STUDIES OF **DELTARETROVIRUS** RNA PACKAGING, INFECTIVITY AND DRUG SUSCEPTIBILITY

DISSERTATION

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By

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ABSTRACT

Deltaretrovirus is a genus of the Retroviridae that includes bovine leukemia virus (BLV) and human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2). These viruses replicate to low titers in their hosts and are poorly infectious in cell culture. Co-cultivation is often used to infect permissive cells. Due to these difficulties, information regarding the molecular details of their life cycle is limited. This dissertation extends the knowledge for three aspects of deltaretrovirus replication: 1) the identification of a deltaretrovirus RNA packaging signal that is necessary and sufficient for packaging of heterologous RNAs; 2) construction of indicator cell lines to detect deltaretrovirus infectivity; and 3) the sensitivity of BLV to nucleoside reverse transcriptase inhibitors (NRTIs).

A minimal BLV RNA packaging sequence (E) required for packaging of heterologous RNAs into BLV particles was analyzed. The hypothesis that a region previously mapped to contain the BLV primary and secondary packaging signal (BLV E) was necessary and sufficient for RNA packaging of heterologous RNA into BLV particles was tested. The BLV E was inserted into a non-viral vector, pLacZ, in order to determine if packaging of the non-viral vector RNA would occur. Reverse transcriptase-polymerase chain reaction (RT-PCR)
analysis of viral RNA from virus particles revealed that non-viral RNA containing the BLV E was packaged into BLV particles, indicating that the BLV E is necessary and sufficient to allow for packaging of a non-viral vector RNA. The ability of a chimeric murine leukemia virus (MLV) retroviral vector (pLN) containing the BLV E to be packaged into BLV particles was also analyzed. RT-PCR analysis showed that packaging of the MLV/BLV chimeric retroviral vector occurred in the presence of the BLV E. To determine if the extended MLV packaging signal (Ψ+) influenced the packaging of the MLV/BLV chimeric retroviral vector RNA, we evaluated the packaging efficiency of the MLV retroviral vector by itself. Interestingly, we found that Ψ+ could be recognized as a packaging signal by the BLV packaging machinery.

In the second study, mammalian cell lines were created to facilitate analysis of deltaretrovirus replication. The hypothesis tested in this study was that the long terminal repeats (LTRs) of BLV, HTLV-1 or HTLV-2 would drive expression of the green fluorescent protein gene (gfp) in cells when transactivated by the BLV, HTLV-1 or HTLV-2 Tax proteins, respectively. The BLGFP, H1GFP and H2GFP cell lines detect virus infection by the expression of GFP due to transactivation of the LTR via the respective Tax proteins. GFP expression was measured by flow cytometry, yielding sensitive and rapid detection of virus infectivity. Interestingly, it was observed that the HTLV-1 and HTLV-2 Tax proteins could transactivate the BLV LTR at levels that were comparable to that of BLV Tax. In contrast, the BLV Tax showed low levels of
transactivation in H1GFP and H2GFP cells. HTLV-1 and HTLV-2 Tax proteins efficiently transactivated both the HTLV-1 and HTLV-2 LTRs.

In the third study, the sensitivity of BLV to NRTIs was analyzed. The hypothesis tested was that BLV is susceptible to some nucleoside reverse transcriptase inhibitors that are used to inhibit human immunodeficiency virus type 1 (HIV-1) replication. BLV was found to be sensitive to low concentrations of dideoxyadenosine (ddA), dideoxyinosine (ddI) and 3'-azido-3'-deoxythymidine (AZT). Interestingly, we observed that BLV was sensitive to (-)2',3'-dideoxy-3'-thiacytidine (3TC) even though it was predicted to be resistant. Drug-resistant BLV was selected by long-term passage in the presence of high concentrations of the antiretroviral drugs. DNA sequencing revealed three amino acid changes in the reverse transcriptase, including a mutation (methionine to valine) in the highly conserved YMDD motif. These observations indicate that BLV is susceptible to NRTIs. In addition, these data indicate 3TC resistance is more complex than the methionine to valine substitution in the YMDD motif.

In summary, this dissertation has determined that a minimal BLV E is necessary and sufficient for RNA packaging of heterologous RNAs into BLV particles. Deltaretrovirus Tax proteins were shown to transactivate each others LTRs and result in GFP expression, which allowed for the development of indicator cell lines for virus infectivity. Finally, this work has demonstrated that BLV is susceptible to NRTIs.
Dedicated to my family
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CHAPTER 1

INTRODUCTION

Retrovirus life cycle

The *Retroviridae* are RNA viruses that utilize a DNA replication intermediate. Retroviruses are characterized by two unique features: 1) the viral genomic RNA is reverse transcribed by the viral enzyme reverse transcriptase (RT) to produce a double-stranded linear DNA copy of the viral genome and 2) the viral DNA integrates into the host cell chromosome through an event catalyzed by the viral enzyme integrase (IN) to form the provirus. The *Retroviridae* contain seven genera that can be divided into simple and complex retroviruses. Simple retroviruses encode only three viral genes, *gag*, *pol* and *env*. The Gag polyprotein contributes the structural genes matrix (MA), capsid (CA) and nucleocapsid (NC), while the Pol polyprotein produces the nonstructural enzymes RT, IN and protease (PR). The *env* gene encodes for both the surface (SU) and transmembrane (TM) domains of the viral envelope (Env) glycoprotein. A typical retrovirus particle is shown in Figure 1.1. The complex retroviruses encode the three basic genes *gag*, *pol* and *env* along with
accessory genes that help regulate the viral replication cycle (Vogt, 1997). This dissertation is focused on molecular studies of bovine leukemia virus (BLV) and human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2), which are complex retroviruses. I will therefore now discuss features of the complex retroviruses.

The replication cycle of complex retroviruses is divided into two phases, early and late. The early phase begins with virus entry into the cell and culminates in the production of an integrated provirus. The Env glycoprotein
binds to specific cell receptors via the SU protein, thereby attaching the virus particle or virion to the cell. After attachment, the TM protein induces fusion of the viral and cellular membranes in order to release the viral core into the cytoplasm. In the cytoplasm, the viral core is uncoated, resulting in a nucleoprotein complex that consists of the viral genomic RNA, the viral proteins MA and NC, and the viral enzymes RT and IN. Reverse transcription of the viral genomic RNA then takes place, producing double stranded linear DNA. During synthesis of the viral DNA, the RNase H domain of RT degrades the viral genomic RNA. The viral DNA remains in the nucleoprotein complex, which is now referred to as the preintegration complex (PIC). The PIC is then transported into the nucleus where IN catalyzes the integration of the viral DNA into the host cell chromosome to create a provirus (Vogt, 1997).

The late phase of the replication cycle involves transcription of the provirus DNA by RNA polymerase II and the subsequent actions leading to the assembly and release of a mature virus particle. Transcription of the proviral DNA initiates in the R region of the 5’-end long terminal repeat (LTR). The LTR contains the cis-acting sequences necessary for the trans-acting cellular transcription machinery to produce viral transcripts. The viral transcripts, either spliced or full-length mRNA, are subsequently transported to the cytoplasm. In the cytoplasm, the spliced mRNAs are translated to produce viral proteins and polyproteins. The full-length mRNAs can either serve as an mRNA or as a viral
RNA destined for packaging into virus particles. The full-length viral mRNAs that will serve as the new genomes interact with Gag polyprotein precursors and together are transported to the plasma membrane where other Gag precursors are present. (The process of RNA packaging will be discussed in a later section.) During this time, production of Env glycoprotein and transport to the plasma membrane occurs. Production of Env is distinct from translation of the other viral transcripts in that it takes place in the rough endoplasmic reticulum instead of on free polyribosomes. After translation, the Env glycoproteins are transported to the plasma membrane via the secretory pathway. At the plasma membrane, the genomic mRNA and Gag molecules assemble into protein complexes prompting the membrane to curve, thus forming a virus bud. The Env glycoproteins are incorporated into the plasma membrane where budding of the progeny virion takes place. Completion of budding occurs and the virus particle pinches off from the plasma membrane releasing an immature virion. Virion maturation occurs immediately after release due to cleavage of the Gag and Gag-Pol polyprotein precursors by PR, leading to core condensation, and the creation of infectious progeny virus (Vogt, 1997). A generalized retrovirus life cycle is shown in Figure 1.2.
Deltaretroviruses

The *Deltaretrovirus* genus of the *Retroviridae* includes BLV, HTLV-1 and HTLV-2. The deltaretroviruses are complex retroviruses that have been shown to be structurally and biologically similar and are characterized together due to genome organization and pathologies. These viruses cause lymphoproliferative diseases in their hosts (Cann and Chen, 1996; Petropoulos, 1997).

Figure 1.2: Retroviral replication cycle. The major steps involved in virus replication are shown. See text for details.
The deltaretroviruses contain the three major viral genes common to all retroviruses, *gag*, *pol* and *env* (Fig. 1.3). In addition, they contain two accessory genes that help regulate virus replication, *tax* and *rex*. Tax is expressed from a doubly spliced *tax/rex* mRNA (Fig. 1.3) and is a transactivator protein. Tax recruits cellular transcription factors to assemble with Tax on the Tax-responsive element (TxRE) in the U3 region of the LTR forming a multiprotein complex that drives gene expression (Bex and Gaynor, 1998; Brooks et al., 1998). The TxRE is composed of three imperfect 21-bp sequences which are necessary for Tax-mediated transactivation. Rex is also expressed from the doubly spliced *tax/rex* mRNA (Fig. 1.3). Rex is a regulator of expression protein and acts at the posttranscriptional level to regulate viral gene expression by enhancing the export of unspliced and incompletely spliced RNAs to the cytoplasm. The Rex-responsive element (RRE) is located in the R region of the 3’ LTR (Cann and Chen, 1996; Derse, 1987; Derse, 1988; Petropoulos, 1997; Tajima and Aida, 2000).

**Human T-cell leukemia virus type 1 (HTLV-1)**

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia/lymphoma (ATL) and is associated with a variety of neurological diseases such as tropical spastic paraparesis or HTLV-1-associated myelopathy (TSP/HAM) (Cann and Chen, 1996; Rosenberg and Jolicoeur, 1997). HTLV-1 infection is endemic in Japan, the Caribbean, South America and Africa
Transmission of HTLV-1 occurs through sexual contact, breast milk, blood products and intravenous drug users (Johnson et al., 2001; Poiesz et al., 2003; Suzuki and Gojobori, 1998). It is estimated that 10-20 million people world-wide are infected with HTLV-1; however, only 3-5% of the infected individuals will develop ATL (Johnson et al., 2001; Mortreux et al., 2003). ATL usually manifests in adulthood, around 20-30 years after infection. Individuals infected early in life with HTLV-1 are more likely to develop ATL than those infected later on in life (Cann and Chen, 1996; Johnson et al., 2001; Mortreux et al., 2003; Poiesz et al., 2003; Rosenberg and Jolicoeur, 1997). ATL disease progression is classified into five different stages: asymptomatic, pre-leukemic, smoldering, lymphoma and acute (Cann and Chen, 1996; Johnson et al., 2001; Mortreux et al., 2003).

The neurological diseases TSP/HAM are characterized by weakness, spastic paraparesis of the lower extremities, and mild peripheral sensory loss (Cann and Chen, 1996; Johnson et al., 2001; Poiesz et al., 2003). The spinal cord is predominantly affected by this disease. Demyelination of the spinal cord and lesions in the white matter of the spinal cord often occur in patients with this disease (Cann and Chen, 1996; Johnson et al., 2001; Poiesz et al., 2003). Additionally, the cerebral spinal fluid of TSP/HAM patients has been shown to be infected by HTLV-1 (Cann and Chen, 1996; Johnson et al., 2001; Poiesz et al., 2003). Interestingly, biological and genetic analyses of viral isolates from patients
with either TSP/HAM or ATL have not revealed any noticeable differences (Cann and Chen, 1996; Johnson et al., 2001; Poiesz et al., 2003; Suzuki and Gojobori, 1998).

Figure 1.3: HTLV-1 and BLV genome organization. A. HTLV-1 proviral DNA. The long terminal repeats (LTRs) are shown at the 5' and 3' ends of the genome. The LTRs are divided into three regions: U3, R and U5. The U3 region contains the tax responsive elements (TxREs) and the sequences necessary for termination and polyadenylation of the mRNAs. The R region is the region of the viral RNA that is repeated at the 5' and 3' ends. The U5 region of the LTR is the first region copied into DNA by RT. The three major viral genes gag, pol and env are shown along with the accessory proteins tax and rex. The dotted line indicates the tax and rex genes are split and that the proteins are translated from a spliced mRNA. B. BLV proviral DNA. Gene descriptions are the same as for HTLV-1.
Human T-cell leukemia virus type (HTLV-2)

Disease associations of human T-cell leukemia virus type 2 (HTLV-2) are not clear. HTLV-2 was first identified in T-cells derived from a patient with hairy-cell leukemia, and subsequent studies have shown a link between HTLV-2 infection and this form of leukemia (Rosenberg and Jolicoeur, 1997). Hairy-cell leukemia that is caused by HTLV-2 infection is often referred to as atypical hairy-cell leukemia. This is because most hairy-cell leukemias are usually of B-cell phenotype and in patients with HTLV-2 infection, the leukemic cells have been found to be T-cells (Cann and Chen, 1996). HTLV-2 infected patients with hairy-cell leukemia have been found to have the following characteristics: T-cell lymphocytosis circulating atypical lymphocytes with characteristic hairy-cell morphology, and bone marrow and splenic pathology that confirms hairy-cell leukemia (Cann and Chen, 1996).

HTLV-2 is prevalent among two groups, intravenous drug users and isolated tribes in parts of Central and South America and in Native Americans (Cann and Chen, 1996; Poiesz et al., 2003; Rosenberg and Jolicoeur, 1997; Trejo and Ratner, 2000; Vandamme et al., 2000). Within the two endemic populations, i.e. tribes and intravenous drug users, virus spread occurs through sexual contact, mother-to-child transmission via breast milk, and needle sharing (Poiesz et al., 2003; Vandamme et al., 2000). A recent report on the evolution and spread of HTLV-2 indicated that intravenous drug users are spreading the
virus in an epidemic wave world-wide due to transmission of the virus through needle sharing. Furthermore, through this mode of transmission, HTLV-2 has been shown to acquire mutations at a much higher rate than that seen in the endemic tribal populations, indicating that new and more aggressive variants of HTLV-2 are on the rise within the intravenous drug population (Vandamme et al., 2000).

**Bovine leukemia virus (BLV)**

Bovine leukemia virus (BLV) is the etiological agent of a chronic lymphatic leukemia/lymphoma in cows (Burny et al., 1985; Burny et al., 1988; Ferrer et al., 1979). Thirty to seventy percent of cattle that are infected by BLV develop persistent lymphocytosis (PL), which is a polyclonal expansion of B cells in response to the presence of BLV antigens (Burny et al., 1985; Burny et al., 1988). Persistent lymphocytosis, regarded as a benign form of leukemia, can remain stable for long periods of time (Willems et al., 2000). Only a small percentage of BLV infected cattle, 1 to 5%, develop lymphoma (Burny et al., 1985; Burny et al., 1988; Ferrer et al., 1979; Schwartz and Levy, 1994). Although persistent lymphocytosis is not a determinant for the development of lymphoma, roughly two-thirds of the animals that developed lymphoma had persistent lymphocytosis (Ferrer et al., 1979; Schwartz and Levy, 1994). The tumor form of BLV infection affects adult cattle with a prevalence around the ages of 5 to 8 years (Ferrer et al., 1979; Schwartz and Levy, 1994). The clinical
signs of malignant lymphoma include enlarged lymph nodes, weight loss, decreased milk production, fever and loss of appetite. These signs become apparent as the tumors metastasize (Ott et al., 2003).

Transmission of BLV occurs horizontally through the transfer of infected lymphocytes, natural transmission via direct contact, milk from infected animals, and occasionally biting insects (Burny et al., 1985; Burny et al., 1988; Schwartz and Levy, 1994; Willems et al., 2000). The majority of virus transmission occurs through the practices of dehorning, and ear tattooing (Schwartz and Levy, 1994; Willems et al., 2000).

