

January 2010

Regulation of Human T-cell Leukemia Virus Type 1 Infection and Replication

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REGULATION OF HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 INFECTION AND
REPLICATION

by

Gunjan Choudhary

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2010

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

Regulation of Human T-cell Leukemia Virus Type 1 Infection and Replication

by

Gunjan Choudhary

Doctor of Philosophy in Biology and Biomedical Sciences
(Molecular Genetics & Genomics)

Washington University in St. Louis, 2010

Professor Lee Ratner, Chairperson

Retroviruses have evolved complex mechanisms to regulate their cellular tropism and gene expression. It is generally accepted that productive infections proceed via interactions between viral envelope molecules and specific receptors on the host cell surface. Currently, there is no known receptor for HTLV-1, though a number of factors that enhance entry have been identified. In an effort to identify a cellular receptor or attachment factor for HTLV-1, we carried out a retroviral cDNA library screen, in which cDNA from permissive HeLa S3 cells was introduced into poorly susceptible NIH 3T3 cells. These cells were selected after infection with HTLV-1 envelope pseudotyped viral particles expressing a drug resistance gene. We isolated approximately 460 cDNAs, of which 20 were prioritized as potential candidates. These candidates are being tested to determine if they participate in viral entry.

In addition to encoding the structural and enzymatic genes common to all retroviruses, HTLV-1 also encodes several accessory genes which contribute to viral replication and the maintenance of gene expression. A newly identified viral gene, HTLV-1 bZIP factor or *hbz*, has been shown to have pleiotropic effects as it functions

differently in its protein and mRNA forms. In an effort to elucidate its role in HTLV-1 replication, we identified a novel function. Mutations that abrogated the *hbz* mRNA or disrupted a stem-loop in *hbz* mRNA, or mutations that eliminated or truncated the HBZ protein were introduced in a functional molecular clone of HTLV-1. The protein and stem-loop mutants had no effect on viral gene expression. However, the mutant that disrupted *hbz* mRNA expressed lower levels of *tax* mRNA, suggesting that *hbz* promotes *tax* expression. We found that this effect of *hbz* was indirect, as *hbz* represses another accessory gene, *p30^{II}*, which is known to sequester *tax* mRNA in the nucleus. These results provide new insights into the regulation of HTLV-1 infection, specifically viral entry and gene expression.

ACKNOWLEDGEMENTS

I would like to thank my mentor Lee Ratner for his support and guidance. He has allowed me to realize my potential as a scientist, and has always been available for helpful discussions. He has let me find my own way, which has not always been smooth, and for this I am truly grateful. I greatly appreciate the opportunity he has given me by allowing me to complete my dissertation work in his laboratory.

I would like to thank the members of my thesis committee – Tim Ley, Jim Skeath, Tim Graubert, John Atkinson, and Dong Yu – for always being supportive. I appreciate the advice and constructive criticism, which has allowed me to make the most of my dissertation work.

I would also like to thank the members of the Ratner lab, both past and present: Brooke, Aneeza, Ajay, Emilia, Esa – without you, lab life would have been tremendously dull; Anna – I am going to miss our lunches together; Dan – thank-you for always answering my questions; John – thank-you for all the laughs; Nancy – thank-you for all the time you spent explaining and teaching; Xiaogang – I will miss our talks; Sirosh – thank-you for your support.

Most importantly, I would like to thank my parents and my sister for their continued love and support. They are the constants in my life, and I appreciate all that they have done to get me to where I am today.

Last, but definitely not least, I would like to thank my boyfriend, Udara Fernando. He has been a constant source of support, love, and laughter, and has always pushed me to do better. I truly appreciate all that he has done for me, despite the cost to him.

