity (2). This result also implies that membrane targeting through LLP also has a direct effect on virus replication. In addition, HIV-1 LLP could also interact with calmodulin with its positively charged amino acids (52, 66, 69). The sequestration of calmodulin by LLP is hypothesized to interfere with the calmodulin-dependent signal transduction pathway.

Virus infectivity depends on the functional and structural integrity of the envelope glycoproteins and the incorporation of viral glycoproteins into budding virions is essential for the production of infectious virus particles (23, 35). The CT domain of the transmembrane protein of HIV has been confirmed to interact with the N-terminal region of the trimeric matrix (MA) protein (19, 44, 45, 57) and promote recruitment of trimeric Env complexes into budding virus particles at the plasma membrane, from which mature virions are released. Deletions in the CT of TM and alterations to the MA protein may

impair Env incorporation into nascent virions (18, 19, 77, 78). In addition, the CT domain is also responsible for regulating the level of envelope proteins expressed on the cell surface and recycling those that are not packaged into virions through specific endocytosis signals in their CT domain (4, 8, 54).

Several cellular factors, which are known to influence the trafficking of proteins to and from the plasma membranes, have also been found to interact with the gp41 cytoplasmic domain of SIV and HIV-1. These include the clathrin-associated adapter complex AP-1 and AP-2 (1), calmodulin (66), p115-Rho GEF (80), α-catenin (37), the prenylated Rab acceptor (21) and Tip47 (3). The envelope proteins of both SIV and HIV-1 are efficiently endocytosed in a clathrin-dependent manner. The cytoplasmic domain of SIV and HIV-1 TMs contains multiple endocytosis signals to mediate clathrin-dependent endocytosis (61, 63).

The CT domain of TM for SIV has been found to be a locus for attenuation of SIV in rhesus macaques (64). Disruption of the tyrosine-dependent sorting signal in the CT domain of SIV TM has been shown to cause attenuation *in vivo* (55). In addition, a leucine zipper motif in the cytoplasmic tail of HIV TM, which is predicted to function for membrane binding, affects the replication and pathogenesis in *SCID-hu thy/liv* transplant mice (36).

Compared with various studies on multiple functions for the CT domain of primate lentiviruses, research on the roles of CT for non-primate lentiviruses is scare. The 226 amino acid CT of EIAV is the longest CT domain of lentiviruses. EIAV is also unique among lentiviruses in that the CT domain of TM gp45 will undergo a further cleavage event to yield a N-terminal glycosylated gp32 and a C-terminal nonglycosylated 20kD

p20 protein with about 175 amino acids, which comprises more than two-thirds of the CT. Amino acid sequencing of the N-terminal p20 shows that the cleavage occurs at His/Leu site for a specific strain (59). The p20 is presumably generated after maturation of virions mediated by viral protease due to the fact that it can only be detected in virions (59). The cleavage event of EIAV within the N-terminal CT domain is similar to that of some oncogenic retroviruses such as murine leukemia virus (MuLV).These retroviruses are also subjected to a post-assembly cleavage resulting in a protein designated the R peptide. This peptide has already been confirmed to be generated within the virions mediated by the viral protease (6, 27, 75). The R peptide of MuLV is 16 amino acids in length and contains 10 conserved amino acids. Cleavage of the R peptide is required for fusion activity and deletion of the R peptide results in increased fusogenicity (7). The precleaved R peptide inhibits fusion and infectivity and this inhibition can be suppressed by introducing mutations to the upstream region of the R peptide (43, 58). EIAV infection does not form large multinucleated syncytia as other lentiviruses. Previous studies in our laboratory have shown that the cleavage of the CT domain of EIAV TM gp45 is not required for virus replication *in vitro* and mutation of the cleavage site to a non-cleavable type does not eliminate virus replication (30). We have also found that expression of p20 is not essential for virus replication. Mutants that do not express p20 still replicate though the replication is delayed compared with parental virus (5).

Genetic intervention with the viral life cycle, commonly called intracellular immunization, has been used as an alternative approach for AIDS therapy (9, 62). Transdominant negative mutants of the HIV proteins Tat, Rev, Gag and Env have been generated and shown to inhibit virus replication in cell culture systems (10,65,67,71).A

transdominant negative Rev mutants is being tested for a human clinical trial (53). The interaction between the cytoplasmic domain of transmembrane protein and the viral matrix protein has been confirmed and this interaction appears to modulate the envelope glycoprotein incorporation into virions (22, 23, 48, 74). Coexpression of a wild type HIV-1 provirus together with a truncated envelope lacking the whole CT domain of the transmembrane protein gp41 could transdominantly interference with viral replication (14). It is reported recently that HIV-1 GST-cytoplasmic tail fusion protein could incorporate into Gag virus-like particles (13). In this study, we used a p20 expression system previously established in our laboratory as the platform to study if the CT domain of EIAV expressed in *trans* can be incorporated into the progeny virions and affect the virus replication.

Materials and Methods

Cells and virus:

Feline embryonic adenocarcinoma (FEA) cells were obtained from Dr. Susan Payne at Texas A&M University. The cells were cultured in Dulbecco modified medium (DMEM) containing 5% fetal bovine serum (FBS). EIAV pSPeiav19 full-length infectious clone is a prototype derived from the cell culture adapted Malmquist strain. Viruses derived from pSPeiav19 are avirulent, replicate both in FEA cells with no cytopathic effect (CPE) and equine monocyte-derived macrophage cultures with CPE.

Construction of p20 expression clone and establishment of FEA cells expressing p20

The p20 expression vector on the background of pSPeiav19 was constructed by Dr. Hall (30). Briefly, a 525bp DNA fragment including the entire coding region of p20 was amplified from pSPeiav19 plasmid using the Ecop20AUG> (5'-CCGGAATTCAAG***ATG***GCAGGCGTGACCGGTGGATC-3') and <Ecop20END (5'-GAGAATTCCTAAACATACTGAGGCATTGATACA-3') and cloned into the EcoR I expression vector pcDNA3 (Invitrogen). For the purpose of protein expression, the first leucine residue of p20 was substituted by a methionine codon for initiation of translation (figure 21A, page 155). After orientation identification and sequencing, one of the positive clones was named pcDNA3p20. FEA cells cultured in 25mm^2 flask were transfected with approximately 1μg pcDNA3p20 by FuGene 6 transfection reagent (Roche). Cells were treated with 500μg/ml G418 at 24 hours after transfection for resistance gene selection. After establishment of the G-418 resistant FEA cells, the concentration of G418 was reduced to 200μg/ml to maintain the cells.

Immunocytochemistry.

FEA and FEA cells expressing p20 in *trans* (p20FEA) were plated in two-well chamber tissue culture slides. At 24 hours post plating, the cells were washed twice with PBS and fixed with methanol-acetone (1:1) for 10 minutes at room temperature. The cells were then stained using Histostain SAP for AP-red, Rabbit primary Kit (Zymed laboratory, Inc) strictly following the manufacturer's protocol with the rabbit anti-p20 serum (1:250) as the primary antibody. The cells were finally mounted with cover slips and visualized by light microscope.

Indirect immunofluorescence assay (IFA)

FEA and FEA cells expressing p20 in *trans* (p20FEA) were plated in two-well chamber tissue culture slides. At 24 hours post plating, the cells were washed twice with PBS and fixed with methanol-acetone (1:1) for 10 minutes at room temperature. Rabbit anti-p20 antibody was diluted to 1:250 by PBS containing 3% FBS and added into the chamber to completely cover the cells. After 60 minutes incubation in moisture box at 37°C, the slide was washed 3 times with PBS (2 minute incubation for each time) and dried at room temperature. Then the slide was covered with 1:100 diluted FITC-conjugated goat anti-rabbit antibodies (Sigma) and incubated in moisture box at 37°C for 30 minutes. The slides were washed twice by PBS and dried. Finally, the slides were mounted and visualized under a fluorescence microscope.

Construction of p20 truncated and p20 C-terminal truncated proviral clone.