Sheep and goats are also susceptible to infection by BLV; however, goats rarely develop lymphoma (Burny et al., 1985; Burny et al., 1988; Schwartz and Levy, 1994). Although not natural hosts for BLV, sheep are often used for experimental infection with BLV due to their high sensitivity to BLV infection. Additionally, their disease progression is markedly distinct from that of cattle. Sheep have a shorter latency period before the onset of leukemia- usually 1 to 4 years, compared to 4 to 10 years for disease progression in cattle. Almost all sheep infected with BLV develop lymphomas (Burny et al., 1985; Burny et al., 1988; Schwartz and Levy, 1994; Willems et al., 2000). Finally, stabilization of the number of infected cells (i.e. persistent lymphocytosis) is not observed in sheep. In sheep, the number of infected cells gradually rise until the onset of leukemia (Rovnak et al., 1993; Willems et al., 2000). Due to these differences, sheep
provide a good model system for BLV infection. Furthermore, the disease is not contagious in sheep, infected animals can be housed with the rest of the herd without transmission of the virus occurring (Willems et al., 2000).

A 1987 estimate of the economic impact of BLV infection was $44 million in the United States. This estimate was mostly based upon the expenses incurred from slaughterhouse condemnation of tumor-bearing carcasses and replacement of the sick or dead cattle (Da et al., 1993). Subsequent studies have added reduced milk production and reduction in reproductive efficiency to the economic losses caused by BLV (Da et al., 1993; Pelzer, 1997). A recent study investigated the association between BLV infection and milk production in US dairy herds. Data was analyzed from the USDA National Animal Health Monitoring System’s Dairy ‘96 study, which surveyed 1006 herds from 20 states (Ott et al., 2003). The study showed that 88% percent of the herds analyzed had at least one cow that was seropositive for BLV (Ott et al., 2003). Milk production among the BLV positive herds was 2.7% less than that of BLV negative herds. From this study it was concluded that in 1995 the total loss to the economy resulting from reduced milk production due to BLV seropositivity was $285 million for the producers and $240 million for consumers for a total of $525 million (Ott et al., 2003). Currently, culling of the herd is the only control strategy for BLV infection, there is no curative treatment.
Deltaretrovirus RNA packaging

The RNA packaging process for retroviruses involves a recognition event of the genome-length viral RNA by the viral Gag polyprotein, as mentioned above in the retroviral replication cycle. Genetic and biochemical analyses have shown that the recognition event involves the interaction of stable RNA secondary structures at the 5’ end of the viral genome and, in many cases, amino acids in the nucleocapsid (NC) domain of the Gag protein (Berkowitz et al., 1995; Certo et al., 1999; Certo et al., 1998; De Guzman et al., 1998; Harrison et al., 1998; Jewell and Mansky, 2000; McBride and Panganiban, 1996; Poon et al., 1998; Schwartz et al., 1997). This viral RNA-protein interaction, leads to the predominant packaging of the genome-length viral RNA, over that of spliced viral RNAs and cellular mRNAs (Swanstrom and Wills, 1997). The RNA sequences necessary and sufficient for the viral RNA packaging process are referred to as the packaging signal (Ψ) or the encapsidation signal (E). Generally, these signals are located in the area of the viral genome that includes the 5’-noncoding region as well as the 5’ half of the gag gene (Swanstrom and Wills, 1997). The packaging signal can enhance the packaging of RNAs that contain the signal as compared to RNAs that do not contain a packaging signal (Berkowitz et al., 1996).

The Gag polyprotein for both complex and simple retroviruses appears to be sufficient for the formation of particles, and Gag-only particles can package
viral RNA (Sakalian et al., 1994). These observations suggest that the genome recognition event involves the full-length viral RNA and the unprocessed Gag polyprotein, and furthermore, indicates that Gag contains the viral protein components needed for specific viral RNA packaging. Attempts to elucidate the RNA packaging process have focused on identifying RNA-binding domains within unprocessed and processed Gag and demonstrating specificity in binding of viral RNA to Gag.

Previously, the region of the BLV genome that contained the BLV packaging signal (BLV E) was mapped by deletion analysis and was found to be located near the 5’ end of the BLV genome and consisted of two important regions (Fig 1.4A) (Mansky et al., 1995). The first region consists of sequences downstream from the primer binding site (pbs) and extends into the beginning of the \textit{gag} gene. This region was found to be necessary for BLV RNA packaging. The second region, found in the \textit{gag} gene, is a 132-bp sequence that enhances BLV packaging in the presence of the first region (Mansky et al., 1995). These results led to the conclusion that the BLV packaging signal necessary for efficient RNA packaging and virus production is discontinuous. Furthermore, the BLV E is located exclusively in the \textit{gag} gene and not in the 5’ untranslated leader region, as is the case for a majority of retroviruses. Structure-function analysis established that the primary packaging signal (E) of BLV was composed of two stable RNA stem-loop structures (SL1 and SL2) located downstream of the start
A secondary packaging signal, consisting of one stable stem-loop structure, was found in the CA domain of Gag (Mansky and Wisniewski, 1998). A global minimum-energy optimal folding of the entire BLV genomic RNA contained the same predicted stable secondary structures of both of the regions of the BLV packaging signal, reinforcing their biological relevance (Mansky and Wisniewski, 1998).

Mutations that disrupted the stems of either or both the stem-loop structures of the primary BLV packaging signal, were found to reduce the replication efficiency by a factor of 7 and 40, respectively (Mansky and Wisniewski, 1998). Equivalent reductions in RNA packaging efficiency were also found with these mutations, indicating a correlation between virus replication and packaging. Additionally, mutations that disrupted the stem-loop structure of the secondary BLV packaging signal were found to have comparable, 4- to 6-fold, reductions in both the replication and RNA packaging efficiencies (Mansky and Wisniewski, 1998).
Figure 1.4: Packaging signal of BLV. A. The 5' end of the BLV genome is shown in the proviral DNA form. The rectangular box at the end of the solid black line represents the 5' long terminal repeat (LTR), containing the U3, R, and U5 regions. The solid black line indicates the viral sequence. The rectangular box above the solid black line represents \textit{gag} and has the matrix (MA), capsid (CA) and nucleocapsid (NC) domains indicated. The jagged line at the end of the viral sequence indicates the end of the viral sequence shown in this diagram. At the bottom, region 1 and region 2 refer to the primary and secondary packaging signals of BLV. B. The predicted secondary structure of the primary packaging signal of BLV. Stem-loop 1 (SL1) and stem-loop 2 (SL2) are indicated. The \textit{gag} AUG start codon is marked with a box.
Mutation of the sequences within the loops of SL1 and SL2, that maintained the same stable secondary structures as the wild type sequence, resulted in reduced virus replication by 10- to 7-fold, respectively. When both loops of SL1 and SL2 contained mutations, a 50-fold reduction in virus production was observed (Mansky and Gajary, 2002). Analysis of the RNA packaging efficiency of the individual and dual mutations found similar reductions, 10- 7- and 50-fold respectively, in RNA packaging indicating a correlation between RNA packaging and virus replication (Mansky and Gajary, 2002). The sequences of the stems were also mutated to assess their importance in virus replication and were found to have a 5-fold reduction in virus replication and a comparable decrease in RNA packaging, as compared to the parental virus (Mansky and Gajary, 2002). These results indicate that the primary sequence of both the loops and stems of SL1 and SL2 are important in maintaining the function of the BLV packaging signal.

The order and efficacy of each stem-loop of the primary BLV packaging signal was also evaluated. A three-fold reduction in virus replication was observed when the order of the stem-loops was reversed. A similar decrease in RNA packaging was also observed, signifying that the reduction in virus replication was due to an RNA packaging defect (Mansky and Gajary, 2002). To determine if one of the stem-loops could compensate for the other, mutants that contained either two copies of SL1 or SL2 were created. Each mutant replicated
five-fold lower than the parental virus (Mansky and Gajary, 2002). Together these results indicate that the order of the stem-loops is important and that SL1 and SL2 are not functionally equivalent.

The conservation of function of packaging signals in the BLV/HTLV genus was tested by Mansky and Wisneiwski. Chimeric viruses were created in which the BLV packaging signal region was replaced with that of either HTLV-1 or HTLV-2. The replication efficiency for these chimeric viruses was found to be three-fourths and one-fifth of that of the parental virus for HTLV-1 and HTLV-2, respectively (Mansky and Wisniewski, 1998). Thus, the predicted packaging signal region of either HTLV-1 or HTLV-2 can replace the BLV primary packaging signal region. The HTLV-1 and HTLV-2 predicted stable RNA secondary structures located downstream of the Gag start codon are similar to each other and to that of BLV. The predicted SL1 and SL2 of HTLV-1 (Fig. 1.4) were used to specifically replace the SL1 and SL2 of BLV in order to test whether the predicted SL1 and SL2 of HTLV-1 could functionally replace that of BLV. The resulting chimeric virus had a five-fold decrease in replication efficiency, as compared to the parental virus (Mansky and Gajary, 2002). These data imply a conservation of packaging signal function within the BLV/HTLV genus. Additionally, they provide evidence that the BLV packaging signal is recognized as a result of its secondary or tertiary structures.
For many retroviruses, it is the NC domain of Gag that is responsible for the interaction of the viral Gag polyprotein with the genome-length viral RNA. The zinc-finger motifs and the basic amino acids in the NC domain have been shown to be involved in RNA packaging of many retroviruses (De Guzman et al., 1998; Jewell and Mansky, 2000; Poon et al., 1996; Schmalzbauer et al., 1996; Schwartz et al., 1997; Zhang et al., 1998). For BLV, it was earlier suggested that the matrix (MA) protein was involved in BLV genome recognition and RNA packaging. Data showed that the BLV MA protein specifically bound to RNAs representing the 5’ end of the BLV genome (Katoh et al., 1991; Katoh et al.,
There were two regions of RNA that the MA protein specifically bound- the
dimer linkage site (dls) and the 5’ end of the *gag* gene. The latter is where the
BLV E was later shown to be located (Katoh et al., 1991; Katoh et al., 1993;
Mansky and Wisniewski, 1998). A recent study of the BLV Gag domains
involved in the packaging process showed that both the MA and NC domains of
BLV Gag played a role in genome recognition and RNA packaging (Wang et al.,
2003). Alanine-scanning mutagenesis of conserved residues in each of the two
zinc fingers in the NC domain resulted in severe packaging defects (less than
15% of wild-type) (Wang et al., 2003). Surprisingly, mutation of basic residues in
the MA domain also led to packaging defects, some as great as those observed
for the NC domain (Wang et al., 2003). Two of the BLV MA mutants that had
significant reductions in packaging efficiency occurred in residues that are
conserved between BLV, HTLV-1 and HTLV-2 (Wang et al., 2003). These data
indicate that the BLV MA plays an important role in viral RNA packaging, which
suggests the BLV genome recognition and RNA packaging process occurs
through a distinct mechanism, compared to that used by other retroviruses.

**Antiretroviral drugs and drug resistance**

Antiretroviral therapy (ART) for the treatment of human immunodeficiency
virus type 1 (HIV-1) infection currently consists of nucleoside reverse
transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors
(NNRTIs) and protease inhibitors (PIs). Use of these three classes of antiretroviral drugs has dramatically reduced the rate of HIV-1 and AIDS related morbidity and mortality (Pillay et al., 2000; Richman, 2001). However the emergence of drug-resistant virus, due to poor patient compliance of therapy, still remains a threat to the success of antiretroviral therapy (Bangsberg et al., 2000; Haubrich et al., 1999). Drug-resistant virus can emerge to any one or a combination of the NRTIs, the NNRTIs or the PIs. For HIV-1, resistance to reverse transcriptase (RT) and protease inhibitors has been shown to correlate to mutations in the genes of HIV RT and protease, respectively (Emini and Fan, 1997; Johnson et al., 2003; Menendez-Arias, 2002).

Mutations in HIV-1 RT that confer drug resistance to either NRTIs or NNRTIs have been mapped to the fingers, palm and thumb subdomains of the catalytic domain of RT (Emini and Fan, 1997; Telesnitsky and Goff, 1997). Within the cell, NRTIs need to be phosphorylated into their triphosphate form so that they can act as a competitive inhibitor to the deoxynucleotide triphosphate (dNTP) substrate (Emini and Fan, 1997; Jonckheere et al., 2000; Menendez-Arias, 2002; Tozser, 2001). Once incorporated into the growing template by RT, the NRTIs act as chain terminators to inhibit DNA synthesis. The NRTIs lack a 3' OH group which prevents further elongation of the template with other dNTPs (Emini and Fan, 1997; Jonckheere et al., 2000; Menendez-Arias, 2002; Tozser, 2001). Some common NRTIs and their structures are shown in Figure 1.6.

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Resistance to NRTIs correlates with substitutions of residues located in the dNTP binding site of RT (Emini and Fan, 1997; Jonckheere et al., 2000; Menendez-Arias, 2002). However, some HIV-1 RT mutations resistant to 3’-azido-3’-deoxythymidine (AZT) are not located near the dNTP binding site. Additionally, it has been shown that AZT resistant mutations in HIV-1 RT work together to augment the selective excision of AZT from the growing template via phosphorolysis (Arion et al., 1998; Boyer et al., 2001; Meyer et al., 1999).

Within the palm domain of HIV-1 RT lies the highly conserved YXDD motif. The two aspartate residues (185, 186) of the YXDD motif, along with another nearby aspartate residue (110), form the polymerase active site of HIV-1 RT (Telesnitsky and Goff, 1997). This region of the enzyme contacts the primer template and binds the incoming dNTP (Kohlstaedt et al., 1993; Telesnitsky and Goff, 1997). In HIV-1 antiretroviral therapy, use of the antiretroviral drug (-)2′,3′-dideoxy-3′-thiacytidine (3TC) revealed that a single amino acid change in the YXDD motif leads to 3TC resistance. HIV-1 contains methionine as the variable amino acid in the YXDD motif (i.e. YMDD) and was found to be sensitive to 3TC. Mutation of the methionine residue at position 184 to valine or isoleucine, creating YVDD or YIDD results in a highly 3TC-resistant virus (Schinazi et al., 1993). The mechanism of 3TC resistance is believed to involve steric hindrance. Placement of a valine or isoleucine in the X position of YXDD
interferes with 3TC binding due to a steric clash between the \(\beta\)-branched amino acids and the oxathiolane ring of 3TC (Sarafianos et al., 1999).

The second class of RT inhibitors, NNRTIs, all bind to a hydrophobic pocket near the polymerase active site of the HIV-1 RT (Emini and Fan, 1997; Frankel and Young, 1998; Jonckheere et al., 2000; Menendez-Arias, 2002; Tozser, 2001). Binding of the NNRTI to the hydrophobic pocket causes a rearrangement in the polymerase active site that locks the RT into an inactive conformation (Frankel and Young, 1998). NNRTIs are a structurally diverse group of compounds, however most can adopt a “butterfly” shape in which one wing is close to and the other wing is distal to the polymerase active site when bound to RT (Emini and Fan, 1997; Jonckheere et al., 2000). Resistant mutations against NNRTIs develop rapidly and often only a single mutation in the RT can cause NNRTI therapy to fail (Jonckheere et al., 2000; Tozser, 2001). The majority of NNRTI resistant mutations are located within the same hydrophobic pocket that the inhibitor binds to, thus cross-resistance within this class of RT inhibitors is higher than that seen with NRTIs (Emini and Fan, 1997; Jonckheere et al., 2000; Tozser, 2001).

The final class of antiretroviral drugs is the PIs. PIs are substrate-based compounds that compete against the viral substrates for the PR (Menendez-Arias, 2002; Tozser, 2001). The inhibitors usually contain a nonhydrolyzable transition-state mimic at the cleavage site of the substrate (Tozser, 2001).
Resistant mutations are found in the substrate-binding pocket of PR (Frankel and Young, 1998; Menendez-Arias, 2002; Tozser, 2001). These mutations alter the specificity and catalytic activity of the enzyme (Frankel and Young, 1998; Menendez-Arias, 2002; Tozser, 2001). Due to similarities in the structures of the inhibitors, cross-resistance between PIs is a problem (Emini and Fan, 1997; Menendez-Arias, 2002).