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Chapter 1

Introduction

HTLV-1 AND DISEASE

HTLV-1 belongs to the *Deltaretrovirus* genus of the Orthoretroviridae family, which also includes the three other types of the human T-cell leukemia viruses – type 2 (HTLV-2), type 3 (HTLV-3), and type 4 (HTLV-4), the three types of the simian T-cell leukemia viruses – type 1 (STLV-1), type 2 (STLV-2), and type 3 (STLV-3), and the bovine leukemia virus (BLV). The HTLVs and STLVs are collectively known as primate T-cell leukemia viruses, or PTLVs. HTLV-1 and -2 were discovered in the early-80s (Gallo, 2005; Kalyanaraman et al., 1982; Poiesz et al., 1980), while HTLV-3 and -4 were discovered in 2005 (Calattini et al., 2005; Mahieux and Gessain, 2009; Wolfe et al., 2005). Of the four HTLVs, HTLV-1 is the only one known to cause disease. It was the first human retrovirus discovered, isolated from a cell-line derived from a patient with cutaneous T-cell lymphoma (Poiesz et al., 1980). It is the etiological agent of adult T-cell leukemia/lymphoma (ATLL), and a variety of inflammatory disorders, including HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP), HTLV-associated uveitis, and infective dermatitis (Proietti et al., 2005). Although the exact number of sero-positive individuals is unknown, an estimated 15-20 million people are infected with HTLV worldwide (de The and Kazanji, 1996). HTLV-1 is endemic to the Caribbean, southern Japan, parts of Africa and South America, and Melanesia; it is also present in Europe and North America. Most HTLV-1-infected individuals never develop any major clinical manifestation; only about 2-6% develop disease after a long latency period, usually several decades.

ORIGINS OF HTLV-1

HTLV-infection is present worldwide, but Africa is the only continent where all seven forms of PTLV exist. Based on this, and phylogenetic studies, the common ancestor of all PTLV is assumed to have originated in Africa (Vandamme, Salemi, and Desmyter, 1998). Based on several analytical methods, three different clades of HTLV-1 exist (Koralnik et al., 1994). The cosmopolitan strain (clade 1) has been transmitted to many populations worldwide, and it represents a very homogeneous group of viruses that probably originated in South Africa (Gallo, Sliski, and Wong-Staal, 1983; Gessain, Gallo, and Franchini, 1992; Koralnik et al., 1994). The second clade (clade 2; HTLV_{Zaire}) was identified in central Africa, and has also been exported to other countries. The third clade (clade 3; HTLV_{Mel}) was identified in remote inhabitants of Papua New Guinea and the Solomon Islands, and in Aborigines from Australia (Franchini, 1995). Evidence indicates that HTLV_{Mel} and the cosmopolitan HTLV-1 probably derived from a common ancestor, but evolved independently. Evolutionary analysis suggests that HTLV_{Mel} is the most divergent clade, preceding the split between the HTLV_{Zaire} and HTLV-1 cosmopolitan groups.

Similar analyses have been carried out on STLV-1 strains of African and Asian origin. STLV, from a single species, was sorted into genetically distinct clades. This suggests recent interspecies transfer between human and primates. In fact, the human clade 2 and the common chimpanzee clade S5 cluster together, suggesting that HTLV_{Zaire} may have resulted from a cross-species transmission of chimpanzee STLV to human.

