Detection of Feline Immunodeficiency Virus DNA Products in Lytic Versus Latent Infection, Including Early Reverse Transcription, Intermediate Reverse Transcription, and Late Circle Junctions

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Abstract

Human immunodeficiency virus type 1 (HIV-1) and other lentiviruses have developed latency as one mechanism to evade the host immune response. As a consequence, a subset of these viruses life-long latency, effectively preventing the eradication of HIV-1 infection. Two types of latency have been proposed: pre-integration and post-integration latency. In pre-integration latency, the pre-integration complex (PIC) composed of newly formed viral DNA, integrase, Vpr, and matrix protein, is prevented from entering the nucleus by host cellular factors. In post-integration latency, the PIC migrates to the nucleus and integrates into the genomic DNA, where it remains as dormant provirus for an indefinite period of time. We recently reported that mucosal administration of low dose cell-associated feline immunodeficiency virus (FIV) (10^2 and 10^3 infected cells) promotes viral latency in peripheral blood mononuclear cells (PBMC) and tissue lymphocytes that can be reactivated by *in vitro* cell stimulation. The current study investigates the type of infection occurring in high- or low-dose cell-associated FIV groups using polymerase chain reaction (PCR). Blood and tissue samples were analyzed

for three different DNA products of FIV infection. Early stage products of reverse transcriptase (163 bp), intermediate stage products (542 bp), as well as late circle junction products (967 bp) of FIV were seen in high-dose groups (10^4~10^6 infected cells) whereas early reverse transcription products were the only, or primary, form detected in low-dose groups (10^2~10^3 infected cells). This is consistent with the development of viral latency in cats challenged with low numbers of infected cells. Because we identified early transcripts in samples from latently infected cats, our findings support one of two theories. (1) Transport of the pre-integration complex into the nucleus is inhibited resulting in pre-integration latency. (2) Late transcription is impaired resulting in post-integration latency. Our data provides evidence that latent infection occurs following mucosal exposure to low-dose FIV and raises the concern that some individuals exposed to low amounts of HIV may also become latently infected. This work helps to focus further investigations into the mechanisms of lentiviral latency.

Introduction

Human immunodeficiency virus (HIV) is the causative agent of AIDS and the largest epidemic in the world today. One of the largest challenges in combating HIV is the presence of latent viral reservoirs. The majority of HIV infected individuals maintain a productive infection and, in the absence of treatment, often progress to AIDS. In contrast, some individuals harbor the virus for months to years without showing symptoms, and a very small subset of exposed individuals remain seronegative indefinitely [9]. However, even in patients that progress to AIDS, there is ongoing development of latent (quiescent) as well as lytic (productive) virus. Antiretroviral

treatments such as the combination of multiple drugs in highly active anti-retroviral therapy (HAART) can dramatically reduce productive HIV replication but are often ineffective against latent virus that re-emerges rapidly after the cessation of therapy. Because latency is a bane for the treatment of HIV, many investigators are actively seeking model systems to better understand the mechanisms of viral latency.

Feline immunodeficiency virus (FIV) is a retrovirus often used as an animal model of HIV. Although more distant from HIV than other primate lentivirus such as simian immunodeficiency virus (SIV), it shares much of its genetic structure and pathogenesis with HIV, and thus is a well-accepted animal model for HIV transmission, immunopathogenesis, and vaccine studies [2]. FIV uses CD134 and CXCR4 as the binding and entry receptor, rather than CD4 and CCR5/CXCR4 as does HIV, but ultimately infects a similar subtype of activated lymphocytes as does HIV [4]. FIV is divided into 5 clades (A-E) [2]. The present study employs the $NCSU_1$ strain of FIV, a clade A virus. FIV exhibits an HIV-like tropism at cellular and tissue levels, resulting in an acute phase with plasma viremia and decreased CD4/CD8 ratios, an asymptomatic period marked by low-level viremia and steady decline of $CD4^+$ cells, and finally development of feline AIDS, in which the cat is left vulnerable to opportunistic infections [2,4,7].