Figure 1.6: Nucleoside reverse transcriptase inhibitors (NRTIs). Structures of common NRTIs that are currently licensed to treat human immunodeficiency virus type 1 (HIV-1) infection are shown.
CHAPTER 2

HETEROLOGOUS RNA CONTAINING THE BOVINE LEUKEMIA VIRUS PACKAGING SIGNAL IS PACKAGED INTO BLV PARTICLES

ABSTRACT

A minimal bovine leukemia virus (BLV) RNA packaging sequence (E) required for heterologous RNAs to be packaged into BLV particles was analyzed. The BLV E was inserted into a non-viral vector, pLacZ, in order to determine if packaging of the non-viral vector RNA would occur. The construct was transfected into cells chronically infected with BLV in order to produce virus particles. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of viral RNA from virus particles revealed that non-viral RNA containing the BLV E was packaged into BLV particles, indicating that the BLV E is necessary and sufficient to allow for packaging of a non-viral vector RNA. We also analyzed the ability of a chimeric murine leukemia virus (MLV) retroviral vector (pLN) containing BLV E to be packaged into BLV particles. RT-PCR analysis showed that packaging of the MLV/BLV chimeric retroviral vector occurred in the presence of the BLV E. To determine if the MLV packaging signal (Ψ+)
influenced the packaging of the MLV/BLV chimeric retroviral vector, we evaluated the packaging efficiency of the MLV retroviral vector, which contains Ψ+. Interestingly, we found that Ψ+ could be recognized as a packaging signal by the BLV packaging machinery. Unexpectedly, it was found that pLNΔ (which does not possess Ψ+) was also packaged into BLV particles. This indicates that an MLV RNA region outside Ψ+ allows for packaging of the MLV RNA into BLV particles.

INTRODUCTION

Among retroviruses, a recognition event between the genome-length viral RNA and the viral Gag polyprotein initiates the viral RNA packaging process. Genetic and biochemical analyses have shown that the recognition event involves the interaction of stable RNA secondary structures at the 5’ end of the viral genome and, in many cases, amino acids in the nucleocapsid (NC) domain of the Gag protein (Berkowitz et al., 1995; Certo et al., 1999; Certo et al., 1998; De Guzman et al., 1998; Harrison et al., 1998; McBride and Panganiban, 1996; Poon et al., 1998; Schwartz et al., 1997). This leads to the predominant packaging of the genome-length viral RNA, over that of spliced viral RNAs and cellular mRNAs (Swanstrom and Wills, 1997). The RNA sequences necessary and sufficient for the viral RNA packaging process are referred to as the packaging signal (Ψ) or the encapsidation signal (E). Generally, these signals
are located in the area of the viral genome that includes the 5’-noncoding region as well as the 5’ half of the gag gene (Swanstrom and Wills, 1997). The packaging signal can enhance the packaging of RNAs that contain the signal as compared to RNAs that do not contain a packaging signal (Berkowitz et al., 1996).

Previously, the region of the BLV genome that contained the BLV packaging signal (BLV E) was mapped by deletion analysis and was found to be located near the 5’ end of the BLV genome. Analysis of the efficiency of RNA packaging revealed two important regions (Mansky et al., 1995). The first region consists of sequences downstream from the primer binding site (pbs) and extends into the beginning of the gag gene. This region was found to be necessary for BLV RNA packaging. The second region, found in the gag gene, is a 132-bp sequence that enhances BLV packaging in the presence of the first region (Mansky et al., 1995). These results led to the conclusion that the BLV packaging signal necessary for efficient RNA packaging and virus production is discontinuous.

Structure-function analysis established that the primary packaging signal of BLV was composed of two stable RNA stem-loop structures (SL1 and SL2) located downstream of the start codon of Gag (Mansky and Wisniewski, 1998). The secondary packaging signal, consisting of one stable stem-loop structure, was found in the capsid (CA) domain of Gag (Mansky and Wisniewski, 1998).
Thus, the BLV E is located exclusively in the *gag* gene and not in the 5’ untranslated leader region, as is the case for a majority of retroviruses (Swanstrom and Wills, 1997). In depth analysis of the two stem-loop structure of the BLV primary packaging signal revealed that the nucleotide sequences of both the stems and the loops were important for efficient virus packaging (Mansky and Gajary, 2002). Additionally, it was found that the relative order of the stem-loops is important and that the two stem-loops (i.e., SL1 and SL2) are not functionally equivalent (Mansky and Gajary, 2002).

For many retroviruses, it is the NC domain of Gag that is responsible for the interaction of the viral Gag polyprotein and the genome-length viral RNA. The zinc-finger motifs and the basic amino acids in the NC domain have been shown to be involved in RNA packaging of many retroviruses (De Guzman et al., 1998; Poon et al., 1996; Schmalzbauer et al., 1996; Schwartz et al., 1997; Zhang et al., 1998). For BLV, it was earlier suggested that the matrix (MA) protein was involved in BLV genome recognition and RNA packaging. Data showed that the BLV MA protein specifically bound to RNAs representing the 5’ end of the BLV genome (Katoh et al., 1991; Katoh et al., 1993). The MA protein bound to two regions of RNA, the dimer linkage site (dls) and the 5’ end of the *gag* gene, or the BLV E (Katoh et al., 1991; Katoh et al., 1993). A recent study of the BLV Gag domains involved in the packaging process showed that both the MA and NC domains of BLV Gag played a role in genome recognition and RNA packaging.
Alanine-scanning mutagenesis of conserved residues in each of the two zinc fingers in the NC domain resulted in severe packaging defects (less than 15% of wild-type) (Wang et al., 2003). Surprisingly, mutation of basic residues in the MA domain also led to packaging defects, some as severe as those observed for the NC domain (Wang et al., 2003). These data suggest that the BLV genome recognition and RNA packaging process occurs through a distinct mechanism compared to other retroviruses.

In this study the BLV E is further analyzed by determining if heterologous RNAs that contain the BLV E can be packaged into BLV particles. The presence of the BLV E on a non-viral vector allowed that vector to be packaged into BLV particles. Packaging of a chimeric Moloney murine leukemia virus (MLV)/BLV retroviral vector into BLV particles was found to occur when the BLV E was in the sense orientation. Interestingly the MLV retroviral vector, pLN, was found to be packaged into BLV particles, suggesting that recognition of the MLV extended packaging signal (Ψ+) had occurred in the BLV containing cells.

**MATERIALS AND METHODS**

**Plasmids.**

pBLVSVNEO is a derivative of p913, in which the region that encodes the \textit{tax} and \textit{rex} genes was replaced by the simian virus 40 (SV40) early promoter fused to the bacterial neomycin phosphotransferase gene (Derse and Martarano,
pLN contains the extended packaging signal (Ψ+) of Moloney murine leukemia virus (MLV) upstream of the neomycin resistance gene (Miller and Rosman, 1989). pLacZ is a mammalian epitope tag expression vector (Boehringer Mannheim, Indianapolis, IN).

**Construction of chimeric plasmids.**

The packaging signal region of BLV (nucleotides 450 to 1183 of the proviral DNA from BLVSVNEO) was PCR amplified and cloned into pCR2.1 (Invitrogen, Carlsbad, CA), a eukaryotic TA cloning vector. The primers used to amplify the BLV packaging signal region are 5’ –GCTCGTCCGGGATTGATCAC-3’ and 5’ -CGCTTCAGCGCGCCGCTATTGC- 3’. DNA sequencing was done to confirm the sequence and orientation of the packaging signal region of BLV in the vector. pLacZBLVE was created by inserting the BLV PCR fragment at the end of the LacZ sequence. The pCR2.1 vector creates EcoRI sites that flank the inserted PCR product. The BLV PCR fragment was released from pCR2.1 by EcoRI digestion and then inserted into the EcoRI site of pLacZ. DNA sequencing was done to confirm the proper introduction of the BLV sequence into pLacZ.

To create the chimeric MLV/BLV retroviral vector, the BLV PCR fragment was released from pCR2.1 by digestion with BamHI and Bcl I. pLN was linearized downstream of the MLV Ψ+ with Bcl I. The BLV sequence was inserted into pLN at the Bcl I site, thus creating a chimeric vector containing both the extended packaging signal of MLV and the packaging signal of BLV. DNA
sequencing was done to confirm the sequence and orientation of the BLV sequence in pLN. pLNB+ denotes the chimeric vector that contains the packaging signal region of BLV in the sense orientation and pLNB- denotes the chimeric vector with the same BLV sequence in the antisense orientation.

To assess the ability of the MLV Ψ+ to be recognized by the BLV packaging machinery, deletions were made in pLN and pLNB+ that removed MLV Ψ+. Both plasmids were digested with Psh AI and Bcl I, filled-in and then religated to create pLN∆ and pLN∆B+.

**Cell culture and transfections.**

Fetal lamb kidney cells chronically infected with BLV (FLK-BLV) were grown in Dulbecco’s modified Eagle medium (DMEM) (GIBCO BRL, Gaithersburg, MD) containing 10% Fetalclone III (Hyclone, Logan, Utah). Transfection of plasmids into FLK-BLV cells was done using SuperFect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Two days posttransfection, cells were placed under G418 selection (900µg/ml) until resistant colonies formed (~3 weeks). Approximately 100 G418-resistant colonies were pooled and used for packaging analysis.

**Determination of RNA packaging efficiencies.**

To prepare virion RNA samples, 200mL of supernatant from pooled stable cell clones was clarified (5min at 700 x g) and the virions were pelleted by ultracentrifugation in a 60Ti rotor at 40,000rpm for 1 h at 4°C. Virion pellets were
resuspended in 140µl of phosphate buffered saline (PBS) prior to extraction of virion RNA. The Qiagen kit, QiaAmp was used to isolate virion RNA and the manufacturer's instructions were followed. Briefly, a lysis solution was added to the resuspended virion pellets and incubated at room temperature for 10 minutes. Ethanol was then added and the precipitated mixture was applied to a column. After two washes, the virion RNA was eluted by the addition of RNase-free water. To prepare cellular RNA, total RNA was isolated from stable cells using the Qiagen kit, RNeasy, and the manufacturer's instructions were followed. Briefly one 100mm plate of stably transfected FLK-BLV cells was trypsinized with 1ml trypsin and the cells harvested (5min at 700 x g). The cell pellet was resuspended in 600µl RLT buffer and then homogenized using a QiaShredder. After homogenization, an equal amount of 70% ethanol was added to the homogenized pellet and then the mixture was placed onto a column. The cytoplasmic RNA was eluted after two washes by the addition of RNase-free water. To control for DNA contamination, the RNAs isolated were treated with DNA-free (Ambion, Austin, TX) and used in reverse transcription-PCR (RT-PCR) without the addition of reverse transcriptase to confirm the absence of DNA.

Detection of the RNA transcript that expresses the packaging signal of BLV and incorporation of RNA into virus particles was done by qualitative RT-PCR. Qualitative RT-PCR was carried out with the Promega Access RT-PCR kit in a 50µl reaction volume. Each tube contained a final amount of 0.5µg RNA,
10µl of AMV/Tfl 5x reaction buffer, 0.2mM each deoxynucleotide triphosphate, 1µM of each primer, 2mM magnesium sulfate, 5units AMV reverse transcriptase, 5units Tfl DNA polymerase, and 1µl Perfect Match (Stratagene). RNA templates were reverse transcribed at 48°C for 45 min followed by a denaturing step at 94°C for 2 min. PCR was then performed with the WT primers 5' - TCGTCTTGGCCACCCTAAACG- 3' and 5' -CGCTTCAGCGGCGGCTATTGC- 3'. These primers amplify an approximately 330-bp fragment of the BLV packaging signal. The PCR protocol consisted of 35 cycles of denaturation (94°C for 1 min), annealing (64°C for 1 min) and elongation (68°C for 1 min). Upon completion of the RT-PCR, 10µl of each sample was loaded onto a 15% polyacrylamide gel that was electrophoresed in 1xTBE for 2.5 hrs at 65 V. The gel was then stained in ethidium bromide and photographed with a BioRad GelDoc system.

Detection of RNA transcripts that express LacZ, both with and without the packaging signal region of BLV, and their incorporation into virus particles was done by qualitative RT-PCR. The same method of RT-PCR and detection of bands that is described above was utilized. PCR was performed with the primers 5' - CCCATTACGGTCAATCCGCCG- 3' and 5' –GCCGCTCATCCGCCACATATC- 3'.

Detection of the RNA transcripts that express the MLV Ψ+, both with and without the packaging signal region of BLV, and that lack the MLV Ψ+, both with
and without the packaging signal region of BLV, and their incorporation into virus particles was done by qualitative RT-PCR. The same method of RT-PCR and detection of bands that is described above was utilized, with only the primer sets differing:

for pLN (MLV Ψ+), 5’ -CGCCTCCTCTTCCATCCG- 3’ and 5’ - CGGAGAACCTGCGTGAATCC- 3’;
for pLN+, 5’ -TCGTCTTGCCACCCTAAACG- 3’ and 5’ - CGGAGAACCTGCGTGAATCC- 3’;
for pLN-, 5’ -CGCCTCCTCTTCCATCCG 3’ and 5’ - TCGTCTTGCCACCCTAAACG- 3’;
for pLNΔ, 5’ -TAGACTGCGTCGCGCGGTAC- 3’ and 5’ - CGGAGAACCTGCGTGAATCC- 3’;
for pLNΔB+, 5’ -TCGTCTTGCCACCCTAAACG- 3’ and 5’ – CGGAGAACCTGCGTGAATCC- 3’.

RESULTS

A non-viral vector containing the BLV E can be packaged into BLV particles

A region encompassing the BLV packaging signal (BLV E) was cloned into a non-viral vector to see if its presence would direct packaging of the non-viral vector RNA into BLV particles. pLacZ, a mammalian expression vector, was used as the non-viral vector (Fig. 2.1B). The BLV E was inserted at the end of
the lacZ gene to create pLacZBLV E (Fig 2.1B). To examine whether the presence of the BLV E would permit the non-viral vector to be packaged by the BLV machinery into BLV particles, pLacZ and pLacZBLV E were transfected into fetal lamb kidney cells that are chronically infected with BLV (FLK-BLV). Because they are chronically infected, FLK-BLV cells contain all of the BLV packaging machinery and will always produce BLV virions. The transfected FLK-BLV cells were placed under G418 selection for three weeks to select for stable integrants, thus creating the two cell lines FLK-BLV pLacZ and FLK-BLV pLacZBLV E. If the BLV packaging machinery recognizes a packaging signal within the non-viral RNAs, then virus particles released from these stable FLK-BLV cell lines will contain LacZ RNA. However, the vast majority of the virus particles released from the stable FLK-BLV cell lines will consist of wild-type BLV RNA since the cells are chronically infected with BLV and because wild-type viral RNA is preferentially packaged over other types of RNA. Packaging of non-viral RNA containing the BLV E has to compete with the wild-type BLV RNA in order to get packaged into BLV particles.

