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# Functional characterization of bovine lentivirus LTRs

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Functional characterization of bovine lentivirus LTRs

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Jun Yang

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This is to certify that the Master's thesis of  
Jun Yang  
has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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## CHAPTER 1. GENERAL INTRODUCTION

### Introduction

Lentiviruses, a subfamily of retroviruses, are a group of exogenous, nononcogenic viruses that cause chronic and variable clinical disease syndromes such as fever, anemia, central nervous system disorders, pneumonia, lymphadenopathy and immunodeficiency. This group of viruses includes human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV) and jembrana disease virus (JDV) (4,18,23,33,39,49,53,69).

BIV occurs worldwide and is associated with chronic immune and central nervous system lesions. R29, the original BIV, was isolated in Louisiana in 1969 from the leukocytes of a cow with persistent lymphocytosis, lymphadenopathy and lesions in the central nervous system (79). Two functional proviruses, R29-127 and R29-106, were sequenced and analyzed and shown to be genetically and antigenically related to HIV (34,39). In vivo studies of R-29 identified no overt clinical signs following experimental infection. Transient clinical signs, such as pyrexia, neutropenia and lymphocytosis, were observed (29,48,62). In 1993, the Florida strain of BIV was isolated from a seropositive dairy herd in Florida (76). In vitro, FL112 had a longer incubation time between passages and a slower lysis of infected cells. FL112-infected cattle had a greater increase in the number of

mononuclear cells and more pronounced histopathological lesions compared to the R-29 -infected cattle (76). Though there are some differences between the two strains, there are no reports of acute fatal disease caused by BIV in naturally or experimentally infected cattle. In general, BIV was found to be a mildly pathogenic bovine lentivirus.

Jembrana disease virus (JDV), the other species of bovine lentivirus, shows differences in in vivo levels of replication and pathogenicity from BIV (74). JDV is only found in some species of cattle in Indonesia, primarily in Bali cattle, and does not cause infection worldwide. After 5 to 12 days of infection, viral titers can reach  $10^8$  units per ml. JDV replicates very fast and causes an acute, fatal disease in infected animals (74). The major clinical signs associated with JDV-infected cattle are fever, lethargy, anorexia and enlargement of the superficial lymph nodes. The mortality rate of experimentally infected cattle is reported to be 17% (74). Though BIV and JDV are both bovine lentiviruses, the different pathogenicities lead us to wonder what are the important factor(s) that contribute to viral pathogenesis. One notable difference between JDV and BIV is the level of virus replication in vivo. Therefore, factors which control virus replication may contribute to differences in viral pathogenicity.

Lentiviruses have a complex genome structure. The long terminal repeats (LTR) flank the structural genes *gag-pol-env*. The LTR is the only promoter in the lentivirus genome and is divided into three regions, U3, R and U5 (19,24,77). In the U3 region, there are many cis-acting sites which function as transcriptional enhancers during virus replication (10,19,24,31,77). Transcription starts at the U3

and R border. The R region contains transactivation response region (TAR). Tat, the virus encoded protein, specifically binds to the secondary loop-stem structure TAR structure, and increases the initiation and elongation of viral transcription (10,31,43,80). Therefore, both viral and cellular proteins contribute to LTR promoter activity. Because the LTR is the only promoter and is an important regulator in viral transcription and replication, the LTR becomes a critical factor in studies of viral pathogenesis.

Sequence comparison between BIV and JDV LTRs showed that the JDV LTR was very divergent. The nucleotide substitutions were dispersed throughout the whole LTR sequence and, except for the common TATA box, there appeared to be few common transcription factor binding sites between BIV and JDV (12,35). Also, the JDV U3 region contained a 192 nucleotide(nt) deletion as compared to BIV. Due to many nucleotide substitutions in the R region, the predicted TAR secondary structure of JDV was different from BIV TAR (12). The sequence information, and the knowledge about pathogenesis and viral replication, led to our hypothesis that the LTR sequence heterogeneity between BIV and JDV differentially regulates the basal and/or Tat-mediated transcription and contributes to the difference in viral replication and pathogenesis. To test the hypothesis, there were three specific aims in the project.

### **Specific Aims**

1. Compare the LTR nucleotide sequences of the three bovine lentivirus, R-29, FL112 and JDV.

2. Determine if the LTRs of R-29, FL112 and JDV exhibit different levels of basal/or Tat-mediated transcription.
3. Determine if the different transcription levels among R-29, FL112 and JDV map to the U3 and/or R-U5 region of the LTR.

## **Thesis Organization**

This thesis consists of a manuscript which is preceded by General Introduction and Literature Review sections and followed by General Conclusion and Literature Cited sections. The format of the manuscript is that of the journal to which it is to be submitted. Bibliographical information corresponding to citations in the whole thesis is located in the Literature Cited section.

## **CHAPTER 2. LITERATURE REVIEW**

### **Pathogenesis of Bovine Lentiviruses**

Lentiviruses are a widely disseminated group of exogenous, nononcogenic retroviruses. The lentiviruses are associated with lifelong, persistent infection and a chronic, variable disease course (40). Few groups of viruses get as much attention as retroviruses because of their importance as human and animal pathogens and also because of their remarkable value as experimental systems. Many lentiviruses have been identified, including human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV) and jembrana disease virus (JDV) (4,18,22,33,39,49,53,69).

Bovine lentiviruses are a group of lentivirus with different pathogenicities in infected cattle. Bovine lentivirus-infected cattle are found in many countries, including the USA, Indonesia, France, Australia, New Zealand, Costa Rica and Germany (45,47,53,64,70,79). At this time, there are two species of bovine lentivirus, bovine immunodeficiency virus (BIV) and jembrana disease virus (JDV).

#### **Bovine immunodeficiency virus (BIV)**

Bovine immunodeficiency virus is found worldwide and causes nonacute, chronic immune and central nervous system lesions. BIV is a mildly pathogenic

lentivirus and there are few reports of overt clinical disease caused by BIV. BIV was largely ignored until studies indicated that BIV was morphologically, serologically and genetically related to HIV, the agent of AIDS (39). Two strains of BIV have been isolated, R-29 and Florida strain.

R29, the original bovine lentivirus, was isolated in 1969 from the leukocytes of a cow in Louisiana with persistent lymphocytosis, lymphadenopathy, lesions in the central system, progressive weakness and emaciation (79). Later studies examined the effects of BIV in experimentally infected calves. Clinical signs following BIV infection were transient and included pyrexia, neutropenia and lymphocytosis (29,63). After 3 months post inoculation (PI), BIV infection was associated with an increase in the lymphocyte blastogenic response to the mitogen phytohaemagglutinin. In addition, neutrophil antibody dependent cell mediated cytotoxicity and neutrophil iodination were decreased (29). The levels of BIV replication in vivo were very low. During peak periods of viral replication in vivo, there were less than 0.03% of peripheral blood mononuclear cells expressing detectable levels of viral RNA by in situ hybridization (8). Though an early decline in Gag-specific antibody reactivity was observed in BIV infection, there was no evidence of increasing viral replication or progression to overt clinical disease (48). All infected animals were clinically normal through 27 months PI.

The Florida strain of BIV includes two isolates, FL112 and FL491, which were obtained from a seropositive cattle herd in Florida in 1993 (76). In vitro, the Florida isolates showed differences in replication characteristics and syncytial appearance as compared to the original R-29. There was a longer incubation time

between passages and slower lysis of the infected cells. In vivo, FL112-infected calves had greater increases in the number of mononuclear cells and more pronounced histopathological lesions than R29 infected cattle (76). However, FL491-infected calves were similar to R29-infected calves. In other lentivirus infections, in vitro characteristics are often correlated with virulence in vivo. In HIV, the more cytopathogenic lentiviral strains in culture are associated with greater in vivo virulence (16,46,56,57). Though there were some differences in pathogenicity among the different BIV isolates, in general, BIV was found to be a mildly pathogenic lentivirus and there have been no reports of acute and fatal disease caused by BIV in naturally or experimentally infected cattle.

### **Jembrana disease virus (JDV)**

The other species of bovine lentivirus, jembrana disease virus (JDV), shows dramatic differences in pathogenicity from BIV. JDV causes an acute, fatal disease in Bali cattle, *Bos javanicus* (74). The major clinical signs in JDV-infected animals are fever, lethargy, anorexia and enlargement of the superficial lymph nodes. Not all of these clinical changes occurred in all infected cattle. The major hematological changes were a leukopenia, lymphopenia, eosinopenia, a slight neutropenia, a mild thrombocytopenia, elevated blood urea concentrations and reduced total plasma protein. The clinical signs persisted for 5-12 days and most infected cattle recovered without recurrence of the disease. The mortality rate in the experimentally infected cattle was 17% (74). The complete nucleotide sequence of the JDV RNA genome was reported (12). The studies showed JDV

morphogenesis, protein structure, antigenic relationship and genome structure were similar with BIV's, and JDV was also classified as a bovine lentivirus (12). Even though BIV and JDV are both bovine lentivirus, the pathogenesis caused by the two bovine lentiviruses are very different. It is not known what factor(s) are responsible for these differences. One notable difference between JDV and BIV is the different levels of virus replication in vivo. Therefore, the factor(s) which control viral replication may contribute to viral pathogenesis.