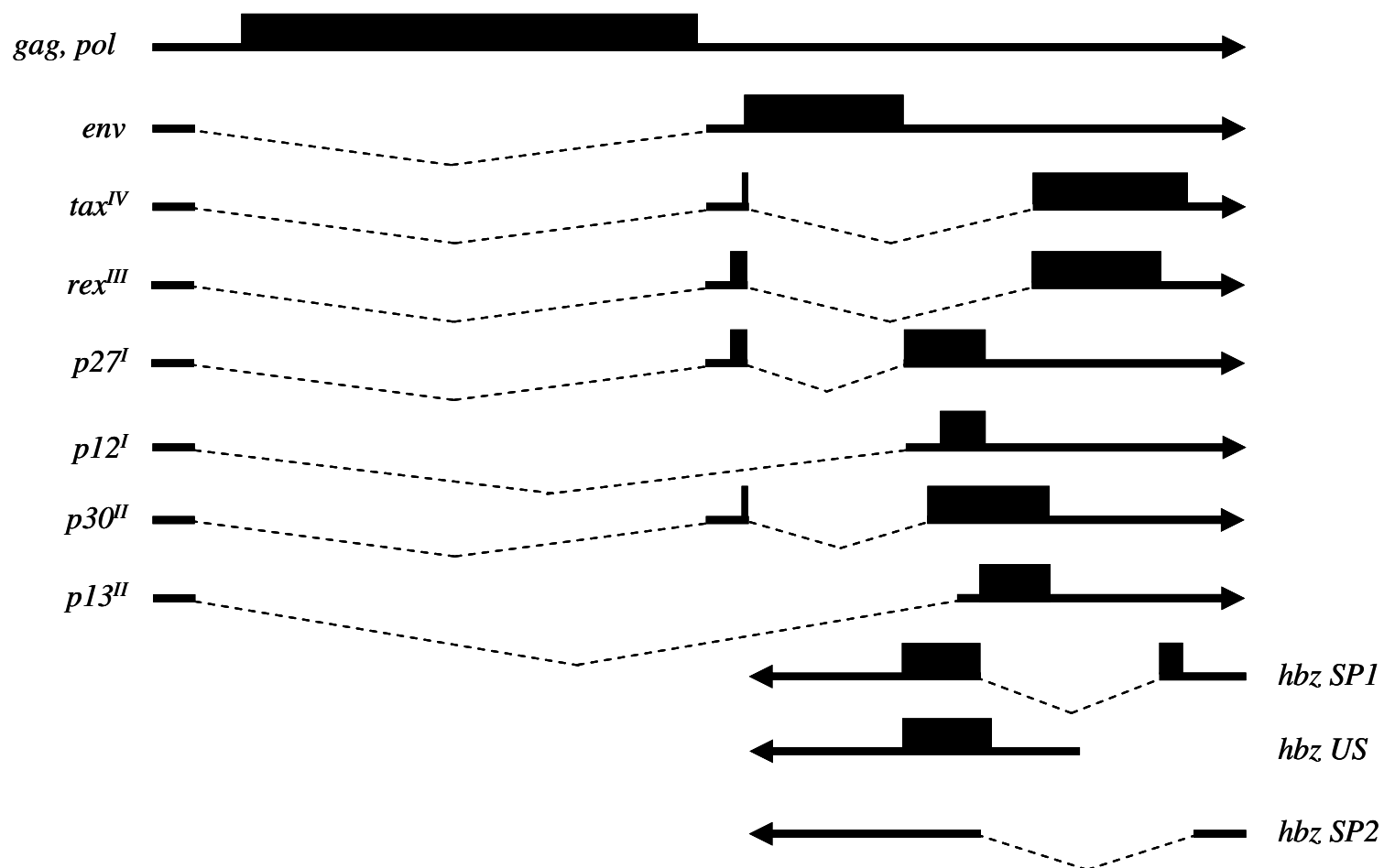
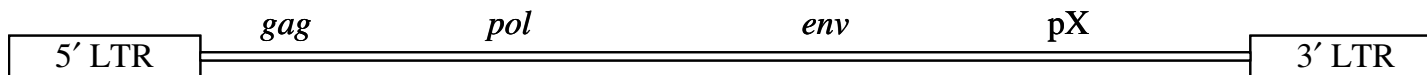
HTLV-1 GENOME

The HTLV-1 genome is 9 kb in length (Fig.1) and represents a typical replication competent complex retrovirus. The viral open reading frames (ORFs) are flanked by two long terminal repeats, the 5' and 3' LTRs, which contain the viral promoter and polyadenylation sequences, respectively. One unique feature of the HTLV-1 genome is that it also initiates transcription from the 3' LTR (Gaudray et al., 2002; Larocca et al., 1989).

The *gag* gene is transcribed and translated as a precursor to the internal structural proteins. It is proteolytically processed into the mature structural proteins capsid (CA), matrix (MA), and nucleocapsid (NC). The *pol* gene encodes the enzymes reverse transcriptase (RT), which has DNA polymerase and RNase H activities, and integrase (IN), which mediates replication of the genome. The *pro* gene encodes the viral protease (PR), which acts late in assembly and proteolytically processes the proteins encoded by *gag*, *pro*, and *pol*. The *env* gene is a precursor to envelope glycoproteins; it encodes the surface (SU) and transmembrane (TM) glycoproteins.

Another unique aspect of the HTLV-1 genome is its pX region, located between the *env* gene and the 3' LTR. It consists of four alternatively spliced ORFs (I to IV) on the sense strand, and two alternatively spliced ORFs on the antisense strand, which encode regulatory and accessory proteins. ORFs III and IV are translated from the same doubly spliced transcript, and encode the Rex and Tax proteins, respectively. Rex is involved in the nuclear export of unspliced or singly spliced viral RNA (Heger et al., 1999; Hidaka et al., 1988), while Tax transactivates the 5' LTR and up-regulates

Fig. 1. Schematic representation of the HTLV-1 genome. The viral mRNAs, their direction of transcription, and location of the open reading frames (black boxes) are shown. The dotted lines represent the introns.