To analyze the packaging efficiency of the non-viral vectors both with and without the BLV E, RT-PCR was performed on both virion and cellular RNA obtained from FLK-BLV pLacZ and FLK-BLV pLacZBLV E cell lines. RT-PCR of the virion RNA samples will identify if LacZ or LacZ BLV E RNA was packaged into BLV virus particles. Use of the cellular RNA in RT-PCR establishes the
presence of the non-viral vector RNA in the cells. To determine if packaging of LacZ or LacZBLV E RNA had occurred, primers that are specific to the *lacZ* gene were used in RT-PCR. These primers are designated as primer set A and their location within the *lacZ* gene is diagrammed in Figure 2.1B and their sequence is given in Table 2.1. Since the FLK-BLV cells are chronically infected, wild-type BLV RNA will always be present in both the virion and cellular RNA samples. In order to detect the wild-type BLV RNA, another set of primers, designated as WT (Table 2.1), was also used in RT-PCR of the LacZ and LacZBLV E RNA samples. Amplification of BLV sequence will always occur with the primer set WT and therefore serves as an internal positive control of the sample.
**Figure 2.1:** BLV packaging signal region. A. The 5’ end of the BLV genome is shown in the proviral DNA form. The rectangular box at the end of the solid black line represents the 5’ long terminal repeat (LTR), containing the U3, R, and U5 regions. The solid black line indicates the viral sequence. The rectangular box above the solid black line represents *gag* with the matrix (MA), capsid (CA) and nucleocapsid (NC) domains indicated. The jagged line at the end of the viral sequence indicates the end of the viral sequence shown in this diagram. At the bottom, region 1 and region 2 refer to the primary and secondary packaging signals of BLV. B. Non-viral vectors used to study BLV packaging. The parental vector pLacZ is shown along with vector pLacZBLVE. The open rectangular box at the end represents the cytomegalovirus (CMV) promoter. The circle represents the N-terminal influenza A virus hemagglutinin (HA) epitope tag. The *lacZ* gene is represented by a light grey filled-in box. The box with the black gradient at the top and bottom represents the BLV packaging signal region (BLV E), which contains the primary and secondary packaging signals of BLV. The open oval represents C-terminal tag of six histidine (His6) residues. The neomycin resistance gene is indicated by a dark grey filled-in box. The small arrows with the letter A on them above the individual constructs refer to the primer set used for RT-PCR analysis on both of the constructs. The arrow pointing to the right designates the forward primer and the arrow pointing to the left designates the reverse primer.
Table 2.1: Primer sets used in RT-PCR of virion and cellular RNA

<table>
<thead>
<tr>
<th>Set</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>5’ CCCATTACGGTCAATCCGCGG 3’</td>
<td>5’ GCCGTCATCCGCCACATAC 3’</td>
</tr>
<tr>
<td>B</td>
<td>5’ CGCCTCTCCTCCTCCACTCCG 3’</td>
<td>5’ CGGAACCTTCGCTGCAATCC 3’</td>
</tr>
<tr>
<td>C</td>
<td>5’ TCGCTTGGCACCCTAAACG 3’</td>
<td>5’ CGGAACCTTCGCTGCAATCC 3’</td>
</tr>
<tr>
<td>D</td>
<td>5’ CGCCTCTCCTCCTCCACTCCG 3’</td>
<td>5’ TCGCTTGGCACCCTAAACG 3’</td>
</tr>
<tr>
<td>E</td>
<td>5’ TAGACTGCCTCGCCGGGTAC 3’</td>
<td>5’ CGGAACCTTCGCTGCAATCC 3’</td>
</tr>
<tr>
<td>F</td>
<td>5’ TCGCTTGGCACCCTAAACG 3’</td>
<td>5’ CGGAACCTTCGCTGCAATCC 3’</td>
</tr>
<tr>
<td>WT</td>
<td>5’ TCGCTTGGCACCCTAAACG 3’</td>
<td>5’ CGCTTCAGCGCCTGCTTATGC 3’</td>
</tr>
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The products of the RT-PCRs were analyzed by electrophoresis to detect the presence or absence of amplified products. The use of primer set A on the cellular LacZ RNA produced a band, indicating that LacZ RNA was present in the cell line. The absence of a band for the virion LacZ RNA sample, when primer set A was used, suggests that either the LacZ RNA did not get packaged into BLV particles or that it was packaged but at undetectable levels for this assay (Fig 2.2A and 2.2B). Use of the WT primer set on both the virion and cellular LacZ RNA samples yielded bands, indicating the integrity of the RNA samples (Fig 2.2A and 2.2B). The LacZBLV E virion and cellular RNA samples both produced a band when primer set A was used in RT-PCR. The presence of a RT-PCR product in the virion RNA sample when primer set A was used signifies
that the LacZBLV E RNA was packaged into BLV particles. These data indicate that the BLV E is necessary and sufficient to allow packaging of a non-viral vector RNA. As a control, the WT primer set was also used for RT-PCR analysis of LacZBLV E RNA samples and a band was detected for both the virion and cellular RNA (Fig 2.2A and 2.2B).

Figure 2.2: Analysis of packaging efficiencies of non-viral vectors. A. Virion RNA. Virion RNA from the supernatant of the stable cell clones of both pLacZ and pLacZBLVE was collected and harvested and then used for RT-PCR analysis. Samples were run on a 15% PAGE gel and stained with ethidium bromide. The top panel is the amplified products from LacZ and LacZBLVE RNA using primer set A, which is shown in Figure 2.1B and listed in Table 2.1. The amplified products from the LacZ and LacZBLVE RNA using the WT primer set are shown in the bottom panel. B. Cellular RNA. Total RNA from the stable cell clones of both pLacZ and pLacZBLVE was harvested and then used for RT-PCR analysis. The top panel is the amplified products from LacZ and LacZBLVE RNA using primer set A and the bottom panel is the amplified products from LacZ and LacZBLVE RNA generated with the WT primer set.
A chimeric vector of MLV and BLV can be packaged by the BLV machinery

To test the packaging ability of a chimeric retroviral vector, the BLV E was cloned immediately downstream of the Moloney murine leukemia virus extended packaging signal (MLV Ψ+) in the MLV based retroviral vector pLN (Fig 2.3). Both the sense and antisense orientation of the BLV E were inserted into the pLN vector. The subsequent chimeric retroviral vectors were named pLNB+ and pLNB- in order to indicate the presence of the BLV E in pLN and it’s orientation within the vector. Using the chimeric retroviral vectors, pLNB+ and pLNB-, we tested whether the presence of the BLV E would permit the chimeric retroviral vectors to be packaged by the BLV machinery into BLV particles. pLNB+ and pLNB- were each transfected into FLK-BLV cells and then placed under G418 selection for three weeks to select for the cells that contained the chimeric retroviral vectors. The resulting resistant colonies were pooled together to create FLK-BLV pLNB+ and FLK-BLV pLNB- cell lines.

To analyze the packaging efficiency of the MLV/BLV chimeric retroviral vectors, RT-PCR was performed on both virion and cellular RNA samples obtained from FLK-BLV pLNB+ and FLK-BLV pLNB- cell lines. A unique set of primers for each chimeric retroviral vector was used to determine if packaging of that particular chimeric retroviral vector RNA occurred. RT-PCR analysis of the LNB+ RNA samples used primer set C, which starts near the 3’ end of the BLV E
and finishes at the 5’ end of the neo gene (Fig. 2.3 and Table 2.1). The packaging efficiency of LNB- RNA samples were analyzed with primer set D, which starts at Ψ+ and goes through the end of the BLV E (Fig 2.3 and Table 2.1). The WT primer set was also used for RT-PCR of the LNB+ and LNB- RNA samples as an internal positive control of the samples.

The subsequent RT-PCR products were analyzed by electrophoresis to detect any amplified products. The LNB+ cellular RNA sample produced a band when primer set C was used in RT-PCR. The generation of a band for the virion RNA sample of LNB+ (Fig. 2.4A and 2.4B) when primer set C was used indicates that the BLV E, in the sense orientation, did allow for packaging of the LNB+ RNA. These data indicates that the presence of the BLV E is sufficient to allow packaging of a MLV/BLV chimeric retroviral vector RNA. The WT primer set on the virion and cellular LNB+ RNA samples also yielded a band when analyzed by RT-PCR, indicating the integrity of the samples (Fig. 2.4A and 2.4B). Analysis of the cellular LNB- RNA sample with primer set D produced a band; however the virion LNB- RNA sample did not produce a band with primer set D (Fig. 2.4A and 2.4B). The absence of a band for the virion LNB- sample indicates that either the LNB- RNA did not get packaged into BLV particles or that it did get packaged into BLV particles, but at levels that were undetectable using this assay. Use of the WT primer set produced bands for both the virion and cellular LNB- RNA samples.
Figure 2.3: Retroviral vectors used to study BLV packaging. The parental pLN vector is shown along with the chimeric and deletion vectors created. The LTRs for MLV are indicated by rectangular boxes at the ends. The solid black line represents the MLV viral sequence. The MLV extended packaging signal is indicated by $\Psi^+$. The neomycin resistance gene is indicated by a grey filled-in box. The box with the black gradient at the top and bottom represents the BLV packaging signal region (BLV E), which contains the primary and secondary packaging signals of BLV. The absence of $\Psi^+$ on the last two constructs, indicate that the MLV extended packaging signal has been deleted. The small arrows with the letters B-F on them above the individual constructs refer to primer sets used for RT-PCR analysis on each of the constructs. The arrows pointing to the right designates the forward primers and the arrows pointing to the left designates the reverse primers.
The MLV $\Psi^+$ can be recognized by the BLV packaging machinery

To distinguish if the packaging of the LNB+ RNA into BLV particles was due to the presence of the BLV E or to $\Psi^+$ or to both of the packaging signals being present, we analyzed the packaging efficiency of three additional retroviral vectors. The first vector we examined was the parental vector, pLN, to determine if $\Psi^+$ was able to be recognized by the BLV packaging machinery as a packaging signal. Next we deleted the $\Psi^+$ region from both pLN and pLNB+ to create pLN$\Delta$ and pLN$\Delta$B+ (Fig. 2.3), respectively. By deleting $\Psi^+$ from pLN the packaging efficiency of a retroviral vector that lacks a packaging signal could be determined. The deletion of $\Psi^+$ from the pLNB+ chimeric retroviral vector allowed us to analyze the involvement of the BLV E in the packaging of the LNB+ RNA.

The three retroviral vectors, pLN, pLN$\Delta$ and pLN$\Delta$B+ (Fig. 2.3), were each transfected into FLK-BLV cells and then placed under G418 selection for three weeks to select for the cells that stably expressed the retroviral vectors. The G418 resistant colonies were pooled together to create FLK-BLV pLN, FLK-BLV pLN$\Delta$ and FLK-BLV pLN$\Delta$B+ stable cell lines. RT-PCR analysis was performed on both virion and cellular RNA obtained from FLK-BLV pLN, FLK-BLV pLN$\Delta$ and FLK-BLV pLN$\Delta$B+ cells. Each retroviral vector had a unique set of primers that was used in the RT-PCR analysis. Primer set B (Fig. 2.3 and Table 2.1) is specific for pLN and amplifies a region of $\Psi^+$ and goes until the 5’ end of the neo
gene. Examination of the LNΔ RNA used primer set E (Table 2.1), which starts downstream of the MLV LTR and finishes at the 5’ end of the neo gene. To analyze the packaging efficiency of LNΔB+ RNA, primer set F (Fig. 2.3), which starts in BLV E and ends at the 5’ end of the neo gene, was utilized. The WT primer set was also used in RT-PCR of the above RNA samples as an internal positive control of the samples.

![Figure 2.4: Analysis of packaging efficiencies of chimeric retroviral vectors. A. Virion RNA. RNA from the supernatant of the stable cell clones for each construct was collected and harvested and then used for RT-PCR analysis. Samples were run on a 15% PAGE gel and stained with ethidium bromide. The amplified products from each reaction using primer sets B and C, respectively, are shown. The amplified products from each construct using the WT primer set is shown in the bottom panel. The primers are listed in Table 2.1 and illustrated in Figure 2.3. B. Cellular RNA. Total RNA from the stable cell clones of each construct was harvested and then used for RT-PCR analysis. The top panel is the amplified products from each construct using the primer sets B and C. The bottom panel shows the amplified products when the WT primer set was used.](image-url)
The RT-PCR products were analyzed by electrophoresis to evaluate the packaging efficiency of the three retroviral vector RNAs. The use of primer set B on both the virion and cellular LN RNA samples resulted in the production of a band (Fig. 2.5A and 2.5B). Primer set E, which is unique to pLNΔ, unexpectedly generated a band for both the virion and cellular LNΔ RNA. These data suggest that the LNΔ sequence might possess an RNA sequence that allows the RNA to be packaged into BLV particles. For the LNΔB+ RNA, both the virion and cellular samples generated products when primer set F was utilized. Use of the WT set of primers in RT-PCR of the LN, LNΔ and LNΔB+ virion and cellular RNA samples produced bands for each sample.
Figure 2.5: Analysis of packaging efficiencies of BLV E and Ψ+ in a BLV system. 
A. Virion RNA. Virion RNA from the supernatant of the stable cell clones for each construct was collected and harvested and then used for RT-PCR analysis. Samples were run on a 15% PAGE gel and stained with ethidium bromide. The amplified products from each construct using primer sets B, E and F are shown. The amplified products from each construct using primer set WT is shown in the bottom panel. The primers are listed in Table 2.1 and illustrated in Figure 2.3. 
B. Cellular RNA. Total RNA from the stable cell clones of each construct was harvested and then used for RT-PCR analysis. The top panel is the amplified products from each construct using primer sets B, E and F. The bottom panel shows the amplified products when primer set WT was used.
DISCUSSION

In this study we have evaluated an RNA sequence that is necessary and sufficient to allow packaging of a non-viral vector that contains the BLV E. In addition, a chimeric MLV/BLV retroviral vector RNA was also found to be packaged into BLV particles. Surprisingly, the MLV vector, pLN, and a mutant vector with the packaging signal deleted were both found to be packaged into BLV particles. This indicates that both Ψ+ and MLV RNA sequence outside of Ψ+ can allow for MLV vector RNA packaging into BLV particles.

Insertion of the BLV E, which comprises both the primary and secondary encapsidation signals (Mansky et al., 1995), into the non-viral vector pLacZ led to efficient packaging of pLacZBLV E. Evaluation of LacZ RNA packaging revealed that either no or undetectable packaging had occurred. The packaging of LacZBLV E RNA confirms the location of the BLV RNA packaging signal (Mansky et al., 1995; Mansky and Wisniewski, 1998) and indicates that these RNA sequences are sufficient for packaging.

A chimeric MLV/BLV retroviral vector was created containing the RNA packaging signals of both viruses. We found that LNB+ RNA was packaged into BLV particles (Fig. 2.4). RNA packaging of virion RNA from a control chimeric retroviral vector containing the BLV E RNA sequence in the antisense orientation, LNB-, was not detected in our assay (Fig. 2.4). This observation agrees with previous work showing that the ability of packaging signals to be
recognized by the packaging machinery is orientation dependent (Adam and Miller, 1988).

LNB+ RNA contains both BLV E and MLV Ψ+, therefore we examined whether the packaging of LNB+ RNA was influenced by Ψ+. We found that the parental vector pLN, which contains the MLV Ψ+, was packaged into BLV particles, indicating that the MLV Ψ+ RNA was recognized by BLV Gag. We further observed that pLNΔ (which does not posses Ψ+) was also packaged into BLV particles, indicating that an RNA sequence outside of Ψ+ allows for MLV RNA packaging into BLV particles. MLV Ψ+ has previously been shown to be recognized by the packaging machinery of another retrovirus. Observations between spleen necrosis virus (SNV) and MLV revealed that SNV Gag can recognize both the SNV E and the MLV Ψ+; however MLV Gag only recognizes the MLV Ψ+ (Certo et al., 1998; Yang and Temin, 1994). The RNA structural motifs of the packaging signal are believed to play a major role in the recognition event between the viral Gag polyprotein and the full-length viral genome. The packaging signals of both MLV and SNV have hairpin pairs that have been shown to be necessary for RNA packaging (Konings et al., 1992; Yang and Temin, 1994) and the primary packaging signal of BLV also has two stable stem-loop structures (Mansky and Wisniewski, 1998). This suggests that both the secondary and tertiary structure of the packaging signals play a role in efficient RNA packaging.
The packaging of LN and LNΔ RNAs into BLV particles raises the question of why LNB- RNA was not detected in the RNA from virions. Although the BLV E was in the antisense orientation, the pLN RNA sequence was still present in the MLV/BLV chimeric retroviral vector. One explanation for why we were unable to detect packaging of LNB- RNA is that LNB- may have an altered secondary structure that prevents the BLV packaging machinery from recognizing either Ψ+ or the MLV RNA sequence outside of the Ψ+ region.

A recent report identified seventeen nucleotides downstream of the env stop codon that were important in MLV packaging (Yu et al., 2000). Both the LN and LNΔ RNAs possess this RNA sequence. Furthermore, it has been recently reported that low levels of replication of a MLV-based vector, in which Ψ+ was deleted, occurs in both SNV- and MLV- based helper cell lines (Beasley and Hu, 2002). This suggests that the SNV Gag can recognize MLV RNA sequences outside of Ψ+. Based upon our results, BLV Gag can also recognize MLV RNA sequences outside of Ψ+.