## **Lentivirus Gene Regulation**

### **Characteristics of lentiviruses**

Lentivirinae are one of the three subfamilies of retrovirus. The other two are oncovirinae and spumavirinae. Oncoviruses are named for their ability to cause neoplastic disease in the infected animals. Viruses originally isolated as tumor-inducing agents, as well as related viruses, are traditionally placed into the subfamily oncovirinae. The oncovirinae includes five groups which are not closely related to one another. The five groups are the Avian Leukosis-Sarcoma Virus (ALSV) group, the Mammalian C-type virus group, the B-type virus group, the D-type virus group, and the HTLV-BLV group. The spumaviruses are agents which cause vacuolation of cells in culture. The spumaviruses are not associated with any known disease and are by far the least well characterized of the retroviruses (19,24,30). Lentiviruses are a group of exogenous viruses with the most complex genome structure.

## Lentivirus genome organization

Retroviral genomes are diploid and composed of two identical positive sense RNAs that are non-covalently bound to each other. The 5' end of genome is modified by the cellular transcription machinery in the form of  $m^7G5'ppp5'G_m p$ . There is a poly(A) sequence in the 3' end which is added as a posttranscriptional modification to newly synthesized transcripts by the cellular mRNA processing machinery. The genomic RNAs, like cellular mRNAs, are modified posttranscriptionally by methylation on the 6 position of occasional adenosine residues. All retroviral genomes share organizational similarities in the special arrangement of the protein-encoding genes, gag-pol-env, which are flanked by the long terminal repeats (LTRs). The gag gene encodes a precursor polyprotein which is subsequently cleaved to several capsid proteins. There are three invariant ones: the matrix protein, the capsid protein, and the nucleic-acid-binding protein. The pol gene encodes the three proteins: the reverse transcriptase, protease and the integrase. The reverse transcriptase, as indicated by its name, functions in transcribing the RNA genome to double strand DNA. The integrase is needed in the integration of the viral DNA into cell DNA. The protease functions in the posttranslation process. The env gene encodes the two envelope glycoproteins. One is the surface protein which is responsible for recognition of cell-surface receptors. The other one is the transmembrane protein which anchors the SU to the virion envelope (19,24,30). Lentiviruses have a variable number of

accessory genes, such as Tat, rev, nef, vif, vpr, and vpu, which play an important role in viral gene regulation and in the viral life cycle (32,43).

### **The life cycle of lentiviruses**

Lentiviruses attach to the host cell by interaction with a specific cell-surface receptor. For HIV, the CD4 cell-surface protein is the main receptor and was the first retrovirus receptor to be identified (22). HIV-1 also uses different co-receptor(s), resulting in a distinctive host range for different virus strains. After binding of the SU protein to its receptor and co-receptor(s), the virus envelope and the cell membrane fuse to release the virion core into the cytoplasm. After the core penetrates into cytoplasm, the process of reverse transcription of the RNA genome into double-strand DNA occurs. During this process, the U3 and U5 regions duplicate to form the long terminal repeat (LTR). The synthesized double-strand DNA translocates into the nucleus and integrates into the cell chromosome by integrase encoded by viral genome. At this phase, the virus is called a provirus. The efficient expression of the provirus starts in the LTR which provides signals recognized by cellular transcription machinery. The provirus is transcribed into viral mRNA and spliced RNAs are transported to the cytoplasm. The early gene expression produces two important regulatory proteins, Tat and Rev (21), which translocate to the nucleus to regulate viral gene expression. Tat functions to increase transcription initiation and elongation by binding a specific RNA structure, termed TAR, which is present on all nascent mRNAs (52,67). While Tat influences virus gene expression overall, Rev exerts a differential effect in the virus life cycle.

Rev protects mRNA from complete splicing (21,28,71). In the late stage, incompletely spliced mRNAs serve as templates for synthesis of the viral structural proteins, including gag, pol and env (21). Gag and Pol are translated from the same polycistronic mRNA by open-reading frame frameshifting. However, Env is translated from a single spliced mRNA. The virus genome replicates itself to many daughter genome RNAs as virus synthesizes its structural units. Then, the structural proteins and genomic RNA assemble into the virion and bud out from host cell. The LTR, the only promoter in the lentivirus genome, plays a critical role in the regulation of viral transcription and replication. The function of LTR is important to the virus life cycle.

## **The Long Terminal Repeats**

The long terminal repeat (LTR) plays a very important role in a number of viral activities, including reverse transcription, integration, transcription, and replication. The LTR is organized into three regions, referred to U3, R and U5 (19,24,77). The U3 region provides signals recognized by cellular transcription factors. Some sequences act as enhancers to increase viral transcriptional efficiency (19,24,77). The TATA box is located in the U3 region, and functions to accurately initiate transcription (6,73). Transcription starts from the R and U5 border. In the nascent RNA, the second structure of TAR is the critical binding site for Tat function (43,80).

## **Basal activity**

Basal activity is the activity of the LTR in the absence of Tat. In HIV, the U3 region, containing many cellular transcription factor binding sites, is the main contributor to the basal activity. In the highly divergent U3 region, the TATA and CCAAT boxes, which are the consensus sequences suggested by the names, are strongly conserved and cannot be deleted, inverted, or moved elsewhere without greatly reducing viral gene expression (6,19,73). Besides the TATA and CCAAT boxes, the other cellular transcription factors, such as NF-kB and SP1, act as enhancers to affect the overall expression of LTR. Enhancers are defined as control sequences or groups of control sequences, usually found upstream of the cap site, which increase the frequency of initiation of transcription but do not themselves specify or provide the sites for transcription. The SP1 and NF-kB are the two critical ones in HIV-1 LTR activity (13,68). SP1, an important transcription factor, not only affects the basal level activity of the HIV-1 LTR but also interacts with the Tat protein and functions in transactivation (41,50,51). Deletion of all the three SP1 sites reduces the enhancer activity (50). NF-kB plays a pivotal role in stimulation of HIV transcription after T-cell activation, though it may not be absolutely required for viral growth. Moreover, NF-kB is the target of several signal transduction pathways involving both cellular and viral proteins (14,59,65). Point mutations in the NF-kB responsive elements decrease dramatically transcriptional activity (65) and may be associated with a longer period of viral latency in vivo (78). There are reports which suggest that deletion of some factor binding sites affects

not only the basal activity but also the transactivation of the LTR (10,31,35).

Therefore, though basal activity in some lentiviruses is much lower than the transactivation, some factors which function in the basal activity also play a role in the viral transactivation.

### **Transactivation**

All viral transcription is activated by some cellular factors which function at the U3 sequence in the proviral LTR. The host cell machinery is not sufficient for efficient viral gene expression and just provides a low, basal level of viral mRNA synthesis. In the presence of the virus encoded transactivator, Tat, the viral transcription level increases dramatically over the basal level of transcription. Although the exact function of Tat in transcription is not well understood, Tat is believed to play a role in two events. One is to increase transcription initiation (54,58,61,75). In this process, Tat possibly acts to facilitate assembly of the RNA pol II complex (58). The other one is to increase transcription elongation efficiency (20,52,54,58,61). The hypothesis that the Tat serves as an anti-terminator is derived from studies showing that Tat had little effect on the level of RNA polymerase density adjacent to the transcription initiation site, but dramatically increased the transcription rate of sequences distal from the HIV LTR (52).

The HIV Tat protein is a 86 amino acid peptide encoded from two separate exons. The first exon, encoding the first 72 amino acids of HIV-1 Tat, contains functional regions of the protein. Tat protein can be separated into different domains according its function (Fig. 1). The N-terminal domain is the first 22 amino

acids with no reported function. Amino acids 22 to 37 contain a cys-rich domain. Any mutations in six of the seven Cys residues abolish Tat function. The next nine amino acids (amino acids 40 through 48) are called the core domain which is conserved in many lentiviruses. Amino acids 49 to 72 contain an arginine-rich (RKKRRQRRR) basic domain, which is responsible for RNA binding and nuclear localization (Fig.1). HIV-1 Tat loosely binds to the bulge region of TAR and cellular



Fig. 1. Organization of functional domains of HIV Tat-1

factors which bind to the loop region in TAR stabilize Tat binding and possibly increase transcription levels by interaction with Tat. The precise TAR structure is very important to Tat function. Any deletion or nucleotide substitution in the bulge, loop or stem region which changes the hairpin structure decreases HIV transactivation levels dramatically (1-4). Results from numerous studies indicate that Tat is not only involved in the transcriptional elongation but also in initiation.