Construction of the p20 truncated clone "pSPeiav19Δp20" on the background of pSPeiav19 clone was described above (page 80). For construction of the pSPeiav19 C-terminal truncated proviral clone "pSPeiav19Δhelix1", a fragment containing the full-

length 3'LTR region plus poly (A) of pSPeiav19 was amplified by using Roche High Fidelity PCR Kit. The upstream primer p20-3'PD-F (5'-GGACTAGTATAATAGAAAAACAAGGGGGGAACTG-3') with Spe I site was paired with downstream primer p20-3'PD-R (5'-GCGAATTCGGATCCTACGATCAGCCAGGTTC-3') with EcoR I site (Figure 20, page 154). The concentration of Mg^{2+} in PCR reaction is 1.5mM with 50ng p19 plasmid as template and the cycling parameters were 94°C 15 seconds, 60°C 30 seconds and 72°C 30 seconds (30cycles). Specific PCR product was purified by QIAquick Gel Extraction Kit (Qiagen) and ligated with pGEM-Teasy T-A clone vector (Promega). Ligation mixture was transformed to DH5α competent cells. Several insert-bearing clones were sequenced. One of the positive clones was cut by Spe I and EcoR I and the inserted fragment was used to substitute the corresponding fragment in pSPeiav19 subclone containing the Sph I and EcoR I fragment in pGEM-Teasy vector, which was used before in constructing the pSPeiav19Δp20 clone. Finally, the Sph I and EcoR I fragment containing physical deletion in the C-terminal p20 region was put back in the pLG338 vector containing other region of the virus genome.

Transfection and preparation of Virus Stock.

The method was as described above for pSPeiav19Δp20 (page 82).

Reverse transcriptase (RT) assay

The procedure was as described above (page 83).

Western Blot

Virions were pelleted from supernatants of infected cells through a 10% sucrose layer in TNE buffer (10mM Tris, 1mM EDTA, 0.15M NaCl) by ultracentrifugation (1 hour,

100,000g, 4 °C) and resuspended in 1X NuPage SDS sample buffer (Invitrogen). Cells were detached by trypsin, washed by PBS and resuspended 1X NuPage SDS sample buffer (Invitrogen). Virions and cell supernatant were heated at 70°C for 10min and resolved on a 4-12% NuPage Bis-Tris Gel. Proteins were transferred onto a polyvinylidene difluoride (PVDF) plus membrane (Omnicron) and the membrane was blocked overnight at room temperature on shaker with TBST containing 10% powdered milk. The membrane was incubated with rabbit anti-p20 antiserum specific for the last 20 amino acids of p20 (1:250) or horse anti-gag (1:500) as the primary antibody diluted in TBST+10% powered milk for 90 min. After two washes with TBST, the membrane was subsequently incubated with 1:5000 horseradish peroxidase (HRP)-conjugated goat anti-horse IgG (H+L) (KPL) or horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (whole Ig) (Amersham Life Science) (1:5000) for 90min. After two washes with TBST, the antigen-antibody complex were detected by ECL Western Blotting Analysis System (Amersham Life Science) and visualized through Kodak X-OMAT X-ray films.

Results:

EIAV p20 expression in FEA cells

Based on our previous studies, the expression of EIAV p20 could be detected in the perinuclear region (trans-golgi) by transfecting the pcDNA3p20 plasmid DNA into FEA cells (5, 30). To test the role of EIAV p20 expressed in *trans* in the replication of CT truncated virus, we reestablished the FEA cell expressing p20 in *trans* (p20FEA). Cellular staining by either immunocytochemistry or indirect immunofluorescence assay (IFA) confirmed a high level p20 expression and golgi localization in approximately 80-90% cells (Figure 21B, 21C, page 155). In addition, p20 could be detected at the

predicted size in a single band from the cell lysis by Western-Blotting, which indicated that p20 expressed in *trans* was not sensitive to the cellular proteases (Figure 21D, page 155).

Replication of p20-truncated virus in p20FEA cells

EIAV maintains the longest CT of transmembrane protein among all lentiviruses with about 226 amino acids and p20 occupies more than two thirds of the C-terminal CT domain with about 175 amino acids (59). Previous studies in our laboratory have shown that virus with truncated p20 still replicates though its replication is delayed and reduced compared with parental virus (5). In the above experiment, the same result was observed that pSPeiav19Δp20 virus replicated at about 2-10 fold lower in FEA cells compared with parental virus (page 94). To test if the p20 expressed in *trans* could affect the virus replication or compensate for the absence of p20 in pSPeiav19Δp20 virus, we used 1,000 cpm pSPeiav19Δp20 to infect FEA or p20FEA cells respectively. At 3, 5, 8 days post infection, 1 ml supernatant was taken out from each well for reverse transcriptase (RT) activity detection. Student t-test analysis indicated that there is no significant difference (p value >0.05) for virus replication between FEA and p20FEA cells at each time point (Figure 22, page 156). The replication of pSPeiav19Δp20 virus in p20FEA cells is still lower than that of parental pSPeiav19 virus in FEA cells (Figure 22, page 156).This indicated that p20 expressed in *trans* did not affect the replication of pSPeiav19Δp20 virus.

Replication of helix1-truncated virus in p20FEA cells

Like primate lentiviruses, the CT domain of the EIAV TM protein also contains two highly hydrophobic domains, with the potential to form amphipathic α-helices designated

lentiviral lytic peptides (LLPs) (47, 72). Both of the amphipathic alpha helix motifs of EIAV CT domain are located in the p20 region. The helix domain closest to the C-terminus of p20 is designated as helix-1 and the other one located nearest the p20 cleavage site is designed helix-2. Since p20 expressed in *trans* did not affect the replication of pSPeiav19Δp20 virus, we intended to further investigate if p20 expressed *in trans* could affect the replication of virus that only expresses the helix-2 region. For HIV-1, each of the helices has been shown to mediate cytoplasmic domain multimerization (41). Because the C-terminal coding region of p20 does not overlap with *rev*, we physically deleted the C-terminal 56 amino acids coding region containing the helix-1 from the virus genome on the background of pSPeiav19 and constructed a new proviral clone named pSPeiav19Δhelix1. Virus derived from the pSPeiav19Δhelix1 clone replicated to an even lower level than pSPeiav19Δp20 virus in FEA cells (Figure 23, page 157). When 1,000 cpm pSPeiav19Δhelix1 virus was used to infect FEA or p20FEA cells, there was no significant difference (p value >0.05) in virus replication with or without p20 expressed in *trans* up to 14 days post infection (Figure 23, page 157). If an interaction does occur between virally expressed TM and p20 expressed in *trans*, then this interaction has little or no effect on virus replication.

No incorporation of p20 expressed in *trans* into progeny virions.

Since it has been reported that HIV-1 GST-cytoplasmic tail fusion protein could incorporate into virus-like particles composed of only Gag proteins (13) and the cytoplasmic domain of HIV-1 gp41 could assemble into a high-ordered structural complex (41), we were interested in determining if the p20 expressed in *trans* could be packaged into progeny virions. Virions of pSPeiav19, pSPeiav19 Δp20 and

pSPeiav19Δhelix1 were obtained by infecting p20FEA cells respectively and subjected to Western-Blot analysis. Antibodies specific for the last 20 amino acids of p20 were used to differentiate the p20 expressed in *trans* in the progeny virions because this region is not expressed by either pSPeiav19 Δp20 or pSPeiav19Δhelix1 virus. No p20 could be detected in the virions of both pSPeiav19Δp20 and pSPeiav19Δhelix1 virus. The results showed that p20 expressed in *trans* was not efficiently incorporated into the progeny virions of CT truncated virus (Figure 24B, page 158). To further test if the p20 expressed in *trans* could be incorporated into progeny virions which maintain the full-length of p20, we used a p20 cleavage-defective mutant, pSPeiav19 P-Pp20, previously constructed in our laboratory (30). For the pSPeiav19P-Pp20 clone, the cleavage site (His/Leu) between gp32 and p20 was changed to a non-cleavage phenotype (Pro-Pro) (30). Under this condition, only the full-length transmembrane protein gp45 could be detected in the virions. The replication of the pSPeiav19P-Pp20 virus was delayed compared with that of parental pSPeiav19 virus (30).When we used pSPeiav19P-Pp20 virus to infect p20FEA cells, the p20 expressed in *trans* still could not be detected by Western-Blot in the progeny virions (Figure 24C, page 158). Also the p20 expressed in *trans* did not significantly affect the replication of pSPeiav19P-Pp20 virus (data not shown).

Discussion

The EIAV p20 region contains both of the amphipathic α-helices designated as lentivirus lytic peptides (LLPs), which are predicted to destabilize cell membranes (50). The toxic properties of the LLPs are not restricted to a specific cell type and may contribute to the cytopathicity of lentiviruses as indicated in the study of HIV-1 (52). However, the EIAV p20 did not induce cytopathic effect (CPE) in either FEA cells or

D17 cells when highly expressed in the absence of other virus proteins (5, 30). It was also observed that truncation of p20 in the virus genome did not affect the occurrence of CPE in equine macrophage cultures (30). This implies that the amphipathic α-helices of EIAV do not contribute significantly to the CPE of the infected cells *in vitro*. In the absence of other viral proteins, p20 is primarily localized in the trans-golgi (5, 30) and both of the amphipathic α-helices are required for the golgi localization (30). Western blot analysis of the concentrated supernatant of the FEA cells expressing p20 revealed that p20 was not released into the supernatant fluid. Under the selection of G418, about 80-90% of the cells were strongly positive for p20 by immunoperoxidase staining (30). The p20 expressed in *trans* maintains the same electrophoretic mobility as seen in virions (Figure 21, page 155) (5).