The data in this report show that heterologous RNAs that contain the BLV E can be packaged into BLV particles. Additionally we found that both MLV Ψ+ and sequences outside of Ψ+ can be recognized by the BLV machinery and packaged into BLV particles. This suggests that BLV Gag recognizes RNA structural motifs during the viral RNA packaging process.
CHAPTER 3

CONSTRUCTION AND CHARACTERIZATION OF DELTARETROVIRUS INDICATOR CELL LINES

ABSTRACT

The Deltaretroviruses, bovine leukemia virus (BLV) and human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) replicate poorly in culture and the molecular details of their life cycles are limited. To facilitate the analysis of virus replication, mammalian cell lines were created with the long terminal repeats (LTRs) of each virus driving expression of the enhanced green fluorescent protein gene (egfp). The BLGFP, H1GFP and H2GFP cell lines detect virus infection by the expression of GFP via the transactivation of the LTR via the Tax protein of BLV, HTLV-1 or HTLV-2, respectively. GFP expression was measured by flow cytometry, yielding sensitive and rapid detection of virus infectivity. Interestingly, we observed that the Tax proteins of HTLV-1 and HTLV-2 could transactivate the BLV LTR at levels that were comparable to that of BLV Tax. In contrast, the BLV Tax showed low levels of transactivation in H1GFP and H2GFP cells. HTLV-1 and HTLV-2 Tax proteins efficiently transactivated both the
HTLV-1 and HTLV-2 LTRs. Finally, spinoculation of BLV resulted in only a 2-fold increase in viral titer. Using this assay system, spinoculation was found to only modestly increase BLV titers.

INTRODUCTION

The determination of virus titer is crucial for the analysis of drug resistance, mutation rates and various steps of the virus replication cycle. End-point dilution and plaque assays for determination of virus titer are time consuming and difficult to implement for large numbers of samples (Gervaix et al., 1997; Kimpton and Emerman, 1992). The development of an indicator cell line to detect and quantify human immunodeficiency virus type 1 (HIV-1) based upon the activation of the $\beta$-galactosidase gene provided a simple and sensitive method to measure virus titer (Kimpton and Emerman, 1992). Multinuclear activation of a galactosidase indicator (MAGI) assay uses the $\beta$-galactosidase gene downstream of the HIV-1 long terminal repeat (LTR) in HeLa cells that express CD4. Transactivation of the HIV-1 LTR by the HIV-1 Tat protein leads to expression of $\beta$-galactosidase. Virus titer is then calculated by counting the number of blue cells (Kimpton and Emerman, 1992).

Other indicator cell lines capable of detecting infectious virus have been subsequently created using various reporter genes (Chackerian et al., 1995; Richman et al., 2002; Vodicka et al., 1997). Gervaix et al. created a HIV-1
indicator cell line that utilizes the green fluorescent protein (GFP) as the reporter
gene (Gervaix et al., 1997). By using GFP as the reporter gene, the cells can be
monitored by fluorescence microscopy without fixation or be monitored by flow
cytometry (Gervaix et al., 1997; Liu et al., 2001; Richman et al., 2002).

The deltaretroviruses include bovine leukemia virus (BLV), human T-cell
leukemia virus type 1 (HTLV-1) and human T-cell leukemia virus type 2 (HTLV-
2). These viruses replicate poorly in cell culture and have low viral titers. Co-
cultivation is often used for infection of permissive cells. Due to these
characteristics, molecular details regarding their life cycle are restricted. To
facilitate the analysis of deltaretrovirus replication, mammalian cell lines were
developed to detect virus infectivity.

MATERIALS AND METHODS

Plasmids.

pBLV913 is an infectious molecular clone of BLV obtained from a fetal
lamb kidney cell line chronically infected with BLV (FLK-BLV). pBLVSVNEO is a
derivative of p913, in which the region that encodes the tax and rex genes was
replaced by the simian virus 40 (SV40) early promoter fused to the bacterial
neomycin phosphotransferase gene (Derse and Martarano, 1990). pHTLVCMVNeo, graciously provided by David Derse (NCI, Frederick), was
derived from an infectious molecular clone of HTLV-1 where the tax, rex, and 3'
region of env were removed and replaced by the neomycin (neo) resistance gene under the control of the CMV promoter (Copeland et al., 1994). pH6NeoCla, provided by Irwin Chen (UCLA), was derived from an infectious molecular clone of HTLV-2 that does not produce Tax nor Rex due to a frameshift mutation at a Cla I restriction site (Green et al., 1995). pBPLX-RSPA contains the BLV tax and rex genes downstream of the Rous sarcoma virus (RSV) promoter. pSE356, provided by Patrick Green (The Ohio State University), expresses the human T-cell leukemia type 1 (HTLV-1) tax and rex genes off the CMV immediate early promoter. pBC20.2, provided by Irwin Chen, contains the human T-cell leukemia virus type 2 (HTLV-2) tax and rex genes downstream of the CMV immediate early promoter.

**Construction of Reporter Plasmids.**

All reporter constructs were constructed similarly. The long terminal repeat (LTR) from each virus was PCR amplified and individually cloned into pCR3.1 (Invitrogen, Carlsbad, CA), a eukaryotic TA cloning vector. The BLV LTR was PCR amplified from pBLVSVNEO using the primers 5’-TGTATGAAAGATCATGCCGAC- 3’ and 5’ -GAGCGCCGGGCAGAGAGG-3’, the HTLV-1 LTR was PCR amplified from pHTLVCMVNeo using the primers 5’ -CTCGCGCGCTGACAATGACCATGAGCCCCA- 3’ and 5’ -AAGGATCCTGTGTACTACGTTTCTCTCCTGG- 3’, and
the HTLV-2 LTR was PCR amplified from H6NeoCla using the primers
5’ –TTGGCGCGCTGACAATGGCGACTAGCCTCC- 3’ and
5’ –GAAGATCTCAATTGTGGTCTTCCCCGGG- 3’. DNA sequencing was done
to confirm the sequence and orientation of each of the LTR within the vectors.
The cytomegalovirus (CMV) promoter of pCR3.1, which lies upstream of the
inserted PCR product, was deleted by removing the SpeI fragment from the
vectors. The resulting vectors were religated, creating pBLVLTR, pHTLV-1LTR
and pHTLV-2LTR, respectively. The enhanced green fluorescent protein gene
(EGFP) was PCR amplified from pIRES2-EGFP (Clontech, Palo Alto, CA) using
primers containing HindIII or EcoRI recognition sites. The primers used were
5’ –AAGCTTCAACAACCATGGTGAGCAAGGG- 3’ and
5’-GAATTCGCGGCCGCTTTACTTGTACAG- 3’. The PCR amplified product was
cloned into pCR2.1 (Invitrogen, Carlsbad, CA) creating pCR2.1GFP. DNA
sequencing was done to confirm the sequence and orientation of the GFP gene
within the vector. To create pBLVLTRGFP, pCR2.1GFP was digested with SpeI
and XbaI to release the GFP gene which was then cloned into pBLVLTR at the
XbaI site. DNA sequencing was done to verify the sequence and orientation of
the GFP gene in the vector. The same strategy was used to create pHTLV-
1LTRGFP and pHTLV-2LTRGFP. P3.1GFP was created by inserting the PCR
amplified EGFP product into pCR3.1. The CMV promoter in pCR3.1 was
removed by digestion with Spe I and the vector was relegated to create p3.1GFP.

**Cell lines, transfections and infections.**

Crandall feline kidney (CRFK) cells and 293T cells were grown in Dulbecco’s modified Eagle medium (DMEM) (GIBCO BRL, Gaithersburg, MD) containing 10% Fetalclone III (Hyclone, Logan, Utah). Transfection of pBLVLTRGFP into fetal lamb kidney cells chronically infected with BLV (FLK-BLV) and CRFK cells was done using SuperFect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. FLK-BLV cells were used to obtain BLV for infections. FLK-BLV cells were grown in DMEM containing 10% Fetalclone III. To obtain infectious BLV, cells were grown to 80% confluency then the supernatant was collected and spun at 1,000 rpm for 5 min. After spinning, 8μl/ml of Polybrene (Sigma, St. Louis, MO), at a concentration of 1mg/ml, was added to the supernatant and 3ml of the supernatant plus Polybrene was used to infect 2 x 10^5 BLGFP indicator cells in a 60-mm diameter petri dish. Transfection of either pHTLV-1LTRGFP or pHTLV-2LTRGFP into FLK-BLV cells and 293T cells was done using SuperFect according to the manufacturer’s instructions. Human T-cell leukemia cells isolated from patients with adult T-cell leukemia (MT-2) (NIH AIDS reagent program) were used to obtain HTLV-1 for infections. MT-2 cells were grown in RPMI containing 10% Fetalclone III. To obtain infectious HTLV-1, MT-2 cells were grown to 80% confluency and then 8μl/ml of
Polybrene, at a concentration of 1mg/ml, was added to the cells. MT-2 cells containing Polybrene were co-cultured with 2 x 10^5 of either H1GFP or H2GFP cells in a 60-mm diameter petri dish.

**Development of an indicator cell lines for infection.**

pBLVLTRGFP was stably transfected into CRFK cells to create BLGFP cells. pHTLV-1LTRGFP and pHTLV-2LTRGFP were each stably transfected into 293T cells to create H1GFP and H2GFP cells, respectively. pBLVLTRGFP, pHTLV-1LTRGFP and pHTLV-2LTRGFP all contain the neomycin resistance gene under the control of the SV40 promoter, thus allowing for selection of stable mammalian cells. Two days post-transfection cells were grown in complete DMEM plus 0.7mg/ml of G418 in order to isolate single cell clones. Cells were placed under drug selection for 3 weeks. Fifty of the G418 resistant colonies were expanded in culture. Subsequently two clonal cell lines were chosen for further study for BLV (BLGFP J and BLGFP 10) and one clonal cell line each was chosen for studies of HTLV-1 and HTLV-2 (H1GFP and H2GFP).

**Spinoculation.**

BLGFP cells were plated in six-well plates coated with 0.01% tissue culture grade poly-L-lysine (Sigma, St. Louis, MO) at a concentration of 1x 10^6 cells per well. Prior to spinning, 3ml of FLK-BLV supernatant plus polybrene was added to the BLGFP cells. The plates were then placed in a Sorvall RT 7
tabletop centrifuge with a RTH-750 rotor and spun at 2,700 rpm at 32°C for 1 h after which the plates were placed in a 37° incubator with 5% CO₂. At specified time points the cells were harvested by addition of 0.5ml trypsin (GIBCO BRL, Gaithersburg, MD) and spun at 1,000 rpm for 5 min. The resulting cell pellet was harvested and analyzed by flow cytometry. For the experiments involving the use of AZT, 24 h after spinoculation, the media was changed to complete DMEM with a final concentration of 0.5µM AZT.

Flow Cytometry.

BLGFP, H1GFP and H2GFP cells were harvested by the addition of 0.5ml of trypsin to the plates and spun at 1,000 rpm for 5 min to pellet the cells. The pelleted cells were then resuspended in 0.5ml of phosphate buffered saline (PBS). A Beckman Coulter Epics XL machine was used for flow cytometry analysis. Prior to sample analysis, the machine was gated at 0.5% fluorescence using negative control cells (i.e. cells with no infection or transfection).

RESULTS

Construction of cell lines for the detection of deltaretrovirus infectivity.

To create cell lines that would allow for sensitive detection of the deltaretroviruses BLV, HTLV-1 and HTLV-2, we constructed retroviral vectors that made use of the long terminal repeats (LTRs) of each virus to drive expression of a reporter gene (i.e., the enhanced green fluorescent protein gene
(EFGP). The LTRs, which contain the U3, R and U5 regions, were PCR amplified and inserted into the eukaryotic TA cloning vector pCR3.1 to produce pBLVLTR, pHTLV-1LTR and pHTLV-2LTR. The CMV promoter in each retroviral vector was deleted ensuring that expression of the reporter gene would be dependent on the transactivation of the virus specific LTR. The EFGP gene was inserted downstream of each LTR, creating the vectors pBLVLTRGFP, pHTLV-1LTRGFP and pHTLV-2LTRGFP (Fig. 3.1A).

The Tax protein, an accessory protein in BLV, HTLV-1 and HTLV-2 that helps regulate deltaretrovirus replication, is a transcriptional transactivator. Tax recruits cellular transcription factors to assemble with Tax on the Tax-responsive element (TxRE) in the U3 region of the LTR forming a multiprotein complex that drives gene expression (Bex and Gaynor, 1998; Brooks et al., 1998). The TxRE is composed of three imperfect 21-bp sequences which are necessary for Tax-mediated transactivation.

To verify that GFP expression could be detected due to transactivation of the BLV LTR by the BLV Tax protein, the retroviral vector pBLVLTRGFP was transfected into chronically infected fetal lamb kidney cells (FLK-BLV). As a negative control, a vector lacking a LTR but containing the GFP gene (p3.1GFP) was also transfected into FLK-BLV cells. Two days post-transfection the FLK-BLV cells were harvested and analyzed by flow cytometry. FLK-BLV cells were found to have a transfection efficiency of 30-40% (data not shown). Transfection
of p3.1GFP into FLK-BLV cells resulted in GFP expression in only 1% of the cells (Table 3.1). The amount of GFP-expressing cells detected when pBLVLTRGFP was transfected into FLK-BLV cells was 14% (Table 3.1), indicating that transactivation of the BLV LTR occurred due to the presence of the BLV Tax protein in the infectious virus.

**Figure 3.1:** Development of indicator cell lines for the sensitive detection of BLV, HTLV-1, or HTLV-2 infectivity. A. The fundamental construct used to create the indicator cells is shown. A basic long terminal repeat (LTR), containing the U3, R and U5 regions, is indicated. LTRs from BLV, HTLV-1 and HTLV-2 were used to create a retroviral vector that was specific for each virus. The box labeled “EGFP” is the *enhanced green fluorescent protein* gene, and the box labeled “pA” represents the bovine growth hormone polyadenylation signal. B. Virus titer was determined by infecting the indicator cells, BLGFP, H1GFP, or H2GFP, and using FACS to analyze the number of GFP-expressing cells.
Due to the relationship between BLV and HTLV-1 and HTLV-2, we investigated whether the BLV Tax protein could transactivate the LTRs of HTLV-1 and HTLV-2. Both pHTLV-1LTRGFP and pHTLV-2LTRGFP were transfected into FLK-BLV cells and analyzed for GFP expression by flow cytometry. The level of GFP-expressing cells detected for each transfection was 3% and 4% for pHTLV-1LTRGFP and pHTLV-2LTRGFP, respectively (Table 3.1). Even though the amount of GFP expressing cells for HTLV-1 and HTLV-2 was only one-fourth of that seen with BLV, these data suggest that the BLV Tax protein can transactivate the LTRs of HTLV-1 and HTLV-2.

<table>
<thead>
<tr>
<th>LTRGFP Construct</th>
<th>% GFP Expression</th>
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<tbody>
<tr>
<td>BLVLTRGFP</td>
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</tr>
<tr>
<td>HTLV-1LTRGFP</td>
<td>4.11</td>
</tr>
<tr>
<td>HTLV-2LTRGFP</td>
<td>3.44</td>
</tr>
<tr>
<td>3.1GFP</td>
<td>1.0</td>
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Table 3.1: Transfection of LTR-driven GFP expression vectors into FLK-BLV cells. FLK-BLV cells are chronically infected with BLV. The LTR from each virus was cloned downstream of the GFP gene to create the three retroviral vectors pBLVLTRGFP, pHTLV-1LTRGFP and pHTLV-2LTRGFP. The percent GFP expression is per 40,000 cells counted by flow cytometry. Each transfection was performed in triplicate. Representative data from one experiment is shown.
To create indicator cell lines for each virus, we needed cells that were permissive to infection by BLV, HTLV-1 and HTLV-2. Previously, we found Crandall feline kidney (CRFK) cells to be permissive to BLV infection and used them to create an indicator cell line for BLV infection. CRFK cells were transfected with pBLVLTRGFP and placed under G418 selection for three weeks in order to obtain stable cell clones. The stable introduction of pBLVLTRGFP into CRFK cells led to the creation of BLGFP cells, for BLV LTR GFP. BLGFP cell clones were isolated, expanded and then screened by fluorescence-activated cell sorting (FACS) to find individual cell clones that yielded maximum levels of GFP expression upon infection with BLV (Fig. 3.1B). The BLGFP cell clones were infected with BLV harvested from FLK-BLV cells. Forty-eight hours post-infection, the individual BLGFP cell clones were pelleted and analyzed for GFP expression by FACS. The majority of BLGFP cell clones isolated yielded extremely low percentages of GFP-expressing cells after infection with BLV (Table 3.2), which is thought to relate to the amount of pBLVLTRGFP present in the cells. Of fifty BLGFP clones evaluated, clones 10 and J had the highest percentage of GFP-expressing cells after infection with BLV (Table 3.2). BLGFP cell clones 10 and J were used in subsequent experiments.