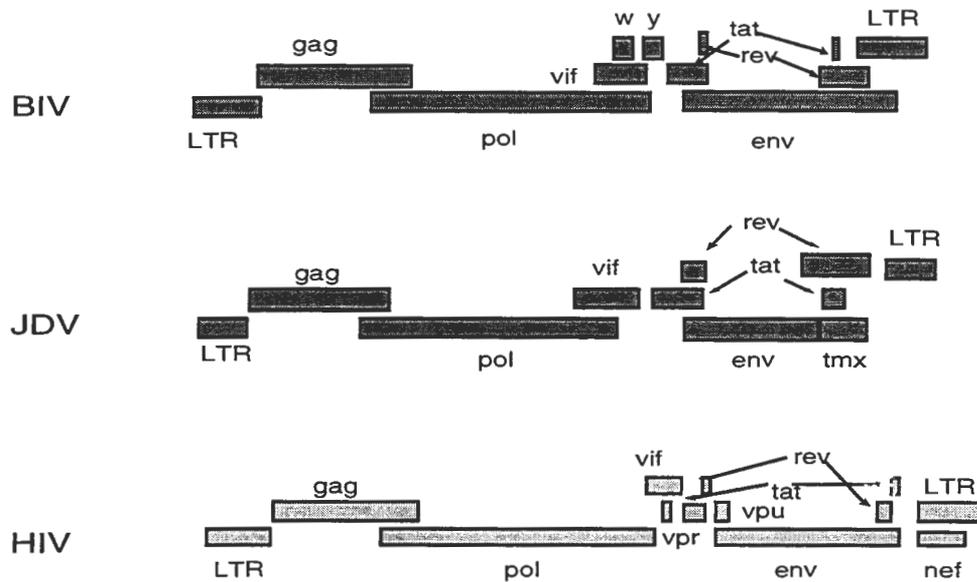


Fig. 2. Comparison of the proviral genome structures of BIV, JDV, and HIV. All identified or predicted open-reading frames (ORFs) of the three lentiviruses are shown. Besides the common LTR-gag-pol-env-LTR structure, they all have Tat, Rev and Vif regulatory ORFs.

## The LTRs of Bovine Lentiviruses

Bovine lentiviruses have a similar overall genome structure as HIV and other lentiviruses (Fig. 2). Flanking the gag-pol-env structural genes are the two LTRs. The BIV R29 LTR has been the only bovine lentivirus LTR studied to date. In the U3 region of the BIV LTR, there are some transcription factor binding sites which contribute to the viral basal activity. Transcription starts from the U3 and R border. In the nascent mRNA, the secondary hairpin structure TAR is critical for Tat transactivation.

In the U3 region, many transcription factor binding sites (TFBS) were identified by computer analysis and their functions were studied by deletion and point mutations (Fig.3, Table 1) (10,31). In the Cf2th cells, CAT activity data indicate that NF- $\kappa$ B, GRE (glucocorticoid responsive element), CAAT, ATF, AP-4, and AP-1 sites are important in transcription. The importance of some factors differs in different cell types. For example, the AP-4 binding sequence was shown to be more important for LTR activation in BLAC-20 cells than in Cf2th or EREp cells. The first SP-1 site (-77) appears to have a negative effect in Cf2th cells, but not in BLAC-20 and EREp cells. The CAAT site is required for LTR function in all of the cell types tested (10,31). Therefore, LTR activity varies in different cell types as a result of the presence and/or absence of cellular factors that interact with TFBS in the BIV LTR.

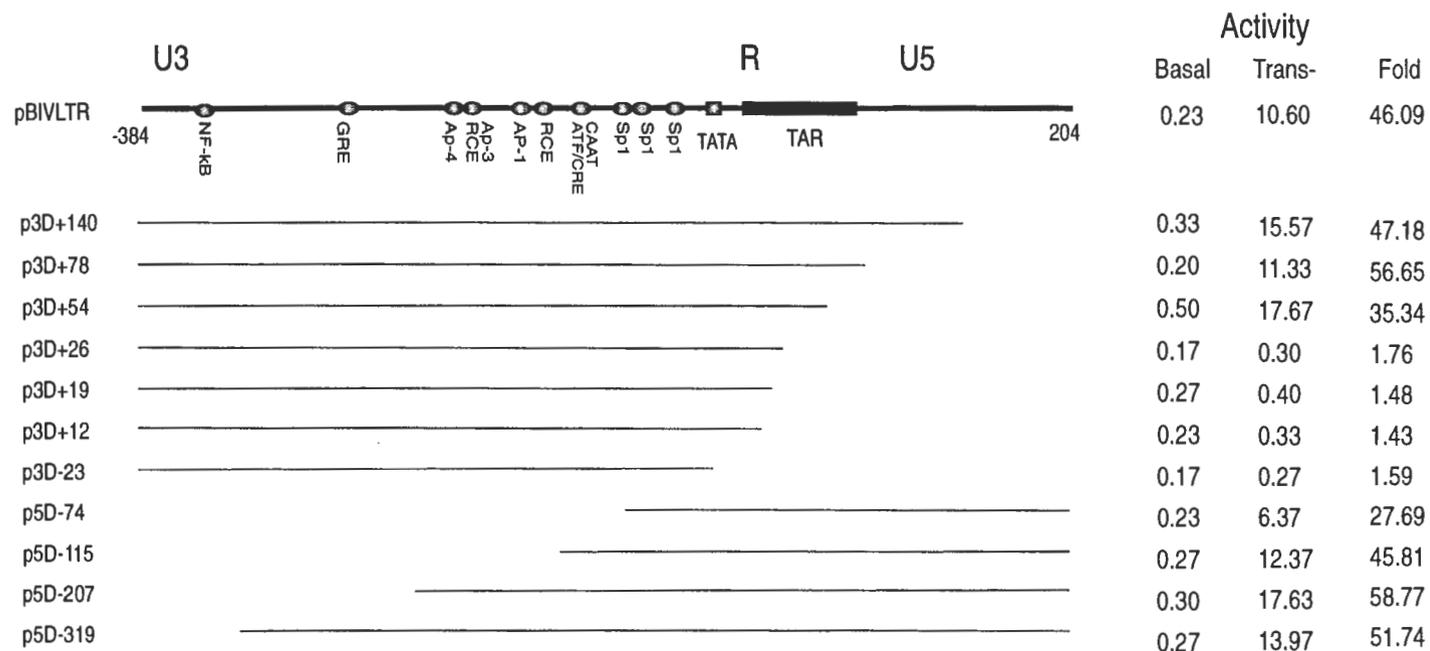


Fig. 3. Summary of the previous studies of BIV LTR activity (10,31). The sequence of the full-length LTR is shown on the top as a solid line. The location of the U3, R and U5 regions are indicated above the line of the full-length LTR. The TAR sequence is bolded. The identified transcription factor binding sites are indicated under the line of the full-length LTR. The plasmids with truncated LTRs are named by the 5' or 3' terminal deletion and numbered with respect to the transcription start site (+1). The BIV LTR activity is measured by CAT activity. Results are given as percentages of [<sup>14</sup>C]chloramphenicol converted to its acetylated form and are means of three transfections. Fold transactivation is determined by the ratio of transactivation to the basal activity.

Besides the activities of these cellular factors in LTR function, Tat-mediated transactivation is important for bovine lentivirus LTR activity. Similar to HIV-1, the binding site of BIV Tat is in the bulge region of the BIV TAR (15). Though BIV Tat has a different way to bind its target site, the secondary hairpin structure of TAR is also important for BIV Tat function. Deletion or point mutations of the TAR sequence showed that changes in the bulge or stem region would decrease Tat-mediated activity dramatically. However, if the deletion or mutation occurred in the loop region, there was little effect on Tat function (10,15,31). These data indicated that the BIV Tat/TAR interactions differed from HIV Tat/TAR. Further analysis showed that BIV Tat binds its TAR tightly, and there is no cellular loop binding protein required to stabilize Tat/TAR interaction (15). The BIV Tat mediated transactivation is not as high as HIV Tat mediated transactivation (15). There is still a lot of work needed to elucidate the role of cellular protein(s) and Tat/TAR interaction in bovine lentivirus transactivation.