As we have previously observed, the delayed and lower level replication of p20-truncated virus compared with parental virus is not caused by impaired envelope glycoprotein incorporation into virions and there is also no severe defect in virus replication (Figure 15, page 121; Figure 16, page 122). Lentivirus envelope protein assembles as an oligomeric structure in rough endoplasmic reticulum (RER). After acquiring defined secondary and tertiary structure in the RER, the Env proteins are transported to the golgi complex and then transported to the cell surface, where virus assembly and budding take place. During its transit through the golgi, the envelope precursor undergoes cleavage by cellular protease into two products, the exterior surface unit glycoprotein and the transmembrane protein (reviewed in 25). A recombinant protein composed of only the cytoplasmic tail of HIV-1 Env or a *β*-galactosidase/cytoplasmic tail fusion protein of HIV-1 (both of which lack gp120, the gp41 ectodomain and the

transmembrane region) localized in a perinuclear membrane region (endoplasmic reticulum) of cells (16). In addition, it is also reported recently that HIV-1 GST-cytoplasmic tail fusion protein could be incorporated into virus-like particles (13). Since the *gag* gene is translated by free-ribosomes and the CT domain of gp41 also does not contain a signal sequence, this interaction probably occurs in the cytoplasm. So if the EIAV p20 expressed in *trans* could be incorporated into progeny virus by interacting with Gag or envelope protein, it might affect the replication of p20-truncated virus. In another words, if p20 plays a role in the post-entry steps during the virus life cycle, the incorporation of p20 expressed in *trans* into the progeny virions could possibly compensate for the absence of p20 in p20-truncated virus when the progeny viruses reentered the cells. Our results indicated that p20 expressed in *trans* did not significantly affect the replication of either p20-deleted or the helix-1 deleted viruses. The p20 expressed in *trans* also could not be detected by Western-Blot in the progeny virions of the two kinds of CT-truncated virus. In addition, even under the presence of full-length p20 region (pSPeiav19 P-P clone) in the virus genome, the p20 expressed in *trans* still could not be incorporated into the progeny virions.

Virus derived from the pSPeiav19Δhelix1 clone replicated to an even lower level than pSPeiav19Δp20 virus in FEA cells. This phenomenon is consistent with that observed in HIV-1 that truncation of the cytoplasmic tail affects the virus replication in a truncation-length dependent manner. For example, if the truncation of the CT domain of HIV-1 is 61 residues, the infectivity of progeny virus in permissive or non-permissive cell is at the lowest point. Either increase or decrease of the number of truncations from 61 residues will increase the infectivity and the increase also seems to be proportional to

the number of increased or decreased residues (32). A deletion of 144 amino acids of human immunodeficiency virus type 1, which comprise most of the cytoplasmic tail, from the C-terminus of gp41 does not affect virus infectivity, Env assembly into virions, and cytopathogenicity in MT-4 cells (68). In the case of envelope incorporation, deletion of 20 to 80 residues from the carboxyl terminus of the SIV TM cytoplasmic tail abrogated the incorporation of the Env glycoprotein into particles and correspondingly impairs the infectivity of the virus. However, further truncation of the SIV TM protein by 100 to 140 amino acids restored the ability of the Env protein to associate with the Gag particles. More interestingly, mutants bearing a 44- or 22- amino acid cytoplasmic domain were incorporated at levels significantly higher than those of the wild type Env and drastically enhanced virus infectivity with respect to that conferred by full-length Env protein (26).

Due to the technique restriction, we do not know where the p20 of parental EIAV virions locates in the cells after virus entry. Because we did not find any significant effect of the p20 in *trans* in CT truncated virus replication, it suggested that the localization of p20 naturally released after virus entry into the cells was probably different from the condition of p20 expressed individually by pcDNA3p20 in the cells. For primate lentiviruses, the change of the CT domain can affect the fusion activity (81) and induce exposure of conserved regions of envelope protein (20). This implies that CT changes could be transmitted to the ectodomain of envelope protein. It is also possible that truncation of the EIAV CT causes conformational changes of the ectodomain of envelope protein. The results of the above experiment (page 96) have indicated that there is no significant difference for the efficiency of virus binding on the cells between p20-

truncated virus and parental virus. This suggests that truncation of the CT domain of EIAV transmembrane protein does not affect the envelope regions that are critical for the cellular receptor (coreceptor) binding. Our further experiments would test the hypothesis if truncation of the EIAV cytoplasmic tail of transmembrane protein affected the neutralization sensitivity of the virus.

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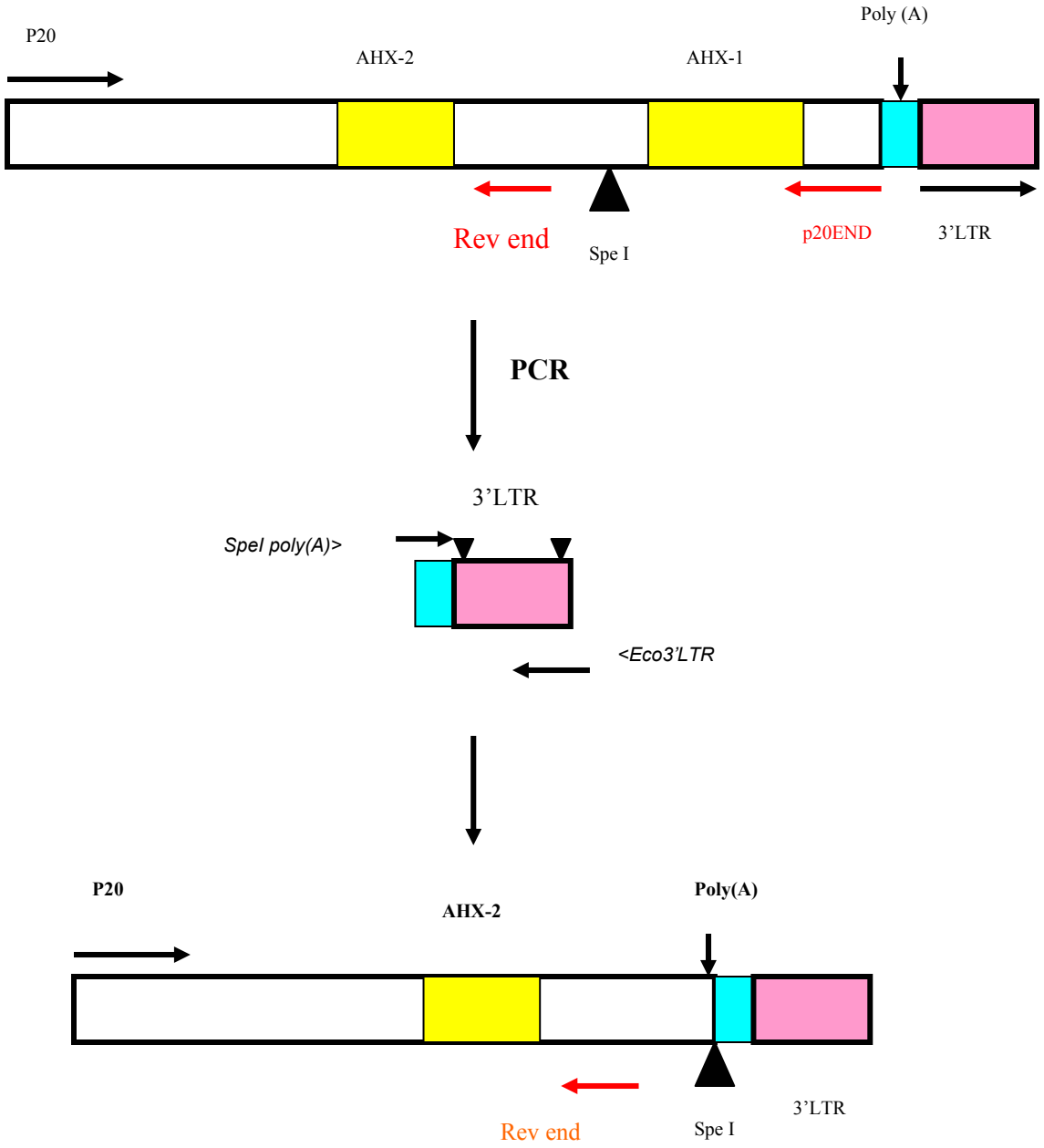

Figure 20: Schematic representation of constructing the pSPeiav19Δhelix1 clone. AHX-1: amphipathic α-helix 1. AHX-2: amphipathic α-helix 1.