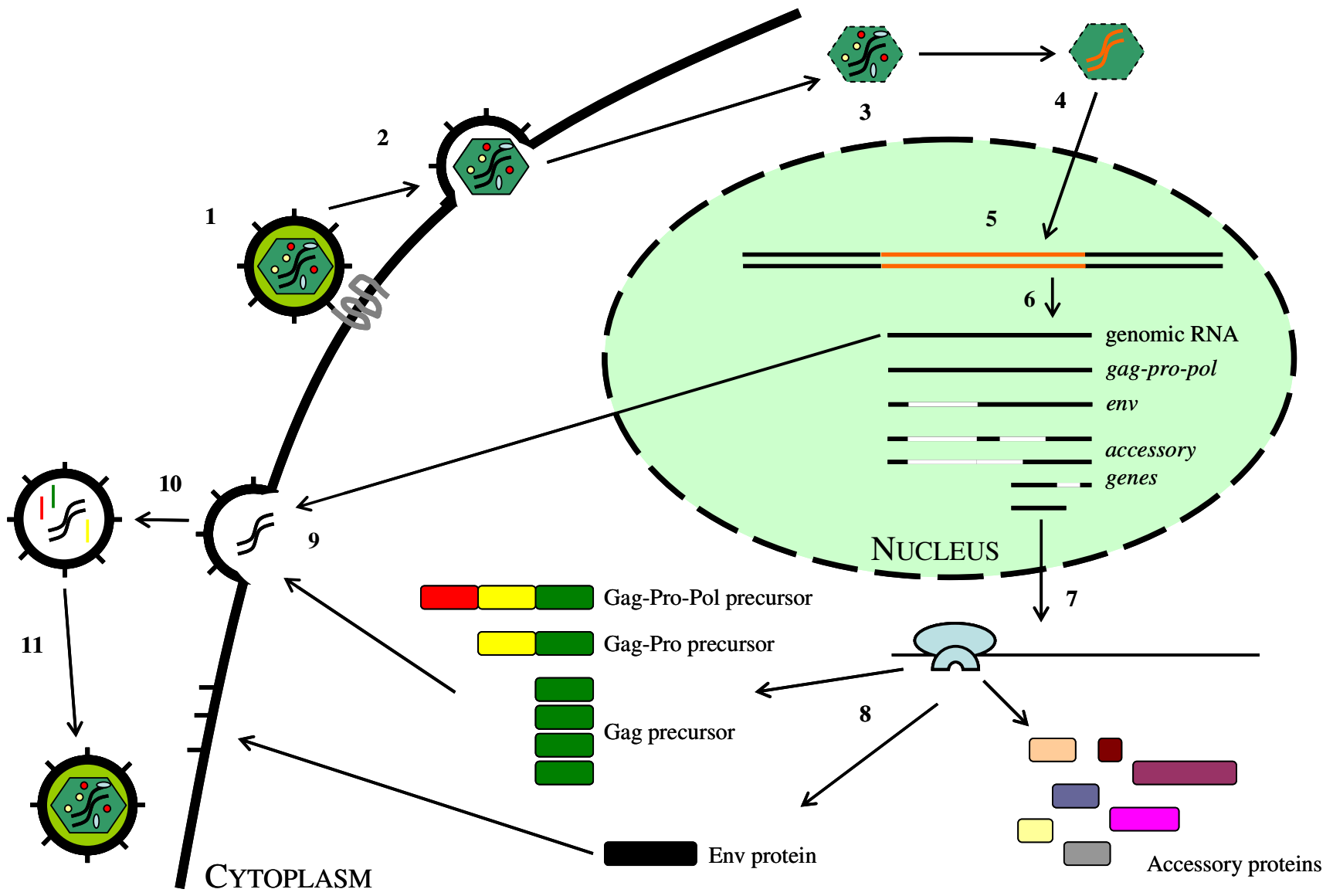


transcription of many cellular genes involved in host cell proliferation (Giebler et al., 1997; Pise-Masison et al., 2001; Yin and Gaynor, 1996). ORF III also encodes a singly spliced truncated form of Rex, p21^{Rex}, which is less well characterized. ORFs I and II produce alternatively spliced mRNA, which encode four accessory genes – p12^I, p27^I, p13^{II}, and p30^{II} (Berneman et al., 1992; Ciminale et al., 1996; Furukawa and Shiku, 1991; Koralnik et al., 1992). The antisense strand transcribes the HTLV-1 bZIP factor, *hbz* (Gaudray et al., 2002). Three alternatively spliced isoforms of *hbz* exist – *hbz SP1* and *hbz SP2* are spliced, while *hbz US* is unspliced (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006). *hbz SP1* and *hbz US* encode proteins that are >95% identical, differing only in the first seven amino acids. These accessory genes are critical for viral infectivity, maintenance of high viral loads, regulation of viral transcription, and viral pathogenesis (Albrecht et al., 2000; Albrecht and Lairmore, 2002; Bartoe et al., 2000; Ciminale et al., 1999; Collins et al., 1998; Lairmore et al., 2000; Matsuoka and Green, 2009; Nicot et al., 2004; Nicot et al., 2001; Satou and Matsuoka, 2007; Zhang et al., 2001; Zhang et al., 2000).

HTLV-1 REPLICATION CYCLE

The HTLV-1 replication cycle (Fig. 2) potentially begins with the binding of the viral envelope (Env) to the host cell receptor(s)/attachment factor(s), which has not been conclusively identified yet. This interaction is thought to trigger a conformational change in Env, which subsequently mediates its fusion to the host cell plasma membrane. Fusion leads to the release of the viral core into the cytoplasm, where its RNA genome is reverse

Fig. 2. HTLV-1 replication cycle. After binding to the host cell receptor, which is still unidentified (1), the viral membrane fuses with the plasma membrane (2), and the viral core is released into the cytoplasm, where it is uncoated (3). The viral RNA genome is reverse transcribed (4), and the viral DNA is integrated into the