Table 1. Functional sequences in BIV LTR<sup>1</sup>

Transcription Factor Binding Sites	Location	Effect of deletion on LTR activity <sup>2</sup>	
		Basal	Transactivation
NF-kB	-348 to -299	40.7	29.9
GRE	-252 to -247	17.0	22.8
Ap-4	-183 to -176	69.9	50.5
Ap-3/RCE	-174 to -168	166.4	123.1
Ap-1	-142 to -135	39.9	34.8
CAAT/ATF	-105 to -94	29.8	13.9
SP-1	-66 to -58	313.3	91.0
1/2 TAR	+32 to +69	155.8	155.2
R-U5	+1 to +204	216.0	4.0
U5	+109 to +204	616.6	598.9

1. Summary from Ref. 31

2. Compared to the wild type of LTR activity which is set to 100 in both basal and transactivation

## **CHAPTER 3. FUNCTIONAL CHARACTERIZATION OF BOVINE LENTIVIRUS LTRs**

A paper to be submitted the Journal of Virology

Jun Yang and Susan Carpenter

### **Abstract**

Lentiviruses, a subfamily of retroviruses, are a group of exogenous, nononcogenic viruses which cause chronic and variable clinical disease. The three bovine lentiviruses that have been characterized so far are the R29 and FL112 bovine immunodeficiency viruses (BIV), and the jembrana disease virus (JDV). These viruses have different pathogenicities and different levels of replication in their hosts. The long terminal repeat (LTR), including U3, R and U5, is the only promoter of lentiviruses and it plays a critical role in the basal and Tat mediated transcription of viral RNA. Comparison of the LTR sequences of R29, FL112 and JDV shows that the differences between R29 and FL112 mainly occur in the U3 region. The difference between JDV and BIV is found throughout the whole LTR. Because of the nucleotide substitutes within the R region, the predicted JDV secondary structure of the transactivation response region (TAR) is different from that of BIV. To determine if the LTR sequence heterogeneity among R29, FL112 and JDV differentially regulates the basal and/or Tat mediated transcription,

the three LTRs were inserted into a CAT reporter plasmid and transfected into Cf2th and 293 cells with or without a Tat expression plasmid. The CAT assay results show that, in the presence of Tat, the activity of FL112 was much higher than R29-4093 or JDV LTR constructs in both cell types. Interestingly, the activity of the JDV LTR construct was less than FL112 and R-29. To map the region important for the lower JDV LTR activity, chimeric LTRs were constructed by replacing either U3 or RU5 of BIV with that of JDV. Results showed that chimeric LTRs had dramatically decreased activity in both cell types. These data suggest that the LTR contributes to differences in BIV replication in vivo, but that other factors may be more important in control of JDV replication.

## Introduction

Bovine lentiviruses are a group of lentiviruses that cause variable pathogenicities in infected cattle (36,38,39,79). There are two species of bovine lentiviruses identified at this time, bovine immunodeficiency virus (BIV) and jembrana disease virus (JDV) (36,38,39,53). BIV, a species of bovine lentivirus with worldwide distribution, contains two known strains, R29 and Florida strain. Most characterized isolates of BIV are related to R29 (39,76). Compared to R29, the Florida strain, in vitro, has longer incubation time between passages and slower lysis of infected cells. In vivo, FL112-infected cattle show increases in the number of mononuclear cells and more pronounced histopathological lesions

(39,76). Despite these differences between R29 and FL112, BIV is a mildly pathogenic bovine lentivirus and there are no reports of overt clinical disease caused by BIV in naturally or experimentally infected cattle. JDV, the other species of bovine lentivirus, is found in some species of cattle in Indonesia, especially in Bali cattle, *Bos javanicus* (26,66). The major clinical signs of JDV-infected cattle are fever, lethargy, anorexia and enlargement of the superficial lymph nodes. The mortality rate of experimentally infected cattle is reported to be 17%. After 5 to 12 days of infection, viral titre can reach  $10^8$  units per ml (26). JDV replicates very fast and causes acute fatal disease in infected animals. Therefore, the three bovine lentiviruses, R29, Florida and JDV, cause quite different clinical disease courses and different levels of viral replication in infected cattle.

In lentiviruses, the long terminal repeat (LTR) is the only promoter and plays an important role in viral gene regulation and pathogenesis (21,37,44). In addition to enhancer elements in the U3 region, transcription increases dramatically in the presence of the viral transactivator, Tat (3,4,10,11,27,60). The interaction between Tat and its binding site, termed transactivation response region (TAR) is specific, and the precise secondary TAR structure is critical to Tat function (5,7,15,17). In HIV, cellular loop-binding factor(s) stabilize the Tat/TAR binding and play a role in tat-mediated transactivation (25,42,81). Though the deletion and point mutation analysis indicate no cellular loop binding factors are required in BIV transcription (10), additional cellular factors are likely to be important for Tat function and LTR activity. Therefore, the contributors to optimal activity of the LTR are the LTR sequence, Tat, and cellular factors. Functional characterization of the LTRs of the

three bovine lentiviruses showing obviously different pathogenicities may demonstrate the contribution of the LTR in virus replication in vivo and pathogenicity.

## **Materials and Methods**

### **Cells and virus**

Two cell lines were used to conduct transient transfection assays for the different LTR and chimeric LTR constructs. The Cf2th (ATCC CRL-1430) cell line is permissive for many lentiviruses, including BIV, and has been used previously in studies of BIV LTR function (31,63). 293 cells (ATCC CRL-1573) are a transformed primary human embryonic kidney cell line and continuously produce adenovirus E1A protein which can increase HIV LTR activity (55).

Fetal bovine lung cells (FBL) were used to cultivate FL112 and R29 bovine lentiviruses for isolation of proviral DNA. All cells were propagated in Dulbecco's minimum essential medium (DMEM) supplemented with antibiotics and 10% fetal calf serum.

### **BIV LTR sequence analysis**

Cell free virus stocks of R29 and FL112 virus were used to infect low passage fetal bovine lung (FBL) cells. R29-4093 isolate was used as R29. After 4-5 days when syncytia were present, the infected cells were collected and total DNA was extracted using SDS-proteinase K (1,8).

The FL112 LTR was PCR amplified with B8260 and B8963C' primers (Table 2) from the FL112 proviral DNA, and the PCR product was cloned into the TA vector (Stratagene). The PCR amplification consisted of a total of 30 cycles: 94°C 2 min; 30 cycles of 94°C 1 min, 50°C 1 min and 72°C 2 mins; 5 mins extension at 72°C. Clones were screened by restriction enzyme analysis and one plasmid was sequenced as the FL112 LTR sequence. The R29 LTR sequence was derived in the same way as that of FL112 LTR. The R29 LTR was amplified by PCR from R29 proviral DNA with B8375 and B8963C' primers and the PCR product was cloned into the TA vector. A correct clone was verified by restriction enzyme analysis and was sequenced.

### **Vector construction**

Tat expression plasmids were constructed by insertion of the BIV Tat exon I or JDV Tat exon I PCR-amplified fragments into a pCR3.1 expression vector. The R29 proviral DNA was as the template for BIV Tat exon I. The oligonucleotides B5602 and B5965C' were used as primers for PCR amplification of the BIV Tat exon I. The PCR amplification conditions were the same as described above. A pUC19 plasmid containing a partial JDV cDNA sequence, including JDV Tat exon1, was supplied by Dr. Charles Wood of the University of Nebraska. The primers J4978 and J5316C' were used for PCR amplification of the JDV Tat exon I. The same amplification conditions described above were used. The 363 bp BIV Tat and 338 bp JDV Tat exon I PCR fragments were ligated separately into pCR3.1

expression vector and transformed into E.coli. The positive clones were identified and verified by sequence analysis.

To construct the LTR-CAT reporter plasmids, the LTRs of R29, FL112 and JDV were amplified by PCR using primers specific to each LTR sequence of the three isolates. They are, respectively, B8375 and B8963C', B6' and B8963C', and J7381 and J159C'. Amplified products were cloned into pCR2.1. Clones with the correct insert and orientation were identified by restriction enzyme analysis and verified by sequencing. A HindIII- XbaI LTR fragment was excised, purified and inserted into the HindIII & XbaI sites in pCAT basic plasmid (Promega) and sequenced.

The PLP ( PCR Ligation PCR ) technique was used to construct the chimeric LTR CAT reporter vectors (Fig.4). The U3 fragment of FL112, RU5 fragment of JDV, and U3 of JDV and RU5 of R29-4093 were amplified with Vent polymerase, creating blunt ends in the amplified products. The primers were B8375 and B384C' for the R29-4093 U3 region; B6' and B384C' for the FL112 U3 region; and J4 and J159C' for the JDV RU5 region; J7381 and J7604C' for the JDV U3 region; and B388 and B8963C' for the R29-4093 RU5 region. The U3 and RU5 fragments were purified and the RU5 fragments were phosphorylated by T4 kinase, then ligated with a high concentration of T4 ligase (50 units per reaction). The chimeric U3 - RU5 was amplified with the 5' primer of U3 and the 3' primer of RU5 by Taq polymerase. Amplified products were ligated to the PCR vector and subcloned into pCAT basic plasmid as described above. All constructs were verified by sequence analysis. Fig.5 summarizes the CAT constructs used in this study.