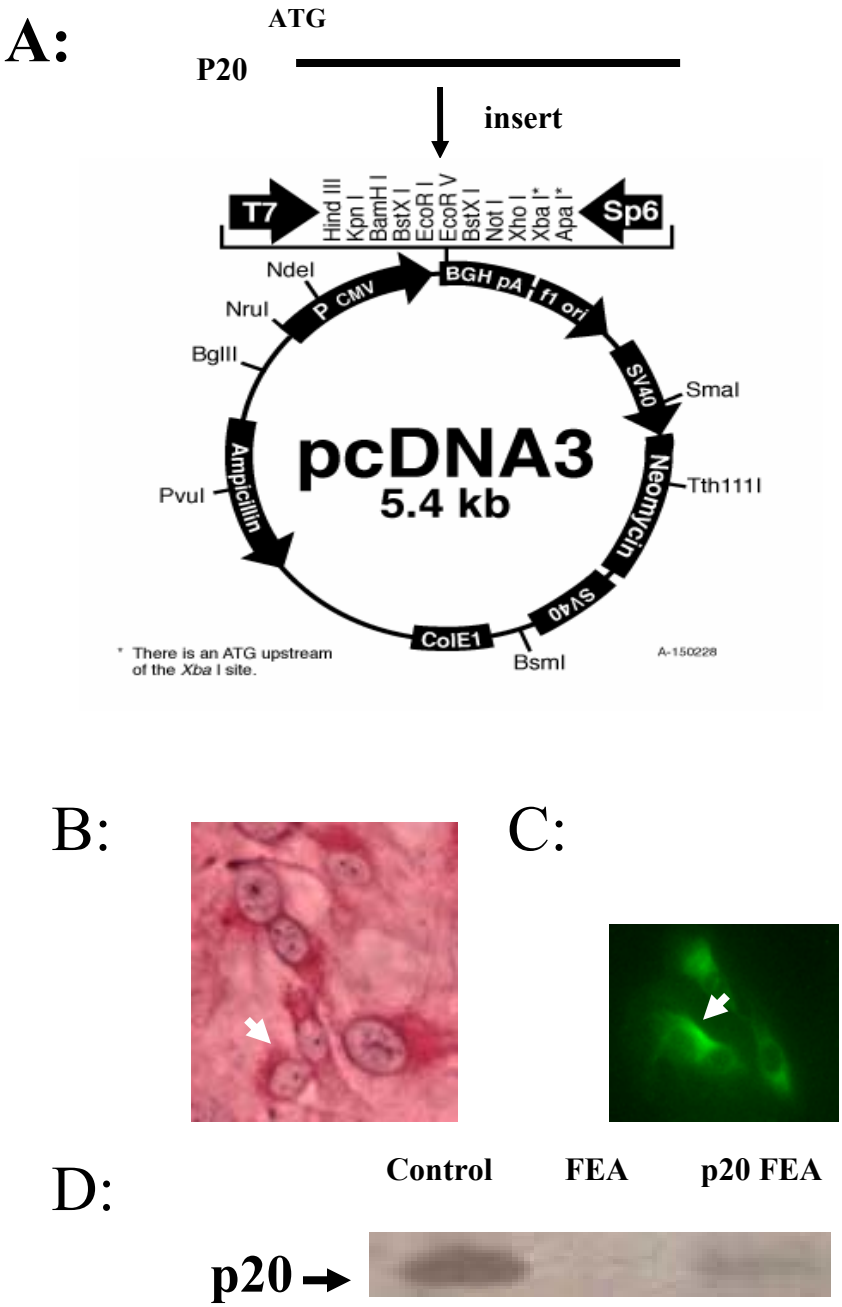

Figure 21: EIAV p20 expression in FEA cells. (A) Construction of p20 expression vector. (B-D) Expression of p20 in FEA cells detected by immunocytochemistry (B) indirect immunofluorescence assay (C) and western blot (D) using anti-p20 antibody. Control: pSPeiav19 virions.

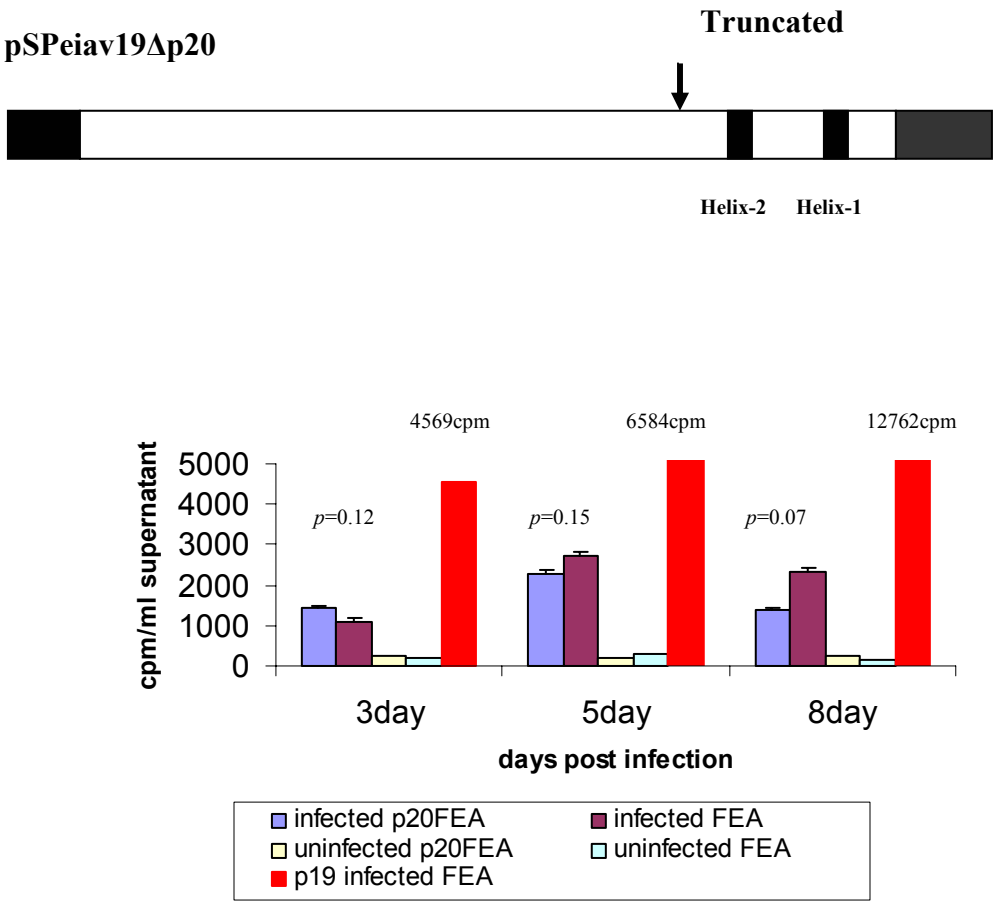

Figure 22: Replication of pSPeiavΔp20 virus in p20FEA cells.1,000cpm pSPeiav19Δp20 virus was used to infected FEA or p20FEA cells established on 24-well plate. At 3, 5, 8 days post infection, 1ml supernatant fluid was taken out from a well for reverse transcriptase (RT) activity detection. Data were analyzed by *student t-test*.