For HTLV-1 and HTLV-2 the human kidney cell line, 293T, has been shown to be permissive to infection (Derse et al., 2001). 293T cells were transfected with either pHTLV-1LTRGFP or HTLV-2LTRGFP and placed under
G418 selection for three weeks in order to obtain stable cell clones. Stable cell clones of HTLV-1 and HTLV-2 were isolated, expanded and then screened by fluorescence-activated cell sorting (FACS) to find the individual cell clones that yielded maximum levels of GFP expression upon infection with HTLV-1 (Fig. 3.1B). The stable cell clones of HTLV-1 and HTLV-2 were co-cultured with HTLV-1 infected T-cells (MT-2 cells). Forty-eight hours post-infection, the individual cell clones were pelleted and analyzed for GFP expression by FACS. Of fifty cell clones evaluated for both HTLV-1 and HTLV-2, only one cell clone was chosen for each virus, resulting in indicator cells named H1GFP and H2GFP (Table 3.2).

Transactivation of deltaretrovirus indicator cells by different Tax/Rex constructs.

The detection of GFP expression after BLGFP cell clones 10 and J were infected by BLV obtained from FLK-BLV cells indicated that the BLV Tax protein, in the context of an infectious virus, was able to transactivate the LTR and drive GFP expression. We decided to investigate if the BLV Tax protein could transactivate the BLGFP cells when it was constitutively expressed from a plasmid instead of from an infectious virus. A BLV tax/rex expression plasmid, pBLPX-RSPA, that contains the BLV tax and rex genes downstream of the Rous sarcoma virus (RSV) promoter, was transfected into BLGFP cell clones 10 and J. Additionally, a BLV infectious molecular clone, p913, was also transfected into
BLGFP cell clones 10 and J and evaluated for GFP expression. Forty-eight hours post-transfection, the cells were harvested and analyzed for GFP expression by flow cytometry. Transfection of both the BLV \textit{tax/rex} expression plasmid and p913 into BLGFP cell clones 10 and J resulted in GFP expression at levels similar to that seen earlier when pBLVLTRGFP was transfected into FLK-BLV cells (Fig. 3.2). Interestingly, whether Tax was expressed from a constitutive promoter or from an infectious molecular clone did not appear to significantly affect the resulting percentage of GFP-expressing cells.

Due to the earlier results of GFP expression when pHTLV-1LTRGFP and pHTLV-2LTRGFP were transfected into FLK-BLV cells, we tested whether the Tax proteins of HTLV-1 and HTLV-2 could transactivate BLGFP cell clones 10 and J and drive GFP expression. We obtained two plasmids that express the HTLV-1 and HTLV-2 \textit{tax/rex} genes off the cytomegalovirus (CMV) immediate early promoter, pSE356 and pBC20.2, respectively. Transient transfection of the HTLV-1 and HTLV-2 \textit{tax/rex} expression plasmids into BLGFP cell clones 10 and J was performed and 48 h later the cells were pelleted and analyzed for GFP expression by flow cytometry. Both HTLV-1 and HTLV-2 Tax proteins were able to transactivate the BLV LTR and surprisingly at levels that were comparable to those of the BLV Tax protein (Fig 3.2).
Table 3.2: Analysis of indicator cell clones for sensitivity to infection. Cell clones were created by the stable introduction of each LTRGFP plasmid into cells permissive to infection by each virus. pBLVLTRGFP was transfected into CRFK cells and HTLV-1 and HTLV-2 were individually transfected into 293T cells. BLV cell clones were isolated and assessed for their ability to express GFP after infection with BLV from chronically infected fetal lamb kidney (FLK-BLV) cells. HTLV-1 and HTLV-2 cell clones were isolated and assessed for their ability to express GFP after infection with HTLV-1 chronically infected MT-2 cells. The cell clones in bold were used in subsequent experiments. The percent of GFP expression is from 40,000 cells counted by flow cytometry.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell Clones</th>
<th>% GFP Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLV</td>
<td>Clone 9</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td><strong>Clone 10</strong></td>
<td><strong>12</strong></td>
</tr>
<tr>
<td></td>
<td>Clone 11</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Clone F</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Clone H</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>Clone J</strong></td>
<td><strong>5</strong></td>
</tr>
<tr>
<td>HTLV-1</td>
<td>Clone 2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Clone 3</td>
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<td><strong>Clone 6</strong></td>
<td><strong>3.2</strong></td>
</tr>
<tr>
<td>HTLV-2</td>
<td><strong>Clone A</strong></td>
<td><strong>4.2</strong></td>
</tr>
<tr>
<td></td>
<td>Clone I</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Clone H</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Figure 3.2: Transactivation of the BLV LTR in BLGFP cells. BLGFP cell clones J and 10 were transiently transfected with a BLV, HTLV-1 or HTLV-2 Tax/Rex expression construct and 48 hours later analyzed by FACS for GFP expression. Two BLV Tax/Rex constructs were used, BLV 913 and BLPX-RSPA. pBLV 913 is an infectious molecular clone of BLV, while pBLPX-RSPA utilizes the Rous sarcoma virus promoter to express BLV Tax and Rex. The HTLV-1 and HTLV-2 Tax/Rex expression constructs used were SE356 and BC20.2, respectively. The percent of GFP-expressing cells is from a total of 40,000 cells counted by FACS.

We next assessed the ability of the tax/rex expression plasmids, BLV Tax/Rex (pBPLX-RSPA), HTLV-1 Tax/Rex (pSE356) and HTLV-2 Tax/Rex (pBC20.2) to transactivate the H1GFP and H2GFP cells. Forty-eight hours post-transfection, the H1GFP and H2GFP cells were harvested and analyzed by flow cytometry for GFP-expressing cells (Fig 3.3). Interestingly, transfection of the BLV Tax/Rex plasmid into H1GFP cells yielded 2-fold less GFP expression than...
seen when pHTLV-1LTRGFP was transfected into FLK-BLV cells. This could be due to a difference in the relative concentrations of the BLV Tax protein obtained from FLK-BLV cells as compared to transfection BLV \textit{tax/rex} expression plasmid, for it has been shown that the FLK-BLV cells harbor four BLV proviruses (Derse et al., 1985a; Derse et al., 1985b). The percentage of GFP-expressing cells when H1GFP and H2GFP were transfected with the HTLV-1 \textit{tax/rex} expression plasmid were considerably higher (2- to 4-fold) than the levels of GFP-expressing cells seen when the same cells were co-cultured with MT-2 cells. This difference is likely due to the low infectivity of virus from the MT-2 cells. Transfection of the HTLV-2 \textit{tax/rex} expression plasmid into H1GFP and H2GFP yielded levels of GFP-expressing cells that were slightly less than those seen with the HTLV-1 \textit{tax/rex} expression plasmid.

**Spinoculation does not result in an increase in BLV infection.**

Based upon data that reported increases in infection efficiencies when spinoculation, or centrifugal infection, was used for HIV-1 and other retroviruses (O'Doherty et al., 2000); (Bahnson et al., 1995; Bunnell et al., 1995), we utilized this technique on BLGFP cell clones 10 and J to determine if an increase in BLV viral titer could be obtained. In the spinoculation protocol, plates are coated with poly-L-lysine. This creates a “sticky” surface that could potentially trap infectious BLV and bring it into contact with neighboring cells, thus contributing to an
increase in viral titer. We first tested whether the addition of poly-L-lysine alone could increase the infection efficiency of BLV. BLGFP cell clones 10 and J were plated out into six-well plates either coated with 0.01% tissue culture grade poly-L-lysine or not coated and then infected with BLV obtained from FLK-BLV cells. It has been shown that beyond 48 hrs there is an increase of GFP expression in the cells (Gervaix et al., 1997) (Liu et al., 2001). In our experiments the cells were harvested and analyzed for GFP expression by flow cytometry at days 2, 3 and 4 post-infection (Fig 3.4). The viral titer of BLV was calculated from the percentage of GFP expressing cells for each experiment. The addition of poly-L-lysine did not have any effect upon the titer of BLV (Fig. 3.4).

Spinoculation was performed on BLGFP cell clones 10 and J. For BLGFP cell clone 10, no increase in viral titer was observed from spinoculation. For BLGFP cell clone J we observed a modest increase in viral titer, approximately 2-fold. To assess whether the increase in GFP expressing cells after 48 hrs was the result of GFP accumulation in the cells or due to virus spread, we repeated the spinoculation experiment but added the reverse transcriptase inhibitor AZT 24 hrs post-infection. AZT was added to the BLGFP cell clones 10 and J at a concentration that we found to completely inhibit replication of BLV (data not shown). The viral titers of BLV at 2, 3, and 4 days post-infection remained relatively unchanged upon the addition of AZT as compared to the spinoculation experiment in which an increase in viral titer was observed at day 3 and 4 post-
infection. These data indicate that the increase in viral titer seen in the spinoculation and infection experiments was due to viral spread.

Figure 3.3: Transactivation of either the HTLV-1 LTR or the HTLV-2 LTR in H1GFP and H2GFP cells. H1GFP and H2GFP cells were transiently transfected with a BLV, HTLV-1 or HTLV-2 Tax/Rex expression construct and 48 hours later analyzed by FACS for GFP expression. Two BLV Tax/Rex constructs were used, BLV 913 and BLPX-RSPA. pBLV 913 is an infectious molecular clone of BLV, while pBLPX-RSPA utilizes the Rous sarcoma virus promoter to express BLV Tax and Rex. The HTLV-1 and HTLV-2 Tax/Rex expression constructs used were SE356 and BC20.2, respectively. The percent of GFP-expressing cells is from a total of 40,000 cells counted by FACS.
Figure 3.4: Effect of spinoculation on BLV titer. BLGFP clones J and 10 were infected by BLV and then spun at 2,700 rpm at 32°C for 1h to determine if spinoculation would lead to an increase in viral titer. For one spinoculation, AZT was added at a final concentration of .5µM 24h post-infection to block BLV replication. Clones J and 10 were also infected by BLV without spinning and either with or without the addition of .01% poly-l-lysine to the plate. The cells were harvested at 2, 3, and 4 days post-infection and analyzed by FACS for GFP-expressing cells. The percentage of cells expressing GFP was used to calculate the virus titer. The percentage of GFP expression was based upon 30,000 cells counted by FACS.
DISCUSSION

We have developed distinct mammalian cell lines for detection of three deltaretroviruses - BLV, HTLV-1 and HTLV-2. Each cell line contains the LTR from one of the viruses to drive expression of GFP upon viral infection. Two BLV cell clones that stably express pBLVLTRGFP were chosen for detailed analysis, BLGFP 10 and BLGFP J. Transfection of either a BLV tax/rex expression plasmid or a BLV proviral clone, p913, resulted in similar levels of GFP-expressing cells (Fig. 3.2). This is in contrast with an earlier report that demonstrated low levels of transactivation of a BLV LTR reporter construct when p913 was present (Derse, 1987). The difference observed in our assay is likely due to the stable expression of pBLVLTRGFP in the BLGFP cells versus transient co-transfection used in the previous study.

We have demonstrated that the Tax proteins of HTLV-1 and HTLV-2 can transactivate the BLV LTR. Furthermore, we observed that the Tax proteins from both HTLV-1 and HTLV-2 were able to transactivate the BLV LTR at levels that were similar to those observed with either infectious BLV or BLV Tax (Fig. 3.2). Previous analysis (Rosen et al., 1985) of the BLV LTR showed that no transactivation activity was detected upon transfection of a BLV LTR reporter construct into cells that are chronically infected with either HTLV-1 or HTLV-2. The difference observed in the two studies could be due to the level of Tax
expression in transfected cells compared to Tax expression in chronically infected cells.

GFP-expressing cells were observed when pHTLV-1LTRGFP and pHTLV-2LTRGFP were each transfected into FLK-BLV cells. This observation is different from previous transactivation studies (Derse, 1987; Rosen et al., 1985). We further observed that transfection of the BLV tax/rex expression plasmid, pBPLX-RSPA, into H1GFP and H2GFP cells resulted in the detection of GFP-expressing cells, indicating that the BLV Tax protein can transactivate the LTRs of HTLV-1 and HTLV-2. These observations differ from previously published studies which we believe is due to differences in the sensitivity of the assays used (i.e., GFP fluorescence vs. CAT activity). A recent report analyzed the sensitivity of a HIV-1 LTR reporter construct that contained either GFP or CAT as the reporter gene. At low levels of Tat transactivated transcription, the GFP reporter had a higher sensitivity of detection as compared to CAT (Kar-Roy et al., 2000).

Our observation that transfection of a HTLV-1 tax/rex expression plasmid was able to efficiently transactivate the LTRs of both HTLV-1 and HTLV-2 is in agreement with two other reports (Ross et al., 1997; Semmes et al., 1996) that assessed the ability of Tax-1 to activate the LTRs of HTLV-1 and HTLV-2. Our data are also in good agreement with reports showing that Tax-2 is able to transactivate not only the HTLV-2 LTR but also the HTLV-1LTR (Cann et al., 1985; Ross et al., 1997).
Deltaretrovirus titers are known to be low both in culture and in their hosts. Due to previous reports (Bunnell et al., 1995; O'Doherty et al., 2000) of viral titer increases through spinoculation, we tested whether spinoculation could increase the viral titer of BLV. We found only a modest (2-fold) increase. Although useful in increasing titer with other retroviruses, spinoculation was not found useful for increasing BLV titers. These data suggest that BLV infectivity may be linked with cell associated virus.

In summary, we have described a cell culture-based assay that detects infection events of the deltaretroviruses BLV, HTLV-1 and HTLV-2. The data presented here show that the Tax proteins of all three viruses are capable of transactivating not only their own LTR, but the LTRs of the other two viruses. Given the difficulties in studying the molecular details of deltaretrovirus replication, these cell lines will greatly facilitate future analyses.
CHAPTER 4

BOVINE LEUKEMIA VIRUS IS SUSCEPTIBLE TO NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

ABSTRACT

Current antiretroviral therapy for the treatment of human immunodeficiency virus type 1 (HIV-1) includes three distinct types of drugs, nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). Comparative studies of drug susceptibility can provide further insight into the mechanisms of drug resistance. In this study, the sensitivity of bovine leukemia virus (BLV) to NRTIs was analyzed. We report the development of a tissue culture-based assay to determine the susceptibility of BLV to NRTIs. We found BLV to be sensitive to low concentrations of dideoxyadenosine (ddA), dideoxyinosine (ddl) and 3’-azido-3’-deoxythymidine (AZT). Interestingly, we observed that BLV was sensitive to (-)2’,3’-dideoxy-3’-thiacytidine (3TC) even though it was predicted to be resistant. Drug-resistant BLV was selected by long-term passage in the presence of high concentrations of the antiretroviral drugs. DNA sequencing
revealed three amino acid mutations in reverse transcriptase, including a mutation of the methionine in the YMDD motif to valine. Our observations indicate that BLV is susceptible to NRTIs. In addition our data indicate 3TC resistance is more complex than the acquisition of a valine residue in the YXDD motif.

**INTRODUCTION**

Antiretroviral therapy (ART) of human immunodeficiency virus type 1 (HIV-1) infection currently consists of nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Use of these three classes of antiretroviral drugs has dramatically reduced the rate of HIV-1 and AIDS related morbidity and mortality (Pillay et al., 2000; Richman, 2001). However the emergence of drug-resistant virus, due to poor patient compliance of therapy, still remains a threat to the success of antiretroviral therapy. Drug-resistant virus can emerge to any one or a combination of the NRTIs, the NNRTIs or the PIs. For HIV-1, resistance to RT and protease inhibitors has been shown to correlate with mutations in the genes of HIV RT and protease, respectively (Johnson et al., 2003; Menendez-Arias, 2002; Smith et al., 1998).

Mutations in HIV-1 RT that confer drug resistance to either NRTIs or NNRTIs have been mapped to the fingers, palm and thumb subdomains of the
catalytic domain of RT (Johnson et al., 2003; Menendez-Arias, 2002; Telesnitsky and Goff, 1997). In the palm domain, two conserved aspartate residues (185, 186) of the YXDD motif, along with another nearby aspartate residue (110) form the polymerase active site of RT (Telesnitsky and Goff, 1997). This region of the enzyme contacts the primer template and binds the incoming deoxynucleotide triphosphate (dNTP) (Kohlstaedt et al., 1993).