Table 2. Summary of the primers used in this study

<b>name</b>	<b>sequence</b>	<b>NT sequence location*</b>
<b>B8963C'</b>	5'-CTGTTGGGTGTTCTTCACCG-3'	187-206
<b>B8260</b>	5'-GGATCTCCTGACCCCTCAAC-3'	7874-7893
<b>B8375</b>	5'-TGTGGGGCAGGGTGGGACCT-3'	7988-8007
<b>B5602</b>	5'-GATTGTGGCAATATGCCCGGA-3'	5216-5236
<b>B5965C'</b>	5'-GGACAGCATTCTGCCAGG-3'	5561-5579
<b>J4978</b>	5'-AACCAAGGAGGGGATCAACC-3'	4978-4997
<b>J5316C'</b>	5'-CCGTGATCTCCAGGGTCCA-3'	5317-5336
<b>B6'</b>	5'-GGGAAGCTTAAAAGGGTGGACTGTGG-3'	7973-7992
<b>J7381</b>	5'-GGGAAGCTTGGTGGACTGTGGGGAGAA-3'	7382-7398
<b>J159C'</b>	5'-GCTTCTAGATTGGGTGGTTCTTGTTCCG-3'	141-159
<b>B384C'</b>	5'-CCCCGTACAGAGTGAAGATAGG-3'	8356-8371
<b>J4</b>	5'-CTCTGGATAGCTGACAGCTCCGAGCCCCCAG-3'	5-35
<b>J7604C'</b>	5'-TGAAGTTGCAGAATGCTCATGTGC-3'	7604-7624
<b>B388</b>	5'-GCTCGTGTAGCTCATTAGC-3'	5-23

\*Nucleotide sequence numbers are based on the BIV127 (Genebank M32690) for BIV primers and on JDV (Genebank U21603) for JDV primers.

### Cell transfection and CAT assays

Cf2th or 293 cells were seeded in triplicate at  $3 \times 10^5$  cells/well in 6-well tissue culture dishes. The following day, cells were transfected with LTR CAT constructs and CAT activity was quantified using methods similar to those previously described (10). Briefly, LTR-CAT construct were transfected with or without BIV/JDV Tat plasmid. All reactions contransfected with 0.5ug beta-

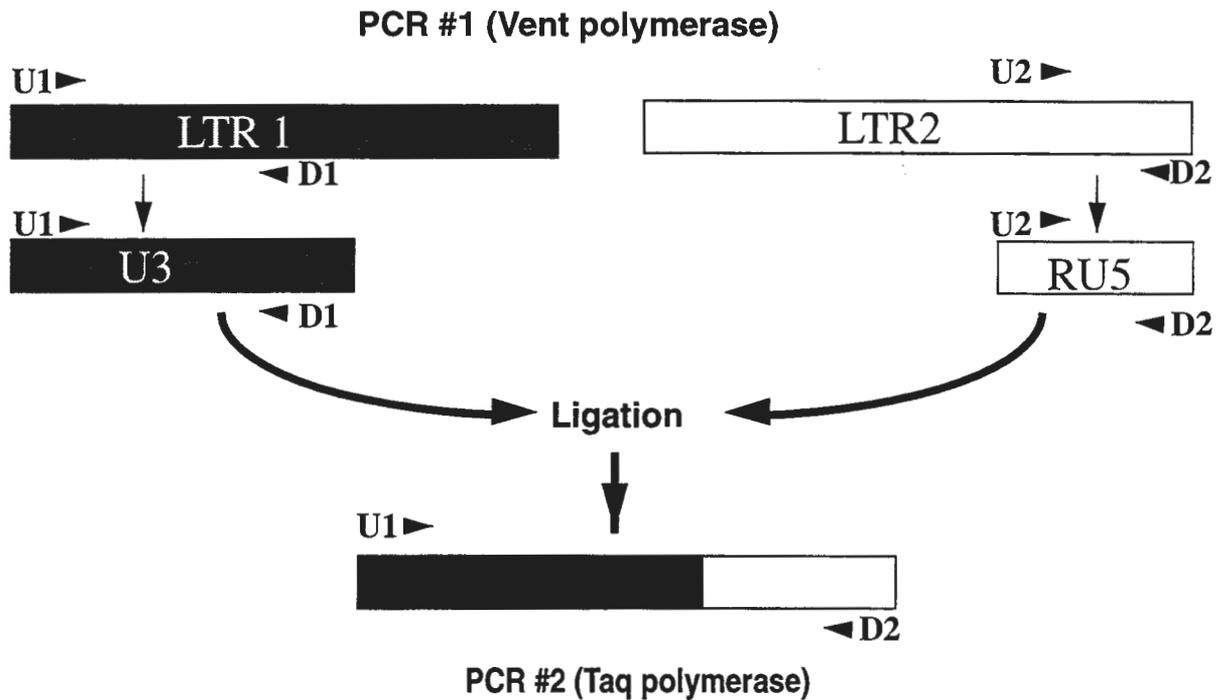


Fig. 4. PLP to construct chimeric LTRs. The chimeric LTR contains the U3 region of the LTR1 and the RU5 region of the LTR2. The U1, D1 and U2, D2 are pairs of primers for PCR amplification of the U3 of LTR1 and the RU5 of LTR2 by Vent polymerase. The purified U3 of LTR1 and the RU5 of LTR2 fragments were phosphorylated and ligated together. Then the chimeric LTR was amplified by Taq polymerase with U1 and D2 primers.

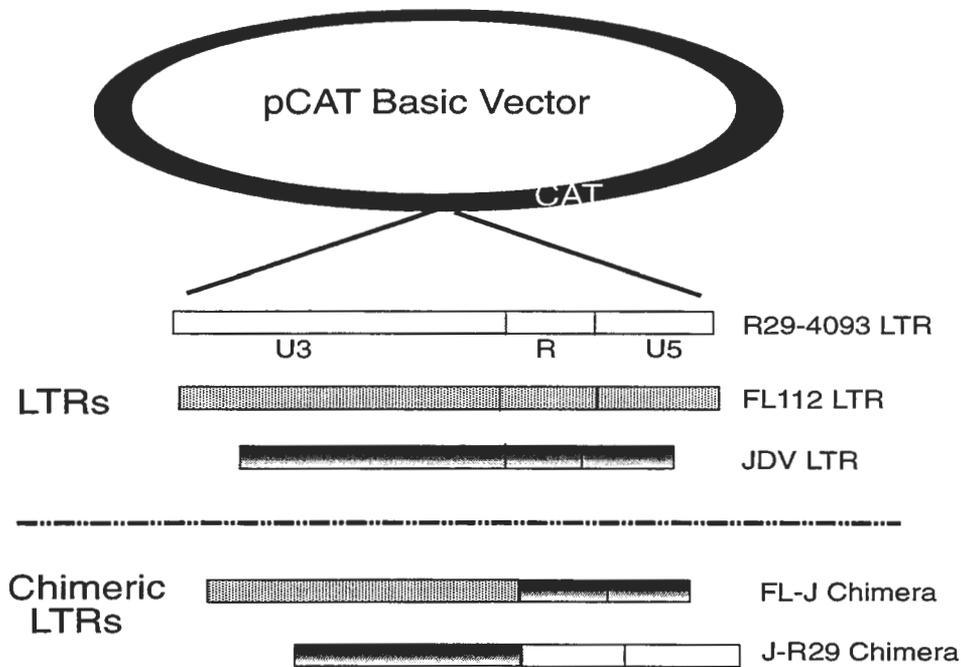


Fig. 5. Summary of the LTR reporter constructs. The three bovine lentivirus LTRs and the chimeric LTRs were inserted upstream of the CAT (chloramphenicol acetyl transferase) reporter gene in the pCAT basic vector. The LTRs were transfected and CAT expression was measured using procedures previously described (10).

galactosidase reporter plasmid pCH110 for measuring transfection efficiency and pUC19 DNA was added to normalize the total amount DNA in each well. Cells were transfected using calcium phosphate co-precipitation and the glycerol shock was performed 3 hours later for Cf2th cells. At two days post-transfection, cells were harvested, washed, resuspended in Tris buffer and then lysed three times by freeze-thawing in a dry ice ethanol bath. 50 ul lysate was assayed for beta-galactosidase analysis and results were used to measure the transfection efficiency and normalize the amount lysates used for CAT assays. Reaction volumes for CAT assays were equalized with 0.25M Tris, pH7.5, to a final volume 92 ul and incubated at 37C with 5 ul 20 mM acetyl coenzyme A and 3 ul of 50 mCi/mmol <sup>14</sup>C-chloramphenicol. Unacetylated and acetylated forms were separated by thin-layer chromatography and quantified using a Molecular Dynamics phosphoimager. The LTR plasmid with the highest CAT activity was used to normalize each experiment, and all experiments were repeated 2-4 times.