Using the FIV model, the Burkhard lab has recently demonstrated that the development of latent infection is at least partly dose-dependent. They reported that mucosal administration of low doses of cell-associated virus (10^2-10^3) promotes latency in the host, while exposure to higher numbers of cell-associated virus results in productive infection [1]. The goal of the current study is to identify whether early and late

viral transcripts are produced in latently infected cats. The information obtained will help focus further investigations into the mechanism(s) of lentiviral latency.

Previous studies have described several DNA intermediates produced by FIV at distinct points of its replication within the host cell [3,4,7,11]. After entering a host cell, the process of reverse transcription from RNA to DNA begins immediately, and can be detected by using LTR-LTR primers that amplify an early DNA product of about 163 base pairs (bp) in length. After reverse transcription, the pre-integration complex (PIC) composed of viral cDNA, integrase, Vpr, and matrix proteins migrates to the nucleus and integrates into the feline's genome. This is detected by amplifying an LTR-Gag product of 542 bp. The third DNA intermediate of interest is an extrachromosomal loop (967 bp) detected by using Env-Gag primers, that indicates productive infection (Fig. 1).

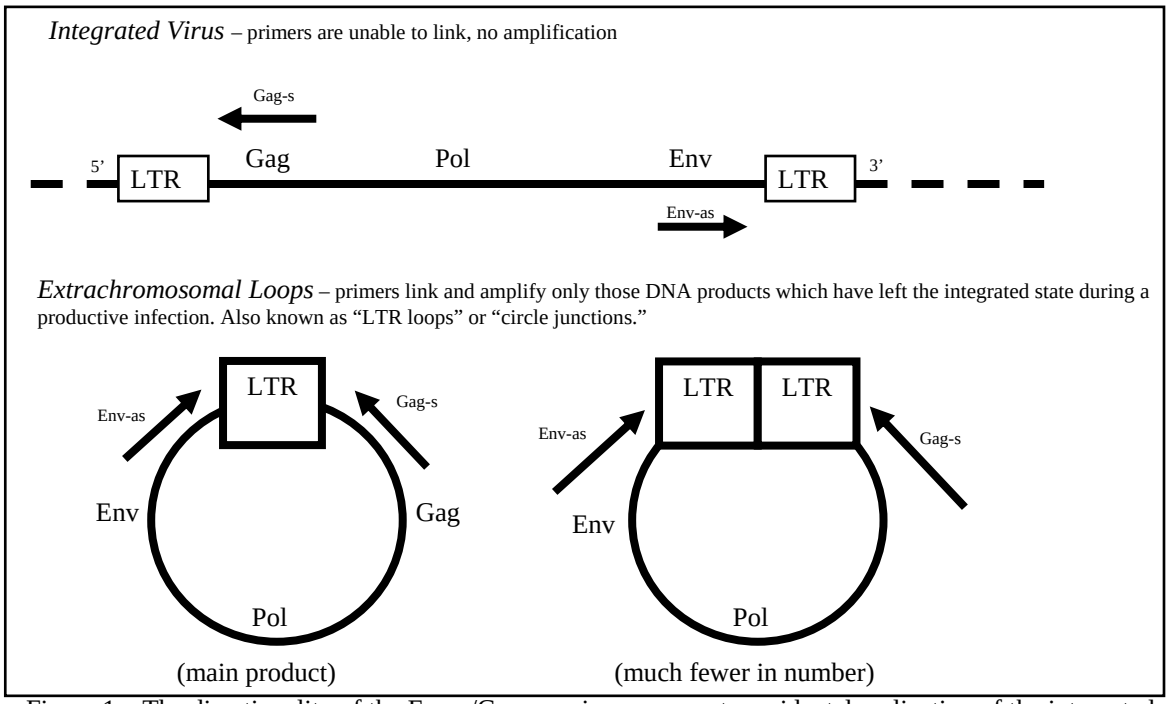

Figure 1 – The directionality of the Env-s/Gag-as primers prevents accidental replication of the integrated virus, ensuring that the amplified product indicates productive infection. This concept has been previously described using similar figures in Frey et al, 2001 and Joshi et al, 2004.