In HIV-1 antiretroviral therapy, use of the antiretroviral drug (-)2',3'-dideoxy-3'-thiacytidine (3TC) revealed that a single amino acid change in the YXDD motif led to resistance to 3TC (Schinazi et al., 1993). Therefore it was concluded that the YXDD motif within RT was a primary determinant of sensitivity to 3TC. HIV-1, which has the motif YMDD, was found to be sensitive to 3TC (Boucher et al., 1993; Schinazi et al., 1993; Tisdale et al., 1993). Mutation of the methionine residue at position 184 to valine or isoleucine, creating YVDD or YIDD, yields a HIV-1 RT that is highly resistant to 3TC (Boucher et al., 1993; Schinazi et al., 1993; Tisdale et al., 1993). Mutation of the methionine to isoleucine or to valine occurs very rapidly, within two weeks, both in vitro and in patients (Gao et al., 1993; Keulen et al., 1997; Tisdale et al., 1993).

Murine leukemia virus (MLV), which has the RT motif YVDD, was shown to be resistant to 3TC (Powell et al., 1999). Surprisingly, when the valine was mutated to methionine or isoleucine, the MLV RT was still resistant to 3TC (Halvas et al., 2000). Furthermore, human T-cell leukemia virus type 1 (HTLV-1)
has the YMDD motif and was found to be resistant to 3TC (Balestrieri et al., 2002; Garcia-Lerma et al., 2001; Macchi et al., 2003). Bovine leukemia virus (BLV) contains YMDD as the wild type motif; however the sensitivity of BLV RT to 3TC has not been examined.

In this study we determined the susceptibility of BLV to the nucleoside reverse transcriptase inhibitors (NRTIs) ddA, ddI, AZT and 3TC. BLV was found to be sensitive to all of the antiretroviral drugs studied. Drug-resistant BLV was selected for by long-term cultivation of BLV in the presence of drug. DNA sequence analysis of virus obtained from the long-term culture revealed three amino acid mutations in the RT region of the BLV pol gene, one of which is associated with drug resistance in HIV-1.

**MATERIALS AND METHODS**

**Cell lines and infections.**

Fetal lamb kidney cells chronically infected with BLV (FLK-BLV) and BLGFP cell clones J and 10 were grown in Dulbecco’s modified Eagle medium (DMEM) (GIBCO BRL, Gaithersburg, MD) containing 10% Fetalclone III (Hyclone, Logan, Utah). BLGFP cell clones J and 10 are Crandall feline kidney (CRFK) cells that stably express pBLVLTRGFP. To obtain infectious BLV, FLK-BLV cells were grown to 80% confluency then the supernatant was collected and spun at 1,000 rpm for 5 min. After spinning, 8µl/ml of Polybrene (Sigma, St.
Louis, MO) was added to the supernatant and 4ml of the supernatant plus Polybrene was used to infect 2 x 10^5 BLGFP indicator cells per 60-mm dish.

**IC_{50} determination for antiretroviral drugs.**

BLGFP cell clones J and 10 were plated at a concentration of 2 x 10^5 per dish and drug was added for 50% inhibitory concentration (IC_{50}) determinations using the BLGFP cells. The antiretroviral drugs used were 3’-azido-3’-deoxythymidine (AZT) (NIH AIDS reagent program and Sigma), (-)2’,3’-dideoxy-3’-thiacytidine (3TC) (NIH AIDS reagent program and Moravek Biochemicals, Brea, CA), dideoxyinosine (ddl) and dideoxyadenosine (dda) (Sigma). Twenty-four hours after the addition of the antiretroviral drugs, the cells were infected with 4ml of supernatant from FLK-BLV cells containing Polybrene and drug. The IC_{50} was determined with five different drug concentrations. One plate each of BLGFP J and 10 cells that were not infected were counted to obtain the number of cells present at the time of infection. Three days post-infection the cells were harvested. The percentage of GFP-expressing cells was determined by flow cytometry and was used to calculate virus titers.

**Selection for mutant BLV RTs resistant to antiretroviral drugs**

Three days post-infection of BLGFP J cells, the determined IC_{50} value of each of the antiretroviral drugs were added to individual plates of infected cells and then the infected BLGFP J cells were returned to 37°C. The infected BLGFP J cells were maintained in the continuous presence of drug at the determined
IC_{50} value for 92 days post-infection. Every three to four days the cells were split and half of the cells were used for flow cytometry analysis to measure the percentage of GFP-expressing cells. At days 13, 31, and 63 post-infection the concentration of the antiretroviral drug in the media of an individual plate was doubled in order to increase selection pressure for drug-resistant virus. This same procedure was used in parallel at the IC_{90} concentration of each drug. As a control, the BLGFP J cells were grown with no drug (0 uM) after the initial infection. The IC_{50} and IC_{90} concentrations of the antiretroviral drugs used were calculated from the plots of the viral titer versus the concentrations of the antiretroviral drugs used for both BLGFP J and BLGFP 10.

Thirty days after maintaining BLGFP J cells at both the IC_{50} and IC_{90} drug concentrations, the supernatant from the BLGFP J cells grown at the IC_{90} concentration of ddA, ddl, AZT and 3TC was harvested and used to infect fresh BLGFP J cells. Prior to infection, the fresh BLGFP J cells were grown for 24 hrs in media that contained the antiretroviral drugs at the IC_{90} concentration. After infection, cells were grown in media containing drugs at the determined IC_{90} concentration. Cells were split every three or four days and half were used for flow cytometry analysis to measure the percentage of GFP-expressing cells. These cells were passaged out to 60 days post-infection.
Flow Cytometry.

BLGFP J and 10 cells were harvested by the addition of 0.5ml of trypsin to plates followed by centrifugation at 1,000 rpm for 5 min to pellet the cells. The pelleted cells were then resuspended in 0.5ml of phosphate buffered saline (PBS). A Beckman Coulter Epics XL machine was used for flow cytometry analysis. Prior to sample analysis, the machine was gated at 0.5% fluorescence using negative control cells (i.e. uninfected cells).

Analysis BLV RT sequence.

Virion RNA was prepared by clarifying (700 x g for 5 min), 200mL of supernatant from BLGFP J cells maintained in media containing the IC$_{90}$ concentration of ddA, ddI, AZT and 3TC for over 100 days. Virus particles were pelleted by ultracentrifugation in a 60Ti rotor at 40,000 rpm for 1 h at 4°C. Virus pellets were resuspended in 150µl of PBS and RNA extracted with The High Pure Viral RNA Kit (Roche, Indianapolis, IN) using the manufacturer's instructions. The virion RNA was treated with DNA-free (Ambion, Austin, TX) to remove any DNA that might be present. Super-Script III Reverse Transcriptase (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions for cDNA synthesis of the viral RNA. The cDNA was then used in PCR to amplify a region of the BLV pol gene, the reverse transcriptase (RT) region, by using a nested set of primers. The first round PCR used primers that amplified a 1.5 Kb region of the BLV genome. Each 100 µl PCR reaction included 10 µl from the
cDNA reaction. The PCR protocol consisted of 35 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min) and elongation (72°C for 2 min). The first round of PCR products were further amplified using a nested set of primers. The nested PCR products were then sequenced for detecting mutations. The primers for the pol gene are as follows, numbering is based upon BLV as determined by Sagata et al (Sagata et al., 1985):

1597+ 5’ -GTCCACACCCAGGGCCCAAG- 3’ ;
2850- 5’ -CATTGTGACCGCTGTCTTTCTTGTA- 3’ ;
2082+ 5’ -AGATACAATTGGCTACAAGGC- 3’ ;
2961- 5’ -CTGCTCATGGACCATTGGTCC- 3’ ;
2251+ 5’ -CTGCTGTAGGCCTCTTTGATA- 3’ ;
3041- 5’ -CTCCTAAGACCGCCTGTAAT- 3’.

RESULTS

Determination of the IC50 values

To determine the concentration of drug that would cause 50% inhibition of BLV replication, we used two previously described BLV indicator cell lines. The BLV indicator cell lines, BLGFP 10 and BLGFP J, were incubated with drug for 24 hrs prior to and during infection with BLV from FLK-BLV cells. The four antiretroviral drugs analyzed were ddA, ddI, AZT, and 3TC. Five different concentrations of each drug were tested in order to determine the 50% inhibitory
concentration (IC$_{50}$) of each drug. The IC$_{50}$ is the concentration of drug that decreased virus titer by 50% as compared to when no drug is added. Three days post-infection, the BLGFP 10 and J cells were harvested and analyzed by flow cytometry to determine the percentage of GFP-expressing cells. The percentage of GFP-expressing cells was used to calculate virus titer. Virus titer was plotted versus drug concentration (Fig. 4.1) in order to determine the IC$_{50}$ of the antiretroviral drugs. The IC$_{50}$ of ddA and ddI was observed to be 5 µM, for AZT 0.1 µM and for 3TC the IC$_{50}$ was found to be 1 µM. The sensitivity of retroviruses to 3TC is shown in Table 4.1

<table>
<thead>
<tr>
<th>Virus</th>
<th>YXDD</th>
<th>3TC Sensitivity Phenotype</th>
<th>References</th>
</tr>
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<td>HIV-1 wt</td>
<td>YMDD</td>
<td>Sensitive</td>
<td>Schinazi et al., 1993</td>
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<tr>
<td></td>
<td>YVDD</td>
<td>Resistant</td>
<td>Schinazi et al., 1993, Boucher et al., 1993, Tisdale et al., 1993</td>
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<tr>
<td></td>
<td>YIDD</td>
<td>Resistant</td>
<td></td>
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<tr>
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<td>YVDD</td>
<td>Resistant</td>
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</tr>
<tr>
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<td>YMDD</td>
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<td>Halvas et al., 2000</td>
</tr>
<tr>
<td></td>
<td>YIDD</td>
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</tr>
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<td>HTLV-1 wt</td>
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<td>Garcia-Lerma et al., 2001, Balestrieri et al., 2002</td>
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<tr>
<td>BLV wt</td>
<td>YMDD</td>
<td>Sensitive</td>
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Table 4.1: YXDD motif in reverse transcriptases and 3TC sensitivity.
Figure 4.1: Determination of IC₅₀ values of antiretroviral drugs to BLV infection. BLGFP indicator cell clones 10 and J were incubated with, ddA, ddl, 3TC or AZT, prior to and during BLV infection. Cells were harvested three days post-infection and analyzed by flow cytometry to determine the percentage of GFP-expressing cells and virus titer. The percentage of GFP expression was based upon 40,000 cells counted by flow cytometry. The IC₅₀ of each drug was determined by plotting virus titer against drug concentration. Each drug was tested in triplicate. Representative data from one experiment is shown.
Selection of drug-resistant BLV

To select for drug-resistant BLV, BLGFP 10 and J cells were infected with BLV from FLK-BLV cells and three days post-infection drug was added at the previously determined IC<sub>50</sub> and IC<sub>90</sub> amounts. For ddA and ddI, 5 µM and 10 µM of each drug was added to infected BLGFP 10 and J cells. AZT was added to infected BLGFP 10 and J cells at final concentrations of 0.1 µM and 0.5 µM, and 1 µM and 5 µM concentrations of 3TC were used. The infected BLGFP 10 and J cells were maintained in the continuous presence of the antiretroviral drugs and were split every three to four days. Half of the BLGFP cells were used to propagate the culture and the other half of the cells were analyzed by flow cytometry to determine the percentage of GFP-expressing cells. GFP expression was used as a measure of BLV infectivity. During long term passage of virus in BLGFP 10 cells, we could not detect GFP-expressing cells past day 31 post-infection (data not shown), indicating that BLV was not actively replicating in the cells. BLGFP J cells were passaged in the presence of the antiretroviral drugs, at both the IC<sub>50</sub> and IC<sub>90</sub> values, for ninety-two days post-infection (Fig 4.2). As a control BLGFP J cells that were infected with BLV but not incubated with any of the antiretroviral drugs were maintained in parallel (i.e., 0 µM) (Fig 4.2). To increase selection pressure for drug resistance, the initial amounts of the drugs were doubled at days 13, 32 and 63 post-infection. Therefore, at day 92 post-infection the final concentrations for both the IC<sub>50</sub> and IC<sub>90</sub> amounts of ddA, ddI,
AZT and 3TC were eight times higher than the drug concentrations at the start of the experiment.

At three weeks post-infection the percentage of GFP-expressing cells for BLGFP J cells had dropped to 2-4%, including cells that were not incubated with drug (Fig. 4.2). At day 66 post-infection of the BLGFP J cells incubated with 3TC, an increase in GFP-expressing cells, up to 10% (IC\textsubscript{50}) and 17% (IC\textsubscript{90}), was observed (Fig. 4.2). BLGFP J cells also showed in an increase (9%) of GFP-expressing cells at day 60 post-infection for the IC\textsubscript{50} value of ddI (Fig. 4.2). The increases in GFP expression suggest that a mutation arose in BLV that permitted virus replication in the higher drug concentrations. Only small increases in the percentages of GFP-expressing cells for BLGFP J cells was observed for ddA and AZT at day 66 post-infection.

**Cell-free infection of fresh BLGFP J cells with virus from long term culture**

To determine if cell-free infectious BLV was still present after maintaining the BLGFP J cells in drug for four weeks, fresh BLGFP J cells were infected. BLGFP J cells were incubated with the IC\textsubscript{90} amount of ddA, ddI, AZT and 3TC for 24 hrs and then infected with supernatant from day 32 of BLGFP J cells maintained at the IC\textsubscript{90} concentration of each antiretroviral drug. The infected BLGFP J cells were maintained in the IC\textsubscript{90} amount of the drugs and were split every three to four days. The percentage of BLGFP J cells that were infected
was determined by flow cytometry. BLGFP J cells were passaged in the presence of the IC$_{90}$ value of each drug for forty-two days post-infection (Fig. 4.3).

It was observed that the BLGFP J cells generated higher amounts of GFP-expressing cells immediately after infection with the day 32 IC$_{90}$ supernatant. Two to three weeks post-infection, the BLGFP J cells produced GFP expression levels that were the same or higher than those observed at the onset of infection with the day 32 IC$_{90}$ supernatant (Fig. 4.3). This increase in the percentage of GFP-expressing cells suggests that a mutation arose in BLV that permitted virus replication in the presence of the higher drug concentrations.
Figure 4.2: Selection of drug-resistant BLV with BLGFP J cells. A. Selection of drug-resistant BLV from BLGFP indicator cell clone J. BLGFP J cells were infected with BLV (from FLK-BLV cells) and 72 hrs later incubated with the indicated antiretroviral drugs at the previously determined IC$_{50}$ and IC$_{90}$ values. Cells were split every three to four days. The percentage of GFP expressing cells was determined by flow cytometry and then plotted versus time. The percent GFP expression was based upon 50,000 cells counted by flow cytometry. The cells were passaged in the presence of drug for 92 days post-infection. At days 13, 31 and 63 the amount of the antiretroviral drug in the media was doubled. As a control, BLGFP J cells that were infected with BLV but not incubated with the antiretroviral drugs were maintained in parallel and are shown as 0 uM.
Figure 4.3: Cell-free infection of fresh BLGFP J indicator cells using virus from long term drug treated cultures. BLGFP J cells were incubated with the IC$_{90}$ concentration of ddA, ddI, AZT or 3T C for 24 hrs and then infected with supernatant from day 32 of BLGFP J cells that were maintained in the IC$_{90}$ concentration of each antiretroviral drug. The percentage of GFP expressing cells was determined by flow cytometry and then plotted versus time. The percentage of GFP expression was based upon 50,000 cells as determined by flow cytometry. The cells were passaged in the presence of the IC$_{90}$ drug concentrations for 42 days post-infection.
Nucleotide sequence analysis of proviral DNA

To identify BLV resistant mutants that may have evolved during drug selection, we sequenced the RT encoding region of the pol gene from virus maintained at the IC$_{90}$ values of ddA, ddI, AZT and 3TC for 92 days post-infection. As a control, non-drug treated virus was also sequenced.