## **Results**

### **LTR nucleotide sequence comparison**

To examine the LTR variability among the three lentiviruses, the LTRs of BIV R29 and FL112 were sequenced and compared to the published JDV LTR sequence (Fig. 6). Differences in LTR sequence between FL112 and R29 were found primarily in the U3 region. Though there was a 16 nucleotide deletion and

many nucleotide substitutions found in the FL112 U3 region, the identified TFBSs were generally conserved between R29 and FL112. In the R-U5 region, there were only two nucleotide substitutions. One was an A to C change at +18 in the loop region of TAR. The other was a C to T change at +34 outside of TAR. From previous data, these changes are not expected to alter the TAR secondary structure or the activity of the LTR (8).

The differences in LTR sequence between JDV and R-29 were more striking (Fig. 6). The JDV LTR is 397 bp long, which is 192 bp shorter than that of R29. This was primarily due to a 157 nt long deletion in the U3 region. There were many nucleotide substitutions, and except for the NF- $\kappa$ B site, the enhancer elements were poorly conserved between the JDV and R29 LTRs. From the transcription factor database, the only Sp-1 site found was farther upstream of TATA box (74nt) than in R29 (19nt). Other transcriptional motifs, including the CAAT sequence, were not found in the JDV U3 region. Because of many nucleotide substitutions in the R region, the predicted JDV TAR secondary structure was different from that of BIV. Since the TAR structure is critical for lentivirus transactivation, this suggested that JDV LTR activity may be significantly different from BIV LTRs.

The result of the sequence comparison suggested that LTR sequence variation contributes to the different levels of viral replication and pathogenesis. The LTR activity and the importance of different LTR regions were tested in the *in vitro* transfection studies using different LTR-CAT, or chimeric LTR-CAT constructs.



### **Comparison of LTR activity in cells permissive for BIV replication**

To test the function of the three bovine lentivirus LTRs in activating downstream gene expression, the three bovine lentivirus LTRs were inserted into a CAT reporter plasmid. The activities of LTRs were assayed by quantifying CAT expression in transient transfection assays. In the Cf2th cells, all LTRs had low, but detectable levels of basal activity (Fig. 8). The basal activity in BIV R29 was 3-5 fold higher than that of FL112 and JDV. In the presence of Tat, all LTRs were more active, however, the range of transactivation differed from 3 to 50 fold over the basal activity. FL112 LTR was most active and had the highest fold of transactivation. The JDV LTR was least active and had lowest fold transactivation. R29 isolates were in intermediate in both transactivation and fold transactivation. Due to the low level of FL112 basal activity, the difference in fold of transactivation between R29 and FL112 was much higher than the difference in the overall level of CAT activity. For example, the activity of FL112 LTR was just about 3 fold higher than R29-4093 in the presence of BIV Tat. However, compared to the fold transactivation, FL112 was 9 fold higher than R29 (Fig. 8).

### **The effect of cell types on LTR activity**

Host cell factors are required for LTR activity. BIV is reported to have a broad host range and it can be cultured in many cell types. However, JDV has never been cultured in vitro. It is possible that BIV LTRs have higher activity than JDV LTR in Cf2th cells because Cf2th cells contain factors which are permissive for

BIV replication, but not for JDV replication. Due to the low activity of the JDV LTR in Cf2th cells, 293 cells were used to test LTR function. 293 cells are a good choice because the transfection efficiency is high in 293 cells and these cells also have high levels of transcription factors, such as the E1A protein, which increase activity of the HIV LTR (55).

Results observed in the 293 cells were similar to that in Cf2th cells. FL112 showed the highest activity and fold transactivation. JDV LTR had the lowest level of activity in the absence or presence of Tat (Fig. 9). Therefore, the 293 cells could not supply the cellular co-factors necessary for JDV LTR activity.

Compared to the data from Cf2th cells, the basal activity of FL112 in 293 cells was similar or even a little bit higher than R29 LTR. More striking, in 293 cells, the difference in activity between FL112 and R29 was greater than that seen in Cf2th. In Cf2th cells, the FL112 LTR was about 3 fold higher than R29 LTR in the presence of BIV Tat; however, in 293 cells, the FL112 LTR was about 12 fold higher than the R29 LTR (Fig. 9). This suggests that the FL112 is more active in 293 cells, possibly due to the presence of cofactors which interact preferentially with the FL112 LTR, and not the R29 LTR.

### **Functional comparison of BIV and JDV Tat**

A possible reason to account for the low activity of JDV LTR is the differences in the predicted TAR structure of JDV and BIV. The differences in TAR secondary structure could affect Tat function dramatically due to the critical role of TAR in Tat activity. To test this possibility, JDV Tat was used in the LTR

transactivation. Unexpectedly, JDV Tat did not transactivate JDV LTR to a high level (Fig. 8,9). So, the heterologous interaction between BIV Tat and JDV TAR was not the main reason for the low level of JDV LTR activity. JDV Tat could transactivate both BIV LTRs, but the transactivation mediated by JDV Tat was about two fold lower than that by BIV Tat in all LTRs in Cf2th cells and about three fold lower than BIV Tat in 293 cells (Table 3). The results indicated that JDV Tat did transactivate BIV LTRs. Also the BIV LTR could be transactivated by the heterologous Tat, although not as efficiently as with the homologous Tat.

### **The activity of chimeric LTRs**

To test which part of LTR, the U3 or the RU5 region, contributes to the low level of JDV LTR activity, the chimeric LTRs were made by replacing JDV U3 or RU5 region with FL112 U3 or RU5 region. The function of the chimeric LTRs was examined as described for the wild type LTRs. Comparing the CAT activity of J/FL standing for (JDV U3 - FL112 RU5 chimeric LTR), FL/J and FL112 LTRs, the FL112 LTR was much higher than the two chimeras (Fig. 10, 11). Both the basal and transactivation of J/FL were too low to be distinguished from the background in both cell types. The FL/J chimera could be activated by BIV Tat or JDV Tat, though the activity level was low. In the presence of BIV Tat, the transactivation of FL112 LTR was about 12 fold higher than FL/J chimera in Cf2th cells, and about 23 fold greater than FL/J in 293 cells (Fig. 10, 11). Therefore, replacement of either U3 or RU5 of FL112 LTR dramatically decreased LTR activity, indicating both the U3 and RU5 regions contribute to the low level of JDV LTR activity.

Table 3. Activity of LTRs in Cf2th and 293 cells

LTRs	Cell lines	Basal Activity	Transactivation	
			BIV Tat	JDV Tat
4093	Cf2th	1.36±0.22	7.31±1.06	4.36±0.45
	293	0.55±0.21	2.84±1.60	1.77±0.02
342	Cf2th	1.28±0.34	15.47±1.95	5.52±0.80
	293	0.48±0.13	3.93±1.03	1.20±0.08
FL112	Cf2th	0.41±0.06	21.07±2.32	9.74±2.08
	293	0.84±0.51	30.00±1.38	8.43±1.10
JDV	Cf2th	0.24±0.12	0.75±0.14	0.39±0.10
	293	0.04±0.01	0.41±0.20	0.02±0.00

## Discussion

The three natural bovine lentivirus, R29, FL112, and JDV, are associated with different pathogenicities and different levels of viral replication in vivo. The LTR activity of the three lentiviruses were examined by inserting wild type LTRs into a CAT reporter plasmid. The LTR activity of FL112 and R29 paralleled the differences of in vivo pathogenicity. In both cell lines, Cf2th and 293, FL112 LTR showed higher transactivation than R29 LTRs, which reflected the increased pathogenicity of FL112 in experimentally infected calves. Therefore, in BIV, the activity of LTR correlated with the level of viral replication and in vivo pathogenicity. However, the activity of JDV LTR was very low in both cell