host genome (5), forming the provirus. The viral genome is transcribed (6) and the unspliced and spliced transcripts are translocated to the cytoplasm (7). Translation of the various transcripts (8) produces the Gag, Gag-Pro, and Gag-Pro-Pol precursor proteins, the Env protein, and accessory proteins. The Gag precursor proteins, Env protein, and viral genome assemble at the plasma membrane (9). Budding occurs, and immature viral particles are released (10). Viral maturation occurs during budding, when the Gag precursors are processed by the viral protease (11). See text for more details.



transcribed to DNA by the viral reverse transcriptase. The DNA copy of the viral genome is then integrated into the host DNA by the viral integrase, forming the provirus, which is the template for transcription of the viral genome. By integrating into the genome of the host cell, the virus is able to take advantage of the host cell machinery to replicate its genome. A number of different transcripts are generated from the provirus, including an unspliced, full-length copy of the viral genome, which (i) serves to encode the viral Gag, Gag-Pro, and Gag-Pro-Pol precursors, and (ii) is encapsidated as the viral genome in progeny particles. Singly spliced transcripts, encoding the Env precursor, and doubly spliced transcripts, encoding the regulatory and accessory proteins, are also generated. The doubly spliced transcripts are readily exported out of the nucleus, while the unspliced and singly spliced transcripts require Rex to bind to their Rex response elements, RxRE, to promote their export. Once in the cytoplasm, these transcripts are translated into their precursor forms, or regulatory/accessory proteins. The Gag, Gag-Pro, Gag-Pro-Pol, and Env proteins then come together at a common site at the cell membrane, and assemble into viral particles. Upon budding from the plasma membrane, these particles are considered immature, as they have not been processed yet. Maturation of the virion occurs through the activation of the viral protease and cleavage of the Gag, Gag-Pro, and Gag-Pro-Pol precursors.