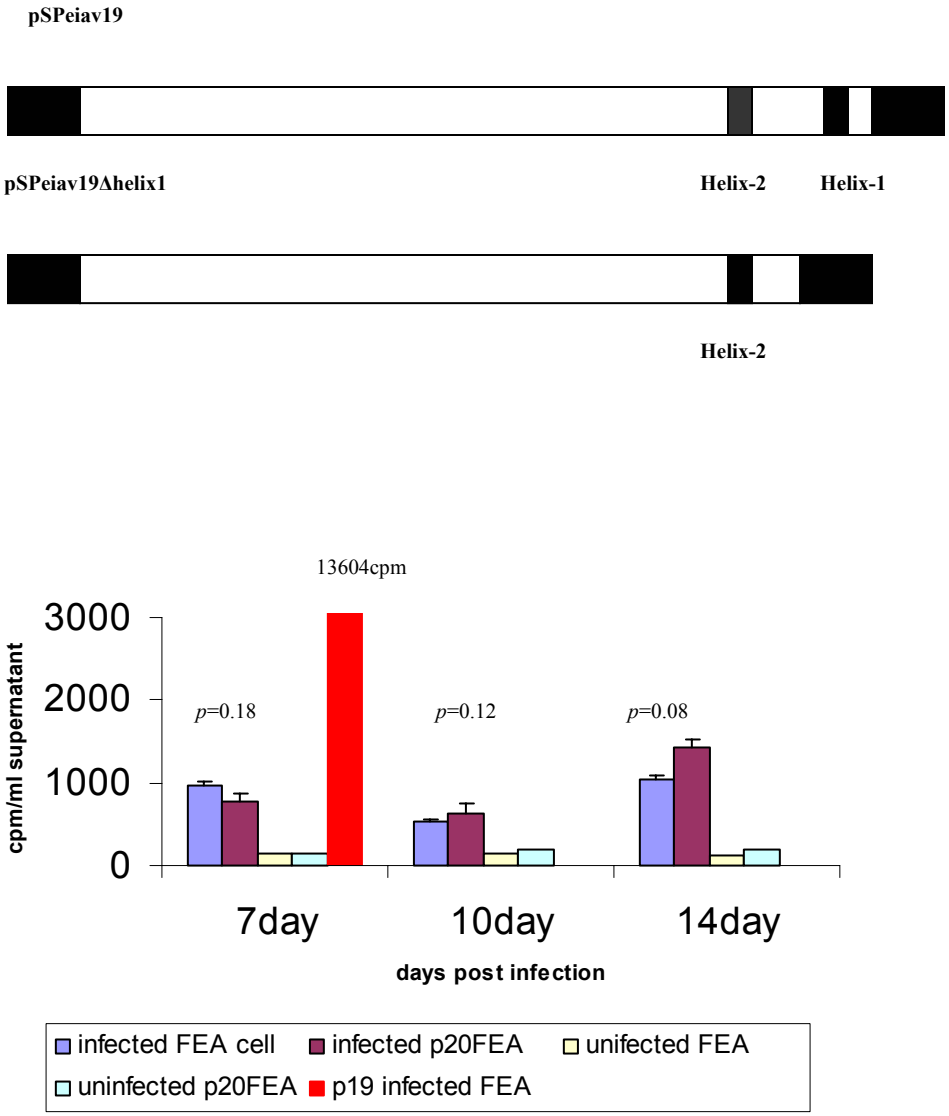

Figure 23: Replication of pSPeiav19Δhelix1 virus in p20FEA cells.1,000cpm pSPeiav19Δhelix1 virus was used to infected FEA or p20FEA cells in 24-well plate. At 7, 10, 14 days post infection, 1ml supernatant fluid was taken out from a well for reverse transcriptase (RT) activity detection. Data were analyzed by *student t-test*.

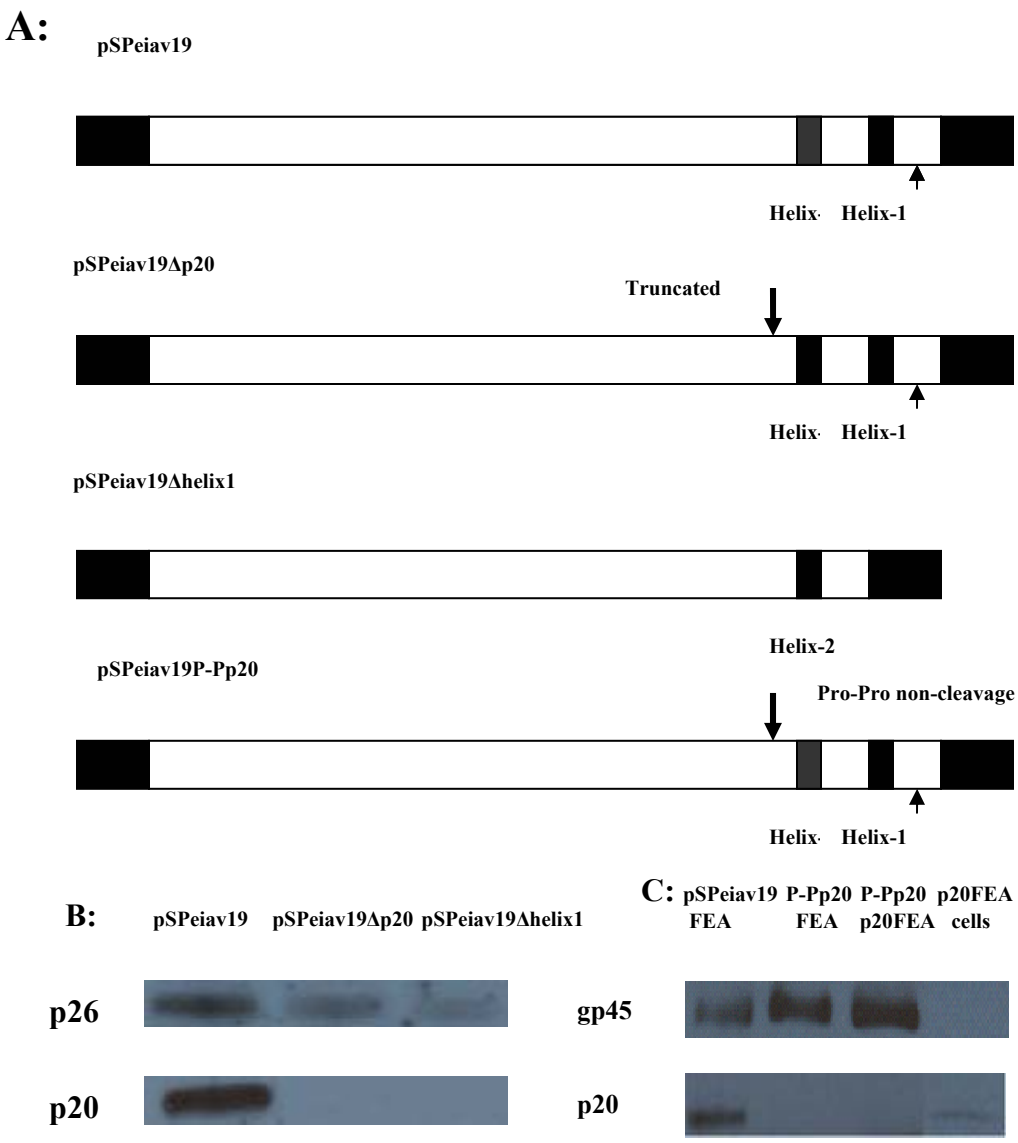

Figure 24: Analysis of incorporation of p20 expressed *in trans* into progeny virions. (A) Virus genome comparison among parental and CT truncated and mutant virus. (B) Virions of pSPeiav19, pSPeiav19Δp20 and pSPeiav19Δhelix1 were obtained by infecting p20FEA cells and subjected to Western-blot analysis. Anti-gag and anti-p20 (specific for peptide of the C-terminal 20 amino acids) were used as primary antibodies. (C). Virions of pSPeiavp19P-Pp20 (P-Pp20) virus were obtained by infecting either FEA or p20 FEA cells and subjected to Western-Blot analysis.Anti-p20 antibody (specific for peptide of the C-terminal 20 amino acids) was used as primary antibodies. "↑ " indicates the site the anti-p20 antibody binds.

APPENDIX

Construction of Recombinant Equine Infectious Anemia Virus (EIAV) Expressing Enhanced Green Fluorescent Protein

Introduction

To insert a reporter gene, which can be traced *in vitro* and *in vivo*, into the virus genome without eliminating the virus production is a useful tool to study the dynamic in early virus infection. For primate lentivirus, recombinant SIV and HIV expressing green fluorescent protein (GFP) have been successfully constructed by inserting the EGFP gene into the *nef* open reading frame (1, 2).The HIVGFP recombinant virus has been used to study the mechanism of apoptosis by using its property to distinguish infected and non-infected cells(1, 3). EIAV has the simplest and shortest virus genome of all lentiviruses. There are only two regulatory genes and an accessory gene in the genome named S1 (*tat*), S2, and S3 (*rev*). Tat and Rev are strictly required for virus genome replication and translation (4). Though the S2 is not required for virus replication (5), it is not an idea place to insert a foreign gene because most of its coding region is overlapped with *tat* and *env*. Previous studies in our laboratory have shown that C-terminal cleavage product (p20) of the EIAV transmembrane protein gp45 is not required for virus replication (6, 7). Since the N-terminal coding region of p20 is overlapped with *rev*, it is also not feasible to insert a foreign gene. In the above experiment, we have found that truncation of the last 56 amino acids of C-terminal p20, which is not overlapped with *rev* any more, does not eliminate the virus replication though the mutant virus replicates at a lower level compared with parental or full-length p20 truncated viruses (Figure 21, page 157). To test if a reporter gene can be inserted into the region and translated with the N-terminal p20 as

a fusion protein, we insert the EGFP gene into this region and expect to construct a recombinant EIAV expressing fluorescent protein.