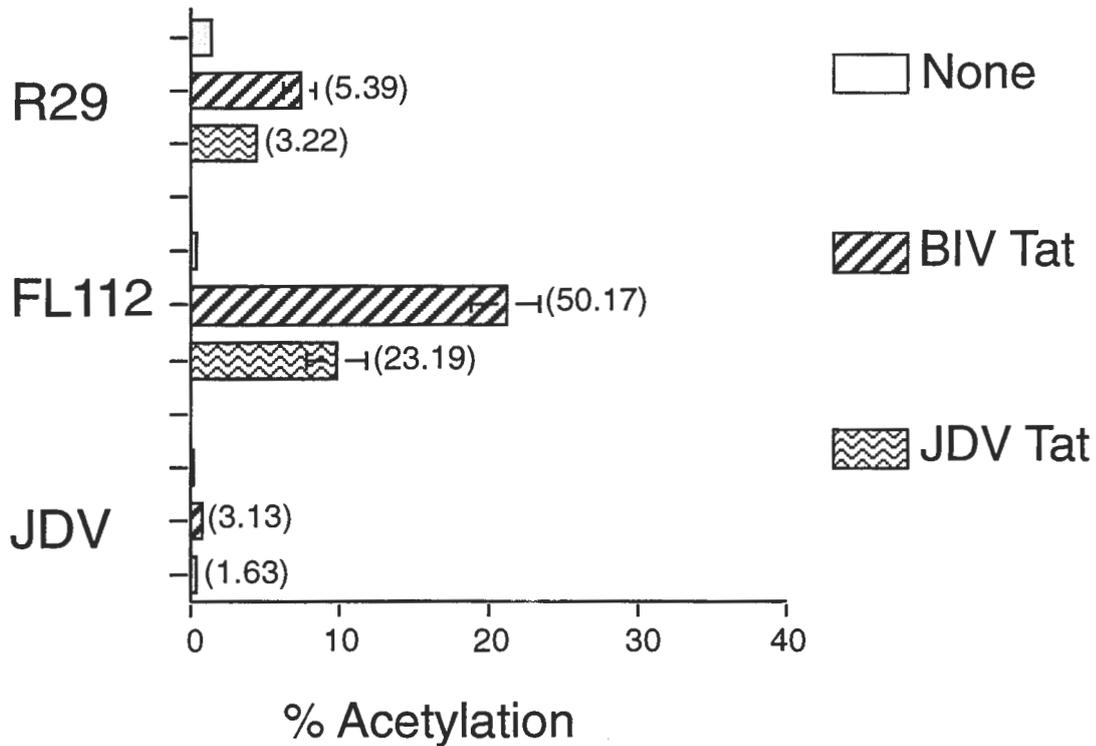


Fig. 8. Activity of LTRs in Cf2th cells. Cf2th cells in each well were transfected with 0.2 ug R29, FL112 and JDV LTR-CAT constructs, 0.5 ug  $\pm$  BIV Tat or  $\pm$  JDV Tat expression plasmid and 0.5 ug pCH110 DNA. % Acetylation is the ratio of the amount of the acetylated chloramphenicol to the total of chloramphenicol (acetylated and unacetylated). The data represent the mean of nine independent transfections  $\pm$  SEM (standard error mean). The numbers in parentheses are the fold of transactivation, calculated as the ratio of the activity in the presence of Tat to the activity in the absence of Tat.

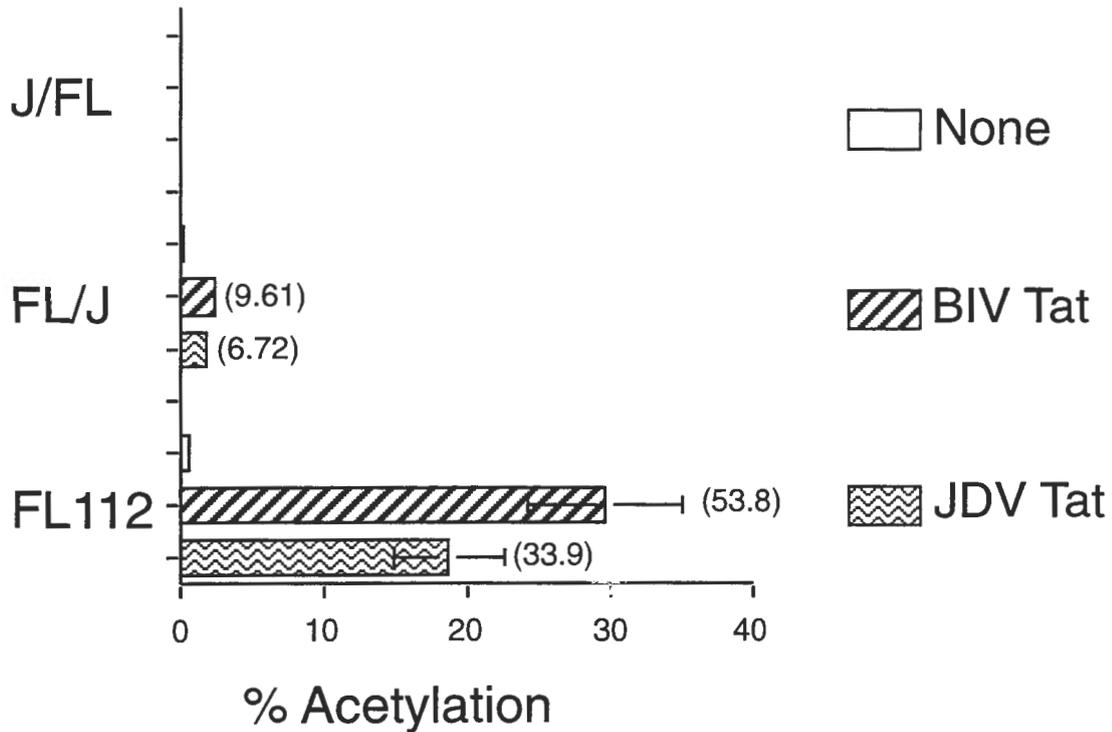


Fig. 9. Activity of LTRs in 293 cells. 293 cells were transfected with 0.1 ug R29, FL112 and JDV LTR-CAT constructs, 0.5 ug  $\pm$  BIV Tat or  $\pm$  JDV Tat expression plasmid and 0.5 ug pCH110. % Acetylation is the ratio of the amount of the acetyled chloramphenicol to the total of chloramphenicol (acetyled and unacetyled). The data represent the mean of nine independent transfections  $\pm$  SEM (standard error mean). The numbers in parentheses are the fold of transactivation, calculated as the ratio of the activity in the presence of Tat to the activity in the absence of Tat.

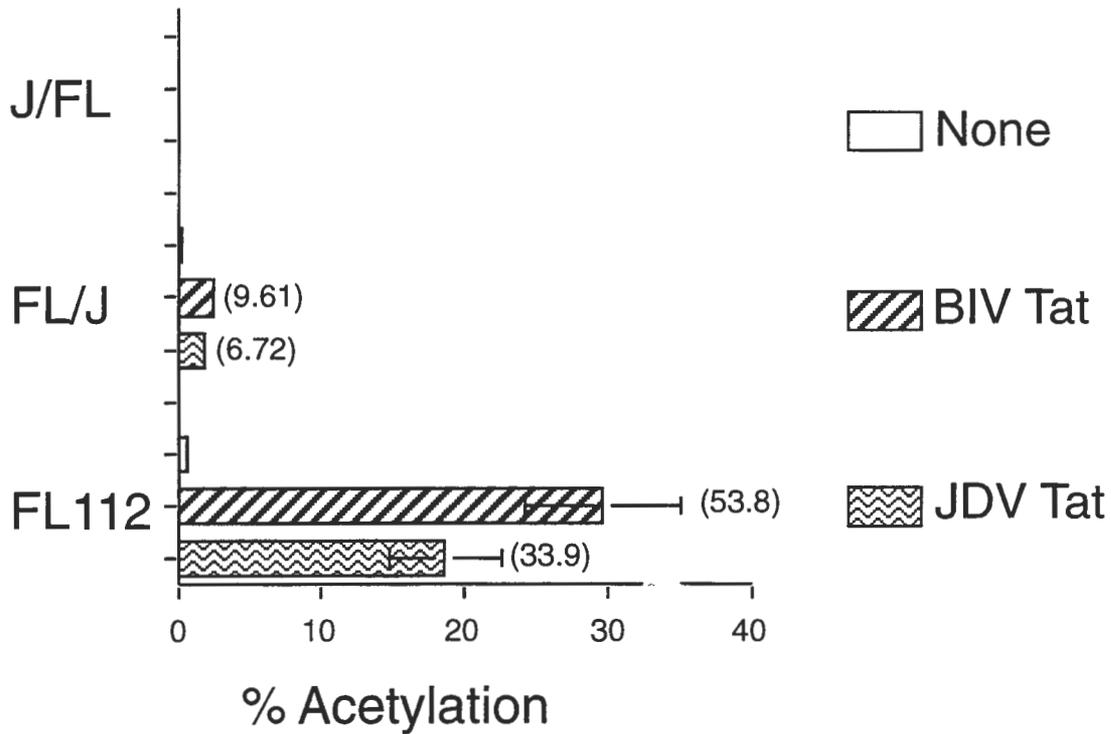


Fig. 10. Activity of chimeric LTRs in Cf2th cells. Cf2th cells in each well were transfected with 0.5 ug FL112 LTR-CAT and FL112 U3-JDV RU5 (FL/J) and JDV U3-FL RU5 (J/FL) chimeric LTR-CAT constructs, 0.5 ug  $\pm$ BIV Tat or  $\pm$ JDV Tat expression plasmid and 0.5 ug pCH110. % Acetylation is the ratio of the amount of the acetyled chloramphenicol to the total of chloramphenicol (acetyled and unacetyled). The data represent the mean of nine independent transfections  $\pm$  SEM (standard error mean). The numbers in parentheses are the fold of transactivation, calculated as the ratio of the activity in the presence of Tat to the activity in the absence of Tat.