Materials/Methods

Samples were obtained from a previously described study cohort [1]. Briefly, eighteen female cats were obtained from Liberty Labs and were housed and cared for in accordance with the standards of the American Association of Accreditation of Laboratory Animal Care and the Institutional Animal Care and Use Committee. Cats were grouped (3 cats/group) and inoculated at 4 months of age with 10^6 naive $CD4^+$ T cells or with serial dilutions of FIV $NCSU_1$-infected $CD4^+$ T cells in 50 μL of lymphocyte medium. $NCSU_1$ is a clade A pathogenic FIV that does not produce high plasma viremia after mucosal transmission but induces high provirus levels and progressive immune dysregulation. Spleen, small intestinal intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL), popliteal lymph node (PLN), mesenteric, lymph node (MLN), medial iliac lymph node (ILN), and peripheral mononuclear blood cells (PBMC) were harvested at 12 weeks post-infection, processed as previously described [1] and stored at 4^oC as DNA until used for this study.

DNA samples were analyzed on a 1% agarose gel for degradation. No noticeable degradation was detected. The concentration of nucleic acids in each sample was then obtained by spectrophotometer (Nanodrop®, Wilmington, Delaware) and the volume standardized so that 100 ng of template DNA would be present in each PCR well.

The primers, first described in Rottman, et al (1995) [8] and later optimized by others [4,7], amplify three distinct DNA intermediates of FIV infection. The early product of reverse transcriptase, indicative of virus entry, was amplified by LTR-sense (5'-GCG CTA GCA GCT GCC TAA CCG CAA AAC CAC-3') and LTR-antisense (5'-GTA TCT GTG GGA GCC TCA AGG GAG AAC TC-3'). The intermediate product,

indicative of integration, was amplified by LTR-sense and Gag-antisense (5'-CGC CCC TGT CCA TTC CCC ATG TTG CTG TAG AAT CTC-3'). The third and final product, indicative of productive infection, was amplified by Env-sense (5'-GGC AAT GTG GCA TGT CTG AAA AAG AGG AGG AAT GAT-3') and Gag-antisense.

PCR was performed on a Mastercycler Gradient© thermocycler (Eppendorf, Germany) using HotStarTaq© Polymerase Master Mix Kit (Qiagen, Germany). The thermal profile included an initial 15 min activation step at 95^{o}C, followed by 40 cycles each containing a 45 sec denaturation step at 95^{o}C, 45 sec annealing step at 60^{o}C, and 1 min lengthening step at 72^{o}C. The profile was completed with a 15 min incubation step at 72^{o}C, after which the products were stored at 4^{o}C until analysis. These conditions were altered during optimization for two particular cases. First, to amplify the product with higher specificity, the LTR-LTR primer pair was amplified using a 1X final concentration of the Q-solution© provided in the HotStarTaq kit. Secondly, because of the particularly low melting temperatures of the primers, the Env-Gag product was amplified with a 57^{o}C annealing step. After amplification, all products were separated according to size on 1% agarose gels for analysis, using SeaKem© agarose powder (Cambrex, Rockland, Maine) and ethidium bromide. The gels were visualized by UV digital photography on a GelDoc2000© machine (BioRad, Hercules, California) using Kodak 1-D© software (Kodak, Rochester, New York).

Results

The initial protocol used real-time PCR that requires use of a reference dye to quantify PCR copies. Through multiple optimization steps, it was found that some of the primer pairs (especially Env-sense and Gag-antisense) had a strong interaction with the

real-time reference dye that inhibited an efficient or specific PCR reaction. This approach was abandoned (data not shown), and standard hot-start PCR was then used to perform the remainder of the analysis.