Materials and Methods

Cells and plasmid

Feline embryonic adenocarcinoma (FEA) cells were obtained from Dr. Susan Payne at Texas A&M University. The cells were cultured in Dulbecco modified medium (DMEM) containing 5% fetal bovine serum (FBS). EIAV pSPeiav19 (abbreviated as p19) full-length infectious clone is a prototype derived from the cell culture adapted Malmquist strain. Viruses derived from p19 are avirulent, replicate both in FEA cell with no cytopathic effect (CPE) and equine monocyte-derived macrophage cultures with CPE.

Construction of EIAV EGFP chimeric clone

Full-length EGFP gene was amplified from pEGFP-C2 vector (Clonetech). Both upstream primer GFP-F (5'-GGACTAGTA***ATG***GTGAGCAAGGGCGAGGA-3') and downstream primer GFP-R (5'-GCACTAG***TCA***TTACTTGTACAGCTCGTCCATGC-3') contains a Spe I site at 5' end. A stop cordon (TGA) contingent to the Spe I site was added to the downstream primer following the last amino acid of EGFP gene on the vector to terminate the translation. Amplification reaction was carried out by Expand High Fidelity PCR system (Roche) with 10ng plasmid as template. The concentration of Mg^{2+} is 1.5mM. Cycling parameter is 95°C 30 seconds, 60°C 30 seconds and 72°C 60 seconds. PCR product was purified by QIAquick Gel Extraction Kit (Qiagen) and ligated with pGEM-Teasy T-A clone vector (Promega). Ligation mixture was transformed to DH5α competent cells and identified by restrictive enzyme cut. One of the positive clones with expected insert was cut by Spe I and purified by QIAquick Gel Extraction Kit

(Qiagen). To avoid the interference of Spe I site on the vector (pLG338) for the EIAV p19 infectious clone, the NcoI and EcoRI fragment which includes full-length envelope gene and 3'LTR was subcloned into the corresponding site of pGEM-Teasy vector (Promega) (8). The EGFP fragment flanked by the Spe I sticky end was inserted into the corresponding site located at C-terminal of TM gp45 of the p19 subclone. After orientation identification, one of the positive clones was cut by Nco I and EcoR I and cloned back in the pLG338 vector containing the rest of the virus genome. The chimeric EIAV p19 clone containing the EGFP gene at its C-terminal cleavage product (p20) of TM gp45 was named as p19GFP. Due to the insertion of EGFP gene, the last 56 amino acids of C-terminal TM gp45 were truncated (Figure 25, page 170).

Transfection and preparation of Virus Stock

The method was as described above for pSPeiav19Δp20 (page 82).

Reverse transcriptase (RT) assay

The procedure was as described above (page 83).

Fluorescence observation

FEA cells cultured in 25mm^2 flasks were transfected or infected with p19GFP plasmid or p19GFP derived virus and observed directly by the Nikon ECLIPSE TE200 Fluorescence Microscope.

Western Blot

The detail procedure was as described above in experiment 1 (page 86). Supernatant containing virus particles by transfecting p19GFP plasmid in FEA cells was briefly centrifuged at 3000cpm for 10 minutes to exclude the cell residue. Then virions were pelleted through a 10% sucrose layer in TNE buffer (10mM Tris, 1mM EDTA, 0.15M

NaCl) by ultracentrifugation (1hour, 100,000g, 4 °C) from 50ml virus stock (>20,000cpm/ml supernatant). The primary antibodies include horse anti-gag (1:500), rabbit anti-p20 (1:100) (specific for the peptide of last 20 amino acids of TM gp45), and rabbit anti-GFP (1:250) (Clonetech). The second antibodies are horseradish peroxidase (HRP)-conjugated goat anti-horse IgG (H+L) (1:5000) (KPL) or horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (whole Ig) (Amersham Life Science) (1:5000).

Recovery of virus genome and RT-PCR

Viral RNA was extracted from virions pelleted from the virus stock (transfecting p19GFP plasmid in FEA cells) by High Pure Viral Nucleic Acid Kit (Roche) according to instruction manual and diluted in 50µl elution buffer. A 10µl RNA samples were added to the reverse transcription mixer containing 2.5µl 10xBuffer without Mg^{2+}, 2.5µl 25mM Mg^{2+}, 7µl 2.5mM dNTP, 1µl RNA inhibitor, 1µl random hexamers and 1µl MuLV reverse transcriptase (50U/µl) from GeneAmp RNA PCR kit. After 5 minutes incubation at room temperature, the mixer was put at 42°C for 15 minutes and then 99°C for 5 minutes to inactivate the enzyme. Primer pairs used for PCR were p20Mu1-F (5'-GGAATTCTGACCGGTGGA***TAA***GGGGAC-3') and LTR-212 (5'-CTCAGACCGCAGAATCTGAGT-3') and the amplified region contains the site at which the EGFP gene was inserted into the virus genome. Amplification reaction was carried out by Expand High Fidelity PCR system (Roche) with 2.5µl cDNA as template. The concentration of Mg^{2+} is 1.5mM. Cycling parameter is 95°C 30s, 60°C 30s and 72°C 60s.

Results

Transfection of p19GFP in FEA cells

To test if the constructed p19GFP chimeric clone could express fluorescent protein and produce virus particles, 10μg p19GFP plasmid DNA was used to transfect FEA cells. At 24 hours after transfection, fluorescent cells could be observed (Figure 26B, page 171). The number of fluorescent cells increased gradually till about 72 hours post transfection and then began to decrease (data not shown). Reverse transcriptase (RT) activity of 1ml supernatant fluid at 3 and 4 days post transfection was more than 10,000cpm and close to 20,000cpm respectively, which indicates that virus particles could be produced (Figure 26A, page 171).

Infection of FEA cells with p19GFP derived virus

Since virus particles could be produced after transfecting the p19GFP clone in FEA cells, we used 20,000 cpm p19GFP transfection supernatant to infect FEA cells cultured in 25mm^2 flasks in order to test if the derived virus was infectious. At 5, 9,12,15,17 days post infection, reverse transcriptase (RT) activity was detected for 1ml supernatant fluid. At all the time points, the RT activity was very low with no more than 1200cpm (Figure 27A, page 172), which indicated that the replication of p19GFP derived virus was extraordinary poor. Results of fluorescence observation for the infected cells showed that no cells were fluorescent (Figure 27B, page 172). We concluded that derived virus from p19GFP clone was not replication competent and EGFP gene was not expressed in the infected cells.

Detection of GFP sequence in the genome of p19GFP derived virus

Failure for p19GFP derived virus to express EGFP in the infected cells may be caused by the absence of EGFP sequence from the virus genome due to selective pressure. This phenomenon has been found in SIVGFP recombinant virus by inserting GFP into SIV *nef* coding region .Though the derived virus from SIVGFP could replicate and express fluorescent protein *in vitro* and *in vivo*, the GFP sequence was lost from the virus genome after 8 weeks post infection (2). To test if the EIAV genome containing the EGFP gene has been successfully packaged into the virions, we recovered the virus genome from the pelleted virions. RT-PCR amplification of the region containing the insertion site for EGFP showed that the product was at the same size as parental p19 virus (Figure 28A, page 173). That means the fragment equal to the length of EGFP was absent from the recovered genome. When EGFP specific primer pairs GFP-F and GFP-R were used, no product could be amplified indicating that the full-length EGFP gene did not exist (data not shown). We concluded that the EGFP sequence was absent from the virus genome before packing into virions. We did not sequence the amplified region to know what kind of change has been occurred after loss of the EGFP sequence.