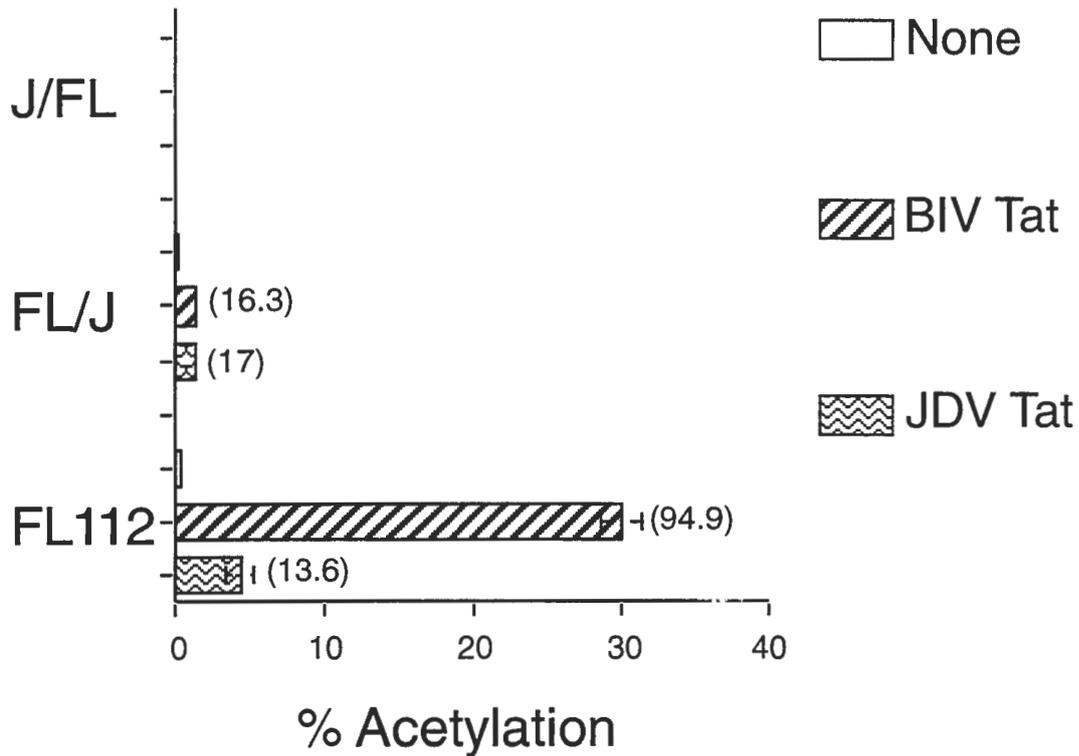


Fig. 11. Activity of chimeric LTR in 293 cells. 293 cells in each well were transfected with 0.5 ug FL112 LTR-CAT and FL112 U3-JDV RU5 (FL/J) and JDV U3-FL RU5 (J/FL) chimeric LTR-CAT constructs, 0.5 ug  $\pm$  BIV Tat or  $\pm$  JDV Tat expression plasmid and 0.5 ug pCH110. % Acetylation is the ratio of the amount of the acetylated chloramphenicol to the total of chloramphenicol (acetylated and unacetylated). The data represent the mean of three independent transfections  $\pm$  SEM (standard error mean). The numbers in parentheses are the fold of transactivation, calculated as the ratio of the activity in the presence of Tat to the activity in the absence of Tat.

types, and did not correlate with the high level of replication *in vivo* and the acute fatal disease course.

To elucidate the reason for the low level of JDV LTR activity, we tested different transactivators (BIV Tat and JDV Tat), different cell types (Cf2th and 293 cells) and U3 or RU5 substituted chimeric LTRs. The consistently low JDV LTR activity in the presence of JDV Tat indicated that the interaction between Tat and TAR was not the critical reason for the low JDV LTR activity in this study. The low JDV LTR activity in 293 and Cf2th cells suggested that both cell types may lack the required cellular factors for JDV LTR activity. The previous JDV infection studies showed that JDV only caused a severe disease in Bali cattle, not in other infected cattle species (71). Therefore, specific host factors may be present in Bali cattle which are required for JDV clinical disease. These factors may also play a critical role in LTR activity. This may account for the low JDV LTR activity in Cf2th (canine thymus cell) and 293 (human kidney cells).

The chimeric LTRs were used to elucidate the critical sites for low JDV LTR activity. Substitution of the JDV U3 for FL112 U3 eliminated activity. This suggests that Cf2th and 293 cells lack some absolutely required factors functioning in the JDV U3 region. These required cellular factors may be specific to host range or cell tropism. In EIAV, the U3 region plays an important role in virus cell tropism for EIAV (63). The low but detectable level of FL/J chimera suggested that the factors functioning in the RU5 region were important for the JDV LTR transactivation, though were not absolutely required. Therefore, both the JDV U3 and the JDV RU5 region accounted for the specific JDV LTR function in which cellular factors played

an important role. The results of JDV LTR sequence analysis showed that the CAAT box, found in most eukaryotic genes, was missing in the JDV LTR. Therefore, the JDV LTR itself may be responsible for the low level of activity. Furthermore, the JDV LTR clone came from the cDNA sequence, and may not represent the *in vivo* active LTR. Therefore, the reasons for the low level of JDV LTR activity are still unknown.

The comparison of the activity between R29 and FL112 in Cf2th and 293 cells also support the importance of cellular factors in LTR activity. The difference in the activity between R29 and FL112 was greater in 293 cells than in Cf2th cells. This suggests that 293 cells support FL112 LTR activity better than Cf2th cells. Therefore, the interaction between host cellular factors and the viral genome is important for LTR activity and for viral pathogenicity.

The activity of JDV Tat was lower than BIV Tat in both cell lines and with all LTRs except the FL/J chimeric LTR. Though the amino acid sequence comparison showed that JDV Tat had the same domains, (N-terminal, Cys-rich, Core, Basic and C-terminal) as BIV Tat, more studies are needed to determine if the low activity is due to the low activity of JDV Tat itself, or to the missing co-factors which were specific to JDV Tat not BIV Tat.

The results of this study showed that the function of BIV LTR paralleled the *in vivo* viral levels of replication and pathogenicity. Though the results of JDV LTR did not correlate with the high viral replication and severe pathogenicity *in vivo*, this study highlights the need for more JDV research, especially the elucidation of the mechanism of JDV LTR function.

## CHAPTER 4. GENERAL CONCLUSIONS

Bovine lentiviruses are a group of lentiviruses with different pathogenicities in cattle. The viral replication level is associated with its pathogenesis. The long terminal repeat (LTR), which flanks the lentivirus structural genes, is the only promoter in the lentivirus genome. Therefore, the function of the LTR is critical to viral replication and viral pathogenesis. This study took advantage of the three natural bovine lentiviruses with different pathogenicities to elucidate the role of the LTR in viral pathogenicity.

The sequence comparison of the three bovine lentivirus LTRs showed that the differences between R29 and FL112 LTR were found in the U3 region and did not alter TAR structure. The differences in LTR sequence contributed to different LTR activities. The results of this study showed that the activity of FL112 LTR was higher than R29 in both Cf2th and 293 cell lines. Therefore, in BIV, the higher LTR activity of FL112 correlated with the more pathogenicity *in vivo*.

However, the low activity of JDV LTR in both cell types did not parallel the severe disease course *in vivo*. Though the reasons for the low JDV LTR activity are still unknown, this study explored some possibilities and raised some interesting ideas. In our system, Cf2th and 293 cells, JDV Tat could activate the three bovine lentivirus LTRs though the activity was lower than BIV Tat. This result suggested that the heterologous interaction between BIV Tat and JDV TAR was not the main

reason for the low activity of the JDV LTR. The absence of required cellular factors was the most likely reason for the low activity of JDV LTR. This is supported by previous data indicating that among different JDV-infected cattle species, only Bali cattle developed severe disease. The results of the activity of chimeric LTRs indicated that the cellular factors functioning in the JDV U3 region were absolutely required for the JDV LTR activity and that the RU5 region also played important role.

Sequence analysis indicated that JDV LTR did not contain a CAAT box which is present in most eukaryote genes. So the JDV LTR itself may also contribute to the low activity. Furthermore, the sequence and clone of the JDV LTR came from a JDV cDNA, and it may not represent the JDV LTRs in vivo. Therefore, the mechanism of the function of JDV LTR is still unknown.

This study explored the contribution of the activity of the LTR to viral pathogenicity in the three bovine lentiviruses which showed different LTR sequences and different pathogenicities in vivo. This initial study of the JDV LTR provided more interesting ideas which would benefit later functional studies of the JDV LTR. Therefore, the results of the study will help to elucidate the mechanism of LTR function and the relationship between LTR function and viral pathogenesis, and may be practical benefit to cattle research.

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