Early Reverse Transcriptase Product (LTR-LTR)

Cats inoculated with high numbers of cell-associated virus developed lytic, progressive infection in multiple tissues [1]. These findings were consistent with the disseminated tissue distribution of LTR-LTR products. The LTR-LTR product was routinely identified in most tissues isolated from the cats inoculated with high numbers (10^6-10^4) of FIV infected cells (Fig. 2). However, two cats (B5 and Z7) within the high dose group exhibited the LTR-LTR product in only two tissues each (ILN and LPL in cat B5, ILN and Spleen in cat Z7). We were also able to detect LTR-LTR products in at least one tissue from each cat inoculated with low numbers (10^3-10^2) of cell-associated virus.

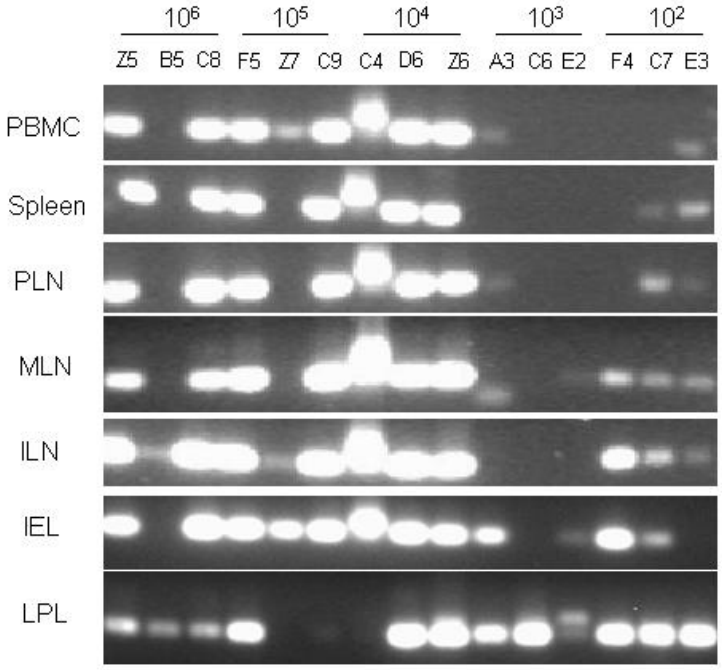

Figure 2 – PCR products of the LTR-LTR primers are shown according to tissue type and cat ID. The concentration of cell-associated virus in the inoculum decreases from left to right. A 100 bp marker was used to identify the correct bands (not shown). LTR-LTR product is 163 bp.

Cats inoculated with high numbers of cell-associated virus developed lytic, progressive infection in multiple tissues [1]. These findings were consistent with the disseminated tissue distribution of LTR-LTR products. Although we did not attempt to quantify the LTR-LTR product in this study, standardized amounts of template DNA were used in each PCR well, along with identical methods of preparing and viewing agarose gels. In cats with high dose exposure and progressive lytic infection, very bright bands, which likely represent a substantial amount of PCR product, are seen in all tissues except the LPL. In contrast, bands are weak or absent in most tissues for latently infected cats with the single exception of the LPL.

Late Reverse Transcriptase Product (LTR-Gag)

The LTR-Gag primers amplify a late reverse transcriptase product, that has been previously used to detect virus integration into the host genome (a crucial step in productive infection). The LTR-Gag product amplified in at least one tissue within each of the high dose cats, although it was not present in all samples (Fig. 3). This supports the previous findings of productive infection in these cats [1]. While seven of the high dose cats produced the late reverse-transcriptase product in at least three tissues, cats B5 and Z7 only exhibited the LTR-Gag product in the LPL.

In contrast, we were only able to detect the LTR-Gag product in two of the low dose cats: in the MLN of cat C6, and in the MLN, ILN, IEL, and LPL of cat F4. The paucity of late reverse transcriptase product in the low dose group (10^3-10^2) is consistent with the absence of detectable infection in these latently infected cats.