HTLV-1 ENTRY

The HTLV-1 envelope protein is synthesized as a 62-kDa precursor glycoprotein, that is cleaved and glycosylated in the endoplasmic reticulum and Golgi (Paine, Gu, and

Ratner, 1994; Pique, Tursz, and Dokhelar, 1990). It is expressed on the surface of infected cells; during the budding process, the viral particles at the cell surface egress and are coated with an envelope that consists of a plasma-membrane derived lipid bilayer and envelope glycoproteins (Env). Env is composed of a 46-kDa surface amino terminal protein, gp46 (SU), and a 21-kDa transmembrane carboxy terminal component, gp21 (TM). SU is entirely extracellular and has four N-glycosylation sites (Delamarre et al., 1996); TM is membrane-anchored, has one N-glycosylation site, and like all retroviral TM, has an amino terminal fusion peptide.

Productive retroviral infections, leading to the formation of viral progeny, depend on interactions between Env and specific cellular receptors present on the cell surface. Analogous to other non-lentiviral retroviruses, it is assumed that an amino terminal receptor binding domain (RBD) in the SU binds to a cell surface receptor, leading to conformational changes and activation of the TM fusion peptide, which initiates membrane fusion (Manel et al., 2005; Weiss et al., 1985).

Although HTLV-1 was discovered 30 years ago, its receptor is still unknown; evidence suggests that the PTLVs have a common receptor (Clapham, Nagy, and Weiss, 1984; Sommerfelt and Weiss, 1990), which is widely expressed (Jassal, Pohler, and Brighty, 2001; Trejo and Ratner, 2000). However, attempts to discover the receptor have been hindered by several factors: (1) the broad *in vitro* tropism has prevented studies from pinpointing a specific factor; (2) Env is highly fusogenic and toxic, leading to a rampant syncytial effect, which makes it challenging to use as a tool in fusion studies; and (3) it is difficult to produce high titers of cell-free virions to use in binding studies.

Despite the difficulties, a host of proteins have been proposed as the receptor for HTLV-1 or an adhesion molecule that promotes infection.

Early reports, based on human-mouse somatic cell hybrids, suggested that the HTLV-1 receptor was encoded on chromosome 17 (Gavalchin et al., 1995; Sommerfelt et al., 1988). However, this was based on the assumption that mouse cells were resistant to HTLV-1; in fact, mouse cells have been shown to be susceptible, albeit poorly, to HTLV-1 (Denesvre et al., 1995; Jassal, Pohler, and Brighty, 2001; Trejo and Ratner, 2000). Before 2003, other factors that had been proposed as the HTLV-1 receptor, or to be involved in viral entry, included the tetraspanins C33 and CD82 (Imai et al., 1992; Pique et al., 2000a), which interact with membrane components; vascular cell adhesion molecule-1 (VCAM-1) (Hildreth, Subramaniam, and Hampton, 1997), which mediates HTLV-1-induced syncytium formation, which can be blocked by anti-gp46 antibodies; intercellular adhesion molecules (ICAMs) (Al-Fahim et al., 1999; Daenke and Booth, 2000; Daenke, McCracken, and Booth, 1999; Uchiyama, Ishikawa, and Imura, 1996); glycosylphosphatidylinositol (GPI)-linked proteins (Niyogi and Hildreth, 2001) and class II histocompatibility (MHC II) molecules (Hildreth, 1998), probably due to their association with the cholesterol in lipid rafts, which has been shown to be important post-binding (Wielgosz et al., 2005); and the heat shock cognate protein HSC70 and phosphatidylglycerol (Sagara et al., 2001; Sagara et al., 1998), which participate in syncytium formation.

In 2003, heparan sulfate proteoglycans (HSPGs) were shown to influence HTLV-1 Env-mediated binding and viral entry (Okuma et al., 2003; Pinon et al., 2003). HSPGs are a type of glycosaminoglycan consisting of a core protein with heparan sulfate