Detection of EGFP protein in the p19GFP derived virions

Since fluorescent cells, which are the indication of EGFP expression, can be observed after transfecting p19GFP plasmid in FEA cells and virus particles can be produced at the same time, we next detect if the EGFP protein is expressed as a fusion protein with N-terminal of TM gp45 and incorporated into the virions as we expected. Virions derived by transfecting p19GFP plasmid in FEA cells were lysed and blotted with anti-GFP antibody. A protein corresponding to the size of about 150kD was found to bind the anti-GFP antibody (Figure 28B, page 173). The size of EGFP is 28kD and the full-length

EIAV envelope is 135kD (gp120). Because the insertion of EGFP gene results truncation of the C-terminal 56 amino acids of TM gp45 with approximate size of 7-8kD, the size of the observed 150kD band is compatible with the predicted size of full-length envelope fused with EGFP. In parental EIAV virions, full-length envelope gp135 only exists as the residue of uncleaved product and most of the envelope component should be surface unit (SU) gp90 and transmembrane protein (TM) gp45. Because EIAV TM gp45 will undergo further cleavage to yield N-terminal gp32 and C-terminal p20 (7), it is expected to observe the bands with the size of about 40kD (N-terminal p20 fused with EGFP) or 65kD (uncleaved N-terminal gp45 fused with EGFP). But to our surprise, no obvious bands can be detected at the sites corresponding to the two molecular weights. We have also tested if the C-terminal 56 amino acids of TM gp45 is actually truncated due to insertion of EGFP by blotting the p19GFP virions with anti-p20 antibody, which only recognizes the last 20 amino acids of C-terminal gp45.The result is, as we have expected, that the anti-p20 antibody can not bind any proteins in the virions (Figure 28C, page 173).

Discussion

In this experiment, we have tested the possibility of generating an EIAV recombinant virus which could express fluorescent protein by inserting the EGFP gene into the C-terminal TM gp45. Though fluorescent protein can be observed in the cells and virus particles can be produced after transfecting the recombinant p19EGFP plasmid in FEA cells, the derived virus is not replication competent and expression of fluorescent protein can not be observed in infected cells. Further identification of the virions obtained by transfecting p19GFP plasmid in FEA cells indicates that the EGFP sequence is absent

from the virus genome before packaging into the virions while EGFP protein can be incorporated into virions mainly in the form of fusion protein with uncleaved full-length envelope product. The absence of EGFP sequence in the virus genome is probably mediated by the selective pressure which favors the original type of virus genome compared with length increased virus genome after insertion. The same phenomenon has been observed for SIV GFP recombinant virus as mentioned above (2). The phenomenon that EGFP is only fused with uncleaved full-length envelope product is probably caused by the instability for N-terminal p20-EGFP or gp45-EGFP fusion protein in the cells. It is also predicted that insertion of EGFP into the C-terminal TM gp45 may prevent the efficient cleavage of envelope product in the cells, which is important for producing replication competent virus. If this is true, it may be part of the reason why the produced virions after transfecting p19GFP plasmid in the FEA cells is replication incompetent. We do not detect the relative content of surface unit gp90 and transmembrane protein gp32 and p20 for the p19GFP virions.

The target site selected for insertion of EGFP in the experiment is within the coding region of a structural protein TM gp45. In the case of primate lentiviruses, successful experience has been got by inserting EGFP gene into *nef* coding region, a non-structural accessory protein that is not required for virus replication. However, compared with non-structural accessory protein, virus structural proteins are critical for virions assembly and infectious ability. Most of them will undergo further processing such as correct folding, glycosylation and cleavage after translation in order to be functional. Insertion of EGFP with molecular weight of 28kD into the structural coding region may interfere with the processing and result in the production of defective virus. From this point of view, the

coding region of structural protein is not a safety choice as the target to insert a foreign gene. Till now, there is also no report of recombinant lentiviruses by inserting a reporter gene into the coding region of structural protein. Though for negative stranded RNA virus, vesicular stomatitis virus (VSV), it is reported that by linking a VSV-G/GFP gene to the downstream of the cytoplasmic domain of envelope glycoprotein (G), the G/GFP protein could incorporated into the virions by forming heterotrimeric protein with G and the produced virus particles are replication competent (11), this condition is different from simply inserting a foreign gene into the coding region of virus structural protein. The G/GFP protein is treated as an additional product with the potential to form oligomeric structure with wild-type G protein. If the G gene is completely substituted by G/GFP gene, virus could not be recovered without the expression of complementing plasmid encoding VSV G. In addition, under this condition, the GFP sequence will be rapidly lost from the virus genome through introduction of a stop codon within the sequence encoding the G cytoplasmic domain, which indicates a strong selection against homotrimeric G protein bearing such a large cytoplasmic domain (11). Even insertion of a reporter gene into the coding region of a non-essential accessory gene for lentiviruses is not always workable. For example, replacement of Vpr, an accessory protein which could incorporate into virions, with EGFP resulted in a SIV genome that did not produce detectable levels of replication-competent virus (1, 2). This result is similar to the findings of our p19GFP plasmid because cells transfected with the SIVΔ*vpr*EGFP are also brightly fluorescent. However, cellular expressed Vpr-GFP fusion protein could be efficiently incorporated into the progeny virions and the fluorescent virus was replication

competent (9, 10). This suggests that EGFP protein produced by virus genome may interfere with the production and modification of other virus proteins.

Though the derived virus of the EIAV p19GFP recombinant clone is not replication competent and EGFP sequence is not existed in the virus genome, the derived virions after transfection is probably still useful because EGFP protein has been found to incorporate into the virions. Because the fluorescent virus produced by cotransfection of Vpr/GFP plasmid and HIV proviral clone has been used to study the intracellular behavior of HIV in living cells (9), the derived virions of p19GFP plasmid with high concentration may also be used to study the early phase of the virus infection such as receptor binding and entry under the electric microscope.

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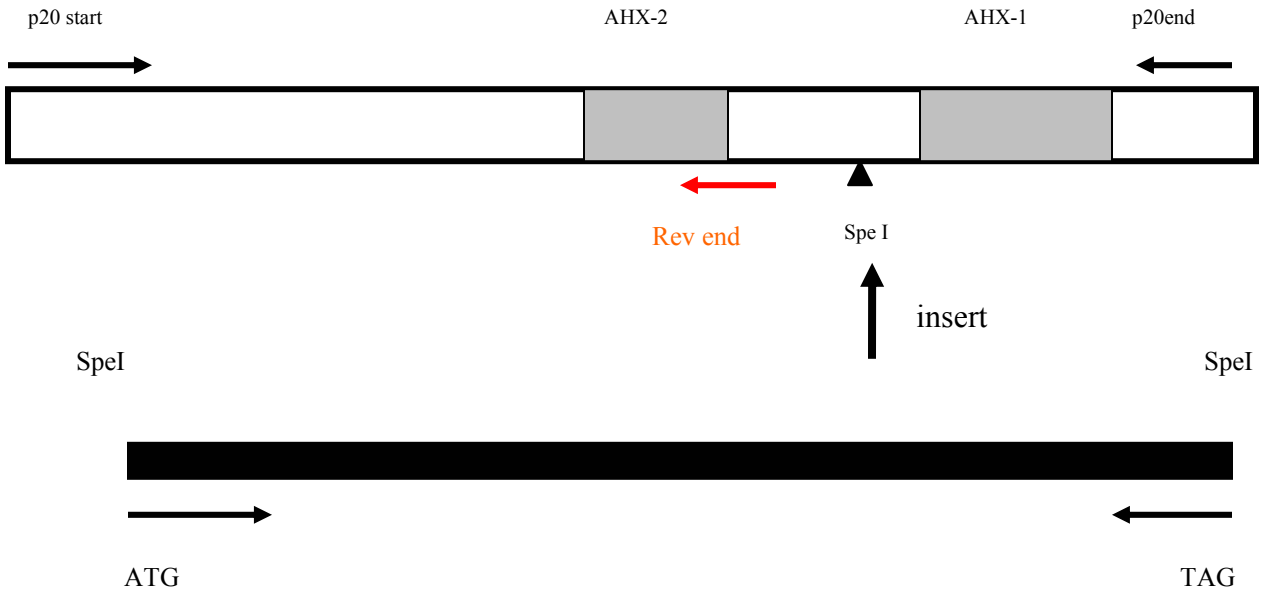
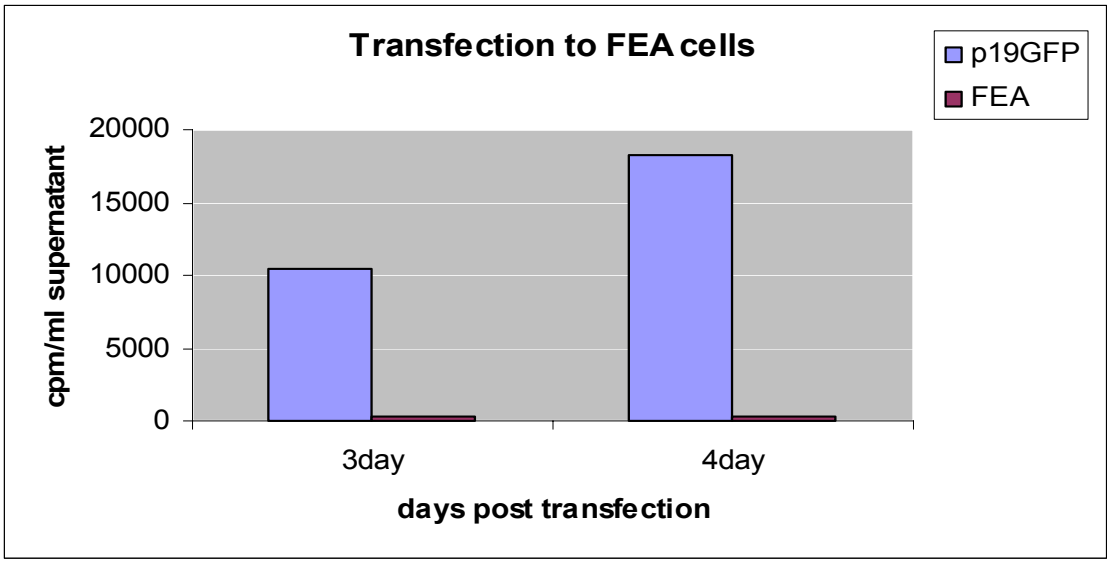