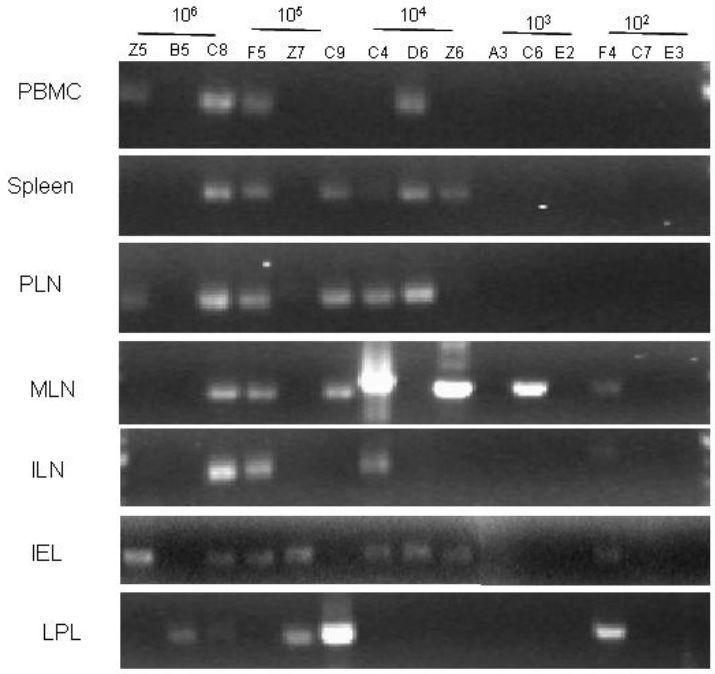

Figure 3 – PCR products of the LTR-Gag primers are shown according to tissue type and cat ID. The concentration of cell-associated virus in the inoculum decreases from left to right. A 100 bp marker was used to identify the correct bands (not shown). LTR-Gag product is 542 bp.

The LTR-Gag product was not uniformly distributed across tissue types. Unlike the LTR-LTR product, a specific pattern of distribution could not be detected. PBMC and ILN tissues showed positive bands in four cats each, whereas the IEL and MLN exhibited bands in eight and seven cats, respectively. The remaining tissues (spleen, PLN, and LPL) displayed bands in five to six cats each.

LTR Loops or "Circle Junctions" (Env-Gag Product)

In support of previous findings [1], the extrachromosomal loops, which indicate productive infection, were not found in any of the seven tissues for any of the six cats exposed to low doses (10^3-10^2) of cell-associated virus (Fig. 4). In contrast, a majority of high dose cats (10^6-10^4) exhibited LTR loops in three or more tissues.

The complete absence of detectable Env-Gag products in B5 and Z7 was unexpected, as both had been exposed to high doses of cell-associated virus. However,

because of the sequential nature of these three DNA products within an infection, the lack of Env-Gag products correlates with the paucity of LTR-Gag product in these animals.

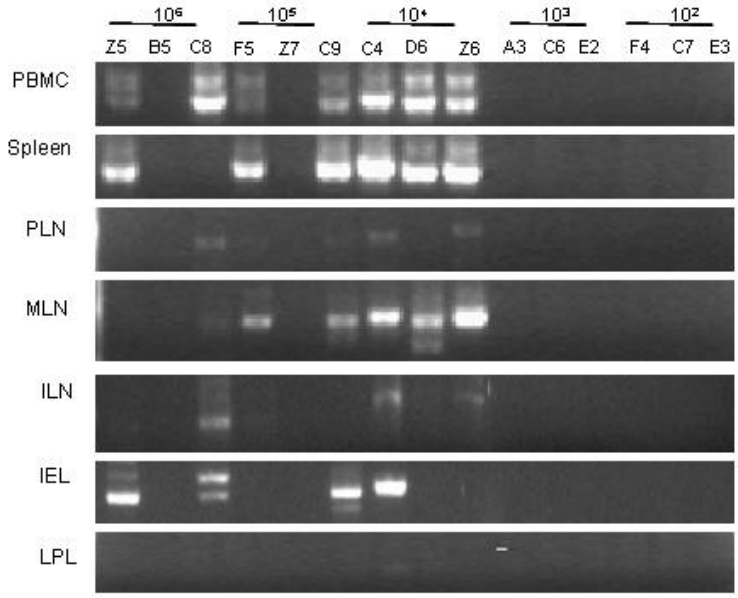

Figure 4 – PCR products of the Env-Gag primers are shown according to tissue type and cat ID. The concentration of cell-associated virus in the inoculum decreases from left to right. A 1 kb marker (not shown) was used to identify the correct band size of 967 bp.