Sequence analysis of virus obtained from BLGFP J cells incubated with ddA, AZT and 3TC revealed four nucleotides that were mutated in the RT region of the pol gene. These four mutated nucleotides resulted in only three amino acid changes, M131V, I136Y and A137D, in the area surrounding the YXDD motif in RT (Fig 4.4). The G to A mutation at position 2800 of the BLV genome (Sagata et al., 1985) changes the methionine residue of the YMDD motif to a valine, thus creating YVDD. Nucleotides 2815 and 2816 of the BLV genome (Sagata et al., 1985) were mutated from TA to AT, creating an isoleucine (I) to tyrosine (Y) substitution. Finally, a C to A transversion mutation occurred at position 2819 of the BLV genome (Sagata et al., 1985), leading to an alanine (A) to an aspartic acid (D) substitution (Fig. 4.4). The amino acid sequence surrounding the YXDD motif of the RT region of pol is shown in Fig. 4.4. No mutations in the pro/pol junction were observed in any samples analyzed.
Figure 4.4: Amino acid sequence of BLV reverse transcriptase (RT) region containing substitutions after drug treatment. The RT-encoding region of the pol gene from virus maintained at the IC$_{90}$ values of ddA, ddI, AZT and 3TC for over 90 days post-infection was PCR amplified and sequenced. Virus not incubated with drug that was passaged for over 90 days was also PCR amplified and sequenced. Amino acids that are underlined for virus incubated with ddA, AZT and 3TC are the mutated amino acids that were revealed by DNA sequence analysis.

**DISCUSSION**

We have determined the sensitivity of BLV to four nucleoside reverse transcriptase inhibitors (NRTIs) using a tissue culture based assay developed to detect BLV infectivity. We selected for drug-resistant BLV by long-term passage of BLV-infected cells in the continuous presence of the NRTIs. Sequence analysis of virus recovered from long-term cultures revealed mutations at four
nucleotide positions. These four nucleotide base changes result in three amino acid mutations in reverse transcriptase region (RT) of the pol gene. One of these mutations altered the YMDD motif in RT to YVDD.

Development of a BLV indicator cell line enabled us to not only readily determine the susceptibility of BLV to antiretroviral drugs but also allowed for the selection of drug-resistant BLV. We found BLV to be sensitive to low concentrations of ddA and ddi. Incubation of BLV in media that contained AZT significantly affected BLV replication, even at low concentrations of the drug. Our finding that BLV is sensitive to low concentrations of AZT is in agreement with studies on the susceptibility of HTLV-1 to NRTIs (Garcia-Lerma et al., 2001; Hill et al., 2003; Macchi et al., 1997).

Surprisingly, we found BLV to be sensitive to 3TC, which is in contrast to reports which found HTLV-1 to be resistant to high concentrations of 3TC (Balestrieri et al., 2002; Garcia-Lerma et al., 2001; Macchi et al., 2003). We determined the IC₅₀ of BLV to 3TC to be 1µM, while a previous study with HTLV-1 RT determined the IC₅₀ for 3TC to be greater than 10µM based upon RT activity (Garcia-Lerma et al., 2001). Another analysis of HTLV-1 sensitivity to 3TC found that the IC₅₀ was 29 ± 2µM (Balestrieri et al., 2002). In our assay, increasing 3TC to 5µM resulted in a 90% decrease in BLV titer. In light of these assays, we believe that HTLV-1 is less sensitive to 3TC than BLV.
A recent report with HTLV-1 RT in which the YMDD motif was altered to YVDD showed that this mutant was highly resistant to 3TC, and allowed for replication in the presence of 100 µM 3TC (Hill et al., 2003). We were not able to determine if a BLV mutant with YVDD was more resistant to 3TC than wild type due to low infectivity of both the wild-type and the mutated BLV proviral clones.

Sequence analysis of virus obtained from BLGFP J cells showed three amino acid substitutions in RT (Fig. 4.4). One of these mutations changes the YMDD motif to YVDD. The methionine to valine mutation of the YXDD motif has been shown to occur rapidly in HIV-1 and is associated with resistance to 3TC (Boucher et al., 1993; Keulen et al., 1997; Schinazi et al., 1993; Tisdale et al., 1993). Another mutation changed the isoleucine (I) at position 167 of BLV RT to a tyrosine (Y). Previously published sequences of different BLV strains have reported either an isoleucine or a tyrosine at this position (Dube et al., 2000). The third amino acid mutation occurs at position 168 of BLV RT and results in an alanine (A) to aspartate (D) change.

Resistance to NRTIs correlates with substitutions of residues located in the dNTP binding site of RT or in the “template grip” (Emini and Fan, 1997; Jonckheere et al., 2000; Menendez-Arias, 2002). HIV-1 RT mutations K65R and L74V, in the fingers subdomain, confer resistance to ddI by repositioning the template/primer which results in a reduced sensitivity to the NRTI (Johnson et al., 2003; Jonckheere et al., 2000; Menendez-Arias, 2002) However, some HIV-1 RT
mutations resistant to AZT are not located near the dNTP binding site. AZT resistance in HIV-1 occurs through the accumulation of several mutations (i.e. K70R, T215Y/F, M41L, D67N, L210W and L219Q/E) in RT in an ordered appearance (Johnson et al., 2003; Jonckheere et al., 2000; Menendez-Arias, 2002). Additionally, it has been shown that AZT resistant mutations in HIV-1 RT work together to augment the selective excision of AZT from the growing template via phosphorolysis (Arion et al., 1998; Boyer et al., 2001; Meyer et al., 1999). None of the known HIV-1 NRTI resistant mutations were observed in virus obtained from long-term passage in the presence of drug with the exception of the M to V mutation in the YMDD motif. This indicates that BLV must differ in its interactions with NRTIs as compared to HIV-1.

The A168D mutation that was observed in BLV is interesting because it occurs just outside the YXDD motif of the catalytic domain of RT. The mutation replaces a small non-polar amino acid with a bulky charged amino acid. Alignment of BLV RT and HIV-1 RT shows that HIV-1 has a glycine residue (190) in the same location that the A168D mutation was found. During treatment of HIV-1 with NNRTIs, a resistant mutation has been shown to occur in which the glycine at position 190 of HIV-1 RT is substituted by either an alanine, a serine or a glutamic acid (Johnson et al., 2003; Menendez-Arias, 2002). NNRTIs have been shown to bind to a hydrophobic pocket near the polymerase active site of the HIV-1 RT (Emini and Fan, 1997; Frankel and Young, 1998; Jonckheere et al.,
The majority of NNRTI resistant mutations discovered in HIV-1 RT are located within the same hydrophobic pocket that the inhibitor binds to (Emini and Fan, 1997; Jonckheere et al., 2000; Tozser, 2001). Our observation of an alanine to aspartic acid substitution at position 168 of the BLV RT that arose during long-term passage of the virus in high concentrations of NRTIs suggests the possibility that BLV RT interacts with NRTIs in a manner that differs from what has been shown with HIV-1.

In summary, we show that BLV is susceptible to NRTIs and that 3TC resistance is more complex than the acquisition of a valine residue in the YXDD motif. Long-term passage of BLV resulted in three amino acid mutations in the RT region of the \textit{pol} gene. One of the mutations has been observed in natural isolates of BLV. One is associated with 3TC resistance in HIV-1 and the final mutation occurs near the YXDD motif of RT. Our data suggest that comparative studies of the susceptibility of various RTs to NRTIs are a valuable approach to understanding the mechanisms of drug resistance.
CHAPTER 5

DISCUSSION

Bovine Leukemia Virus Packaging

A minimal bovine leukemia virus (BLV) RNA packaging sequence (E) required for packaging of heterologous RNAs into BLV particles was analyzed. The BLV E comprises both the primary and secondary encapsidation signals that were previously mapped by deletion analysis (Mansky et al., 1995). The data in this report shows that heterologous RNAs containing the BLV E can be packaged into BLV particles. Packaging of the non-viral vector RNA, LacZBLV E, confirms the location of the BLV RNA packaging signal (Mansky et al., 1995; Mansky and Wisniewski, 1998) and indicates that these RNA sequences are both necessary and sufficient for packaging. A chimeric MLV/BLV retroviral vector RNA was also packaged into BLV particles. Surprisingly, we found that both MLV Ψ+ and sequences outside of Ψ+ can be recognized by the BLV machinery and packaged into BLV particles.

MLV Ψ+ has previously been shown to be recognized by the packaging machinery of another retrovirus. Observations between spleen necrosis virus
(SNV) and MLV revealed that SNV Gag can recognize both the SNV E and the MLV \( \Psi^+ \); however MLV Gag only recognizes the MLV \( \Psi^+ \) (Certo et al., 1998; Yang and Temin, 1994). The RNA structural motifs of the packaging signal are believed to play a major role in the recognition event between the viral Gag polyprotein and the full-length viral genome. The packaging signals of both MLV and SNV have hairpin pairs that have been shown to be necessary for RNA packaging (Konings et al., 1992; Yang and Temin, 1994). Structure-function analysis established that the BLV primary packaging signal was composed of two stable RNA stem-loop structures (SL1 and SL2) (Mansky and Wisniewski, 1998). The hairpin pair of \( \Psi^+ \) is structurally similar to the two stem-loops of the BLV primary packaging signal, thus BLV Gag could recognize the packaging signal based upon the secondary RNA structures.

Recent data has shown that an upstream flanking region of \( \Psi^+ \) is required for MLV Gag-mediated packaging and that optimal MLV Gag-mediated packaging occurred when three regions of MLV were present. Those regions are the upstream flanking sequence, the hairpin pair and the downstream flanking sequence of the packaging signal (Beasley and Hu, 2002). These data indicate that packaging of viral RNA occurs through a cooperative effort between different regions surrounding the packaging signal. BLV Gag could also recognize more than one region surrounding the packaging signal. The packaging of a MLV
vector RNA lacking \( \Psi^+ \) lends support to the suggestion that more than one area of the viral genome can lead to efficient packaging of the viral genome.

A recent report identified seventeen nucleotides downstream of the \textit{env} stop codon that were important in MLV packaging (Yu et al., 2000). Furthermore, it has been recently reported that low levels of replication of a MLV-based vector, in which \( \Psi^+ \) was deleted, occurs in both SNV- and MLV- based helper cell lines (Beasley and Hu, 2002). This suggests that the SNV Gag can recognize MLV RNA sequences outside of \( \Psi^+ \). Based upon our results, BLV Gag can also recognize MLV RNA sequences outside of \( \Psi^+ \).

Further analysis into the packaging of both \( \Psi^+ \) and MLV sequences outside of \( \Psi^+ \) by BLV would provide insight into the mechanism of recognition between the viral Gag polyprotein and the full-length viral genome. A possible experiment for the future would be to replace the BLV packaging signal region with either \( \Psi^+ \) or the seventeen base pairs downstream of the \textit{env} stop codon and then measure both the packaging efficiency of the RNA and the viral titer compared to wild type BLV. This experiment would be similar to a previously published experiment in which the BLV packaging signal region was replaced with that of either HTLV-1 or HTLV-2 (Mansky and Wisniewski, 1998).

An additional experiment would be to determine if both matrix (MA) and nucleocapsid (NC) play a role in the packaging of MLV RNA. A recent study of the BLV Gag domains involved in the packaging process showed that both the
MA and NC domains of BLV Gag played a role in genome recognition and RNA packaging (Wang et al., 2003). A hybridization study involving both MA and NC proteins from BLV and MLV Ψ+ RNA would determine if the same mechanism used to packaging BLV viral RNA is also used in the packaging of the MLV RNA.

**Deltaretrovirus Indicator Cell Lines**

We describe mammalian cell lines that were created using the long terminal repeats (LTRs) of BLV, HTLV-1 and HTLV-2 to drive expression of the enhanced green fluorescent protein gene (egfp) in cells when transactivated by the BLV, HTLV-1 or HTLV-2 Tax proteins, respectively. The BLGFP, H1GFP and H2GFP cell lines detect virus infection by the expression of GFP due to transactivation of the LTR after infection. The data presented here show that deltaretrovirus Tax proteins can transactivate each others’ LTRs and result in GFP expression.

Titers of deltaretroviruses are known to be low both in culture and in their hosts; therefore the BLV indicator cell line was used to test whether spinoculation could increase the viral titer of BLV. Previous reports have shown that spinoculation can increase the viral titers of other retroviruses (Bunnell et al., 1995; O'Doherty et al., 2000). However, spinoculation was not found to be useful for increasing BLV titers; we found only a modest (2-fold) increase.
Future experiments with the deltaretroviruses indicator cell lines could be extended to the analysis of samples from BLV positive cows and patient samples of individuals that are infected with either HTLV-1 or HTLV-2. The indicator cells could determine the viral titer in the infected individuals and be used to monitor the progression of treatment (if any is given). In addition, deltaretrovirus indicator cell lines could also be used to determine if treatments for the diseases associated with these viruses will result in a decrease in viral infectivity. Furthermore, these cells can be used to assess the effect of mutations that are introduced into the viral genome. Given the difficulties in studying the molecular details of the replication cycle of deltaretroviruses, these cell lines will greatly facilitate future analyses.

**Bovine Leukemia Virus Susceptibility to Nucleoside Reverse Transcriptase Inhibitors**

We determined the sensitivity of BLV to nucleoside reverse transcriptase inhibitors (NRTIs) using a tissue culture based assay developed to detect BLV infectivity. BLV was found to be sensitive to relatively low concentrations of dideoxyadenosine (ddA), dideoxyinosine (ddI) and 3’-azido-3’-deoxythymidine (AZT). Interestingly, we observed that BLV was sensitive to (-)2’,3’-dideoxy-3’-thiacytidine (3TC) even though it was predicted to be resistant due to studies that found HTLV-1, which also has the YMDD motif, to be resistant to high
Drug-resistant BLV was selected by long-term passage in the presence of high concentrations of antiretroviral drugs. DNA sequencing revealed three amino acid mutations in reverse transcriptase (RT) region of the pol gene, including a mutation (methionine to valine) in the highly conserved YMDD motif. Based upon studies with HIV-1 and HTLV-1, mutation of the YMDD motif to YVDD yields a virus that is highly resistant to 3TC (Hill et al., 2003; Schinaze et al., 1993; Tisdale et al., 1993). We were unable to determine if a BLV molecular clone with YVDD was more resistant to 3TC than wild type due to low infectivity of the mutated proviral clone. Growing virus in extremely high concentrations (>100µ M) of the antiretroviral drugs could provide further evidence for the development of drug resistance. Use of the BLV indicator cells would determine if the mutant virus was actively replicating at such high drug concentrations, thus proving it was resistant. DNA sequencing of the virus could then be performed again to determine if new mutations had arisen to allow the virus to replicate at these higher drug concentrations or if the mutation of the YMDD motif was sufficient to allow virus replication at these drug concentrations. For example, AZT resistance in HIV-1 occurs with the acquisition of a series of mutations in RT, which confer higher levels of drug resistance when linked.
The A168D mutation that was identified when BLV was passaged with drug for over 90 days post-infection is interesting because it is located just outside of the catalytic domain of RT. The mutation replaces a small non-polar amino acid with a bulky charged amino acid. Alignment of BLV RT and HIV-1 RT (Fig. 5.1) shows that HIV-1 RT has a glycine residue (position 190) in the same location that the A168D mutation was found. During treatment of HIV-1 with NNRTIs, a resistant mutation has been shown to occur in which the glycine at position 190 is substituted by either an alanine, a serine or a glutamic acid (Johnson et al., 2003; Menendez-Arias, 2002). NNRTIs have been shown to bind to a hydrophobic pocket near the polymerase active site of the HIV-1 RT (Emini and Fan, 1997; Frankel and Young, 1998; Jonckheere et al., 2000; Menendez-Arias, 2002; Tozser, 2001). The majority of NNRTI resistant mutations are located within the same hydrophobic pocket where the inhibitor binds (Emini and Fan, 1997; Jonckheere et al., 2000; Tozser, 2001). The observation that an alanine to aspartic acid substitution at position 168 of the BLV RT arose during long-term passage of the virus in high concentrations of NRTIs suggests that BLV RT interacts with NRTIs in a manner that differs from what has been shown with HIV-1. Further analysis of this mutation should include whether or not it confers resistance to NNRTI's. One method to test this would be DNA sequence analysis of BLV that was maintained in the presence of NNRTIs after long-term passage of the virus.
Alignment of the BLV and HIV RTs (Fig. 5.1) revealed only 50% homology. Additionally, some of the resistance mutations that arose in HIV-1 during treatment with NRTIs are wild type amino acid residues in BLV. Intriguingly, BLV is susceptible to these NRTIs. These observations suggest that BLV and HIV-1 have distinct interactions with antiretroviral drugs and provide an argument that comparative studies of drug susceptibility can provide further insight into the mechanisms of drug resistance.
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