Figure 25: Schematic representation of constructing a p19EGFP proviral clone

A:



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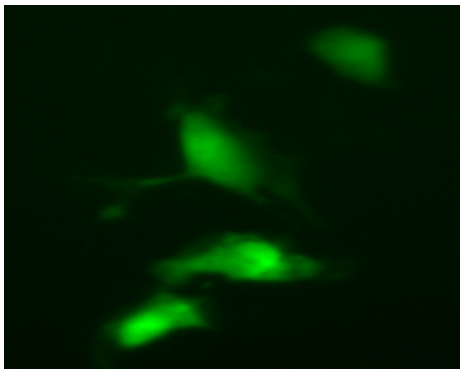
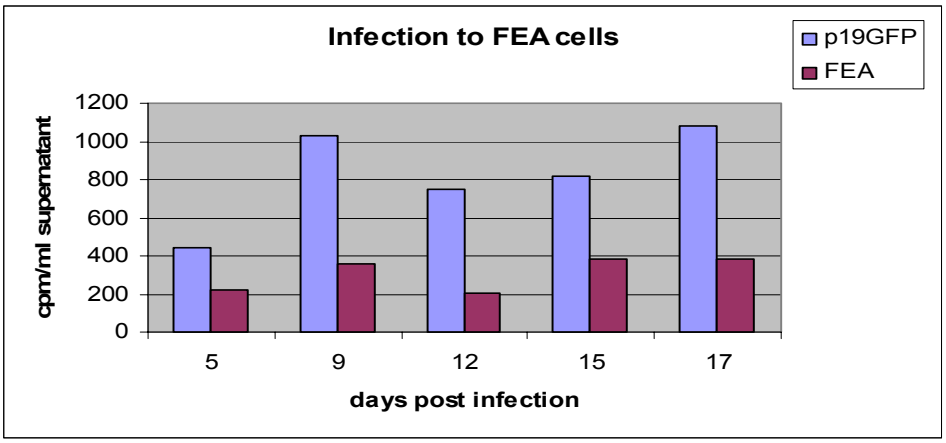


Figure 26: Transfection of 10µg p19GFP plasmid in FEA cells (A) Reverse transcriptase (RT) activity of 1ml supernatant fluid at 3 and 4 days post transfection. (B) Observation of cells at 48 hours after transfection under fluorescence microscope with 400-fold magnification

A:



B:

Figure 27: Infection of FEA cells with 20,000cpm virus derived by transfecting p19GFP plasmid. (A).Reverse transcriptase (RT) activity at different time points after infection. (B).Observation of FEA cells after infection by p19GFP virus under fluorescence microscope with 400 folds magnification.

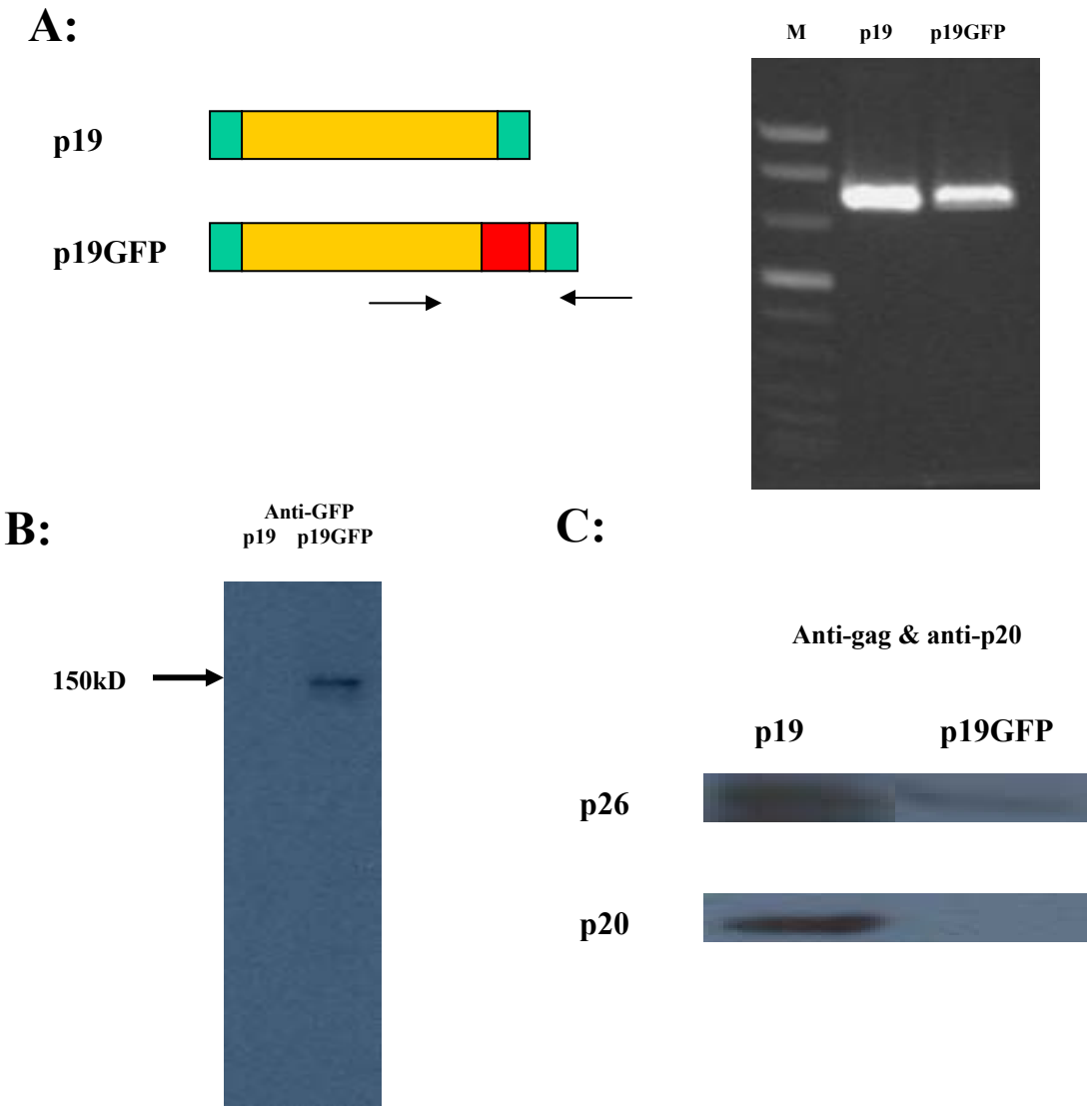


Figure 28: Properties of virions derived by transfecting p19GFP plasmid in FEA cells. (A). PCR amplification of the region containing the site for insertion of EGFP gene by RT-PCR. Primers p20Mu-1F (upstream) and LTR-212 (downstream) were complemented with the N-terminal p20 and 3'LTR coding region of EIAV genome. Plasmid p19 was used as a control. (→←) indicated the region the primer pair covered. (B, C). The p19GFP virions were analyzed by Western-Blot with anti-GFP (B), anti-gag or anti-p20 (specific for the peptide of last 20 amino acids of EIAV TM gp45) (C) as the primary antibodies. Virions derived from p19 clone were used as a control.