The late product was most prevalent in the PBMC, where seven of nine of the high-dose cats displayed very bright bands. Similarly, the spleen samples elicited bright Env-Gag bands in six of nine of the cats, and MLN displayed six bands, albeit less bright in appearance. The PLN, ILN, and IEL samples each amplified in only four cats. No Env-Gag product amplified to a detectable degree in the LPL samples.

Discussion

In this study, we examined samples from a cohort of cats previously described to have either lytic or latent FIV infection [1]. We hypothesized that when exposed to a low viral load, these low-dose cats would tend to develop a pre-integration latency, where the PIC is held in the cytoplasm preventing the virus from entering the nucleus for immediate integration. We found that samples from low dose, latently infected cats contained early

(LTR-LTR) transcription products, infrequent intermediate reverse transcriptase products (LTR-Gag), and the complete absence of Env-Gag LTR loops. The absence of LTR loops supports previous inability to detect productive infection in these animals. The occasional presence of LTR-Gag products suggests that, at least in some animals, virus was transported into the nucleus and integrated into the genomic DNA. However, in the majority of tissues from most low dose cats, this intermediate product was not detected.

This lack of an intermediate product in low dose cats may represent innate inhibition of viral DNA transport into the nucleus. In HIV, some have argued that the host APOBEC proteins are responsible for an innate restriction process [6], but this has not been tested in the FIV system. Others have demonstrated a similar state can be induced in FIV infection in vitro by zidovudine (AZT) treatment, a main component of HAART [4]. AZT prevents the virus from forming late DNA products of infection, and may promote a latent state. However, similar findings would be seen if there was inhibition of late stage transcription.

Because gut-associate lymphoid tissue (GALT) is a strong and early target for the HIV [5], SIV [12], and FIV [2] viruses, infection leads to rapid depletion of lamina propria lymphocytes [5,13]. This early and massive depletion of lamina propria lymphocytes is not reflected in the lymph nodes or PBMC, the latter of which is commonly used to monitor HIV disease progression [5]. The rapid depletion of LPL creates an absence of target cells in the gut tissues, which may be the reason that detection of all transcription products (LTR-LTR, LTR-Gag, and Env-Gag) was limited and/or absent in the LPL of high-dose cats with lytic infections. Consistent with previous studies that have examined early FIV infection and progression to disease, PBMC were

infected but not depleted to the point of preventing the amplification of clear Env-Gag products.

Our findings suggest a basic exposure threshold for the number of cell-associated virus (somewhere between 10^3 and 10^4) required for FIV to induce a productive infection within 12 weeks of exposure at the vaginal mucosa. At lower doses, latency occurs with lack of transcription progression to intermediate and late transcripts, especially circle junctions, which indicate the presence of active viral replication. Because we did not detect intermediate products in most tissues from most cats with latent infection, our data most likely supports the hypothesis that exposure to low-dose cell-associated FIV results in pre-integration latency. Preliminary work by others in the laboratory has identified a potential protein-protein interaction similar to the interaction between APOBEC host proteins and the HIV PIC, previously described in Harris and Liddament (2004) [6]. Those findings support our working hypothesis of pre-integration latency, although further research is necessary to identify the exact mechanism of latency, and to exclude an AZT-like block of late transcription.

In summary, we examined transcriptional DNA intermediates in a cohort of cats with latent and lytic FIV infection. In latently infected cats, we found early, but generally not intermediate or late transcription DNA products. We also found that the gut, primarily the lamina propria, was the major target for latent infection in these cats. This data most likely supports our hypothesis that exposure to low numbers of infected cells results in pre-integration latency, although it could also be explained by post-integration latency via a transcriptional block. This system provides a new model to examine HIV latency.

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