polysaccharide chains and have been implicated in the binding and receptor-mediated entry of numerous viruses, including a number of herpes simplex viruses, flaviviruses, picornaviruses, and HIV (Bobardt et al., 2003; Spillmann, 2001). At around the same time, glucose transporter 1 (GLUT-1) was identified as the receptor of HTLV, based on the observation that cells transfected with HTLV-1 or -2 Env blocked acidification of the media by altering lactate production and glucose transport (Manel et al., 2003). However, the role of GLUT-1 as the receptor has been questioned since then, as one study showed evidence that activated CD4⁺ T cells that were susceptible to HTLV-1 infection, expressed undetectable levels of surface GLUT-1 (Takenouchi et al., 2007). However, they did suggest that GLUT-1 may be required in conjunction with other factors, as it did enhance HTLV-1 cell-to-cell transmission. In 2005, HSPGs were implicated once again, as a study showed that they mediated HTLV-1 attachment and entry into CD4⁺ T cells (Jones et al., 2005). Another protein that has been shown to influence HTLV-1 entry is the coreceptor for certain isoforms of vascular endothelial growth factor (VEGF), neuropilin-1 (NRP-1). Evidence suggests that HTLV-1 uses HSPGs and NRP-1 for entry (Lambert et al., 2009) and GLUT-1 and NRP-1, either individually or collectively, to infect a variety of cell types (Ghez et al., 2006). The most recent study analyzed these three factors, GLUT-1, NRP-1, and HSPGs, and suggested that a fourth molecule, DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN), plays a critical role in HTLV-1 binding, transmission, and infection (Jain et al., 2009).

Taken together, it is evident that these factors enhance entry and/or cell-to-cell transmission of HTLV-1, but are not the receptor for HTLV-1. It is possible that they

work cooperatively, and that they may even form a complex, as evidenced by Ghez et al. (2006). Chapter 2 summarizes our efforts to identify the HTLV-1 receptor/attachment factor using an unbiased functional cDNA library screen.

HTLV-1 TRANSMISSION

HTLV-1 is transmitted by three routes: vertical – from mother to child, predominantly through breastfeeding, horizontal – via sexual intercourse, and parenteral – via infected blood transfusions or the sharing of needles and syringes (Manns, Hisada, and La Grenade, 1999). Interestingly, physiological transmission requires live HTLV-1-infected cells, since fresh frozen plasma from sero-positive donors does not transmit HTLV-1 (Okochi, Sato, and Hinuma, 1984).

In vitro experiments have shown that transmission by cell-free virions is very inefficient; in fact, free virus is not detected in the serum of infected patients (Asquith and Bangham, 2008). Efficient transmission relies on direct contact between HTLV-1-infected and uninfected cells, polarization of the microtubule-organizing center (MTOC), which is triggered by the viral Tax protein, and formation of the virological synapse, which allows the spread of viral proteins and genomic RNA into the target T lymphocyte (Igakura et al., 2003; Majorovits et al., 2008). Like HIV, HTLV-1 can also efficiently infect dendritic cells, which can subsequently transmit the virus to CD4⁺ T-cells (Jones et al., 2008). To facilitate its transmission, HTLV-1 takes advantage of the pleiotropic functions of its proteins, especially Tax, to clonally expand the population of infected

cells (Gatza, Watt, and Marriott, 2003; Jeang et al., 2004; Matsuoka and Jeang, 2005; Yoshida, 2001).

HTLV-1 REGULATORY GENES

As the work conducted for this dissertation involves Tax, HBZ, and p30^{II}, the following sections will focus on those three factors, and not discuss the other regulatory genes – Rex, p12^I, p27^I, and p13^{II}. Rex is a RNA-binding post-transcriptional regulator that binds specifically to the Rex response element (RxRE), located at the 3' end of sense viral mRNAs. It promotes the nuclear export of unspliced and singly-spliced viral mRNAs and inhibits the splicing and export of doubly spliced mRNAs (Boxus and Willems, 2009; Kashanchi and Brady, 2005). The pX ORF I mRNA encodes two accessory proteins – the 152-amino-acid p27^I and the 99-amino-acid p12^I. Translation of p12^I is initiated at an internal methionine codon in the p27^I ORF. The p27^I protein is not very well studied, although evidence suggests that it is produced during the course of natural infection *in vivo* (Ciminale et al., 1996; Pique et al., 2000b). The p12^I protein is involved in a number of processes, which suggest that it is likely to promote T-cell proliferation and viral escape from immune surveillance (Fukumoto et al., 2009; Nicot et al., 2005; Nicot et al., 2001). The pX ORF II mRNA encodes the two accessory proteins p30^{II} and p13^{II}. Translation of p13^{II} is initiated at an internal methionine codon in the p30^{II} ORF. The p13^{II} protein localizes to the mitochondria and alters membrane potential, which causes an increase in the production of reactive oxygen species (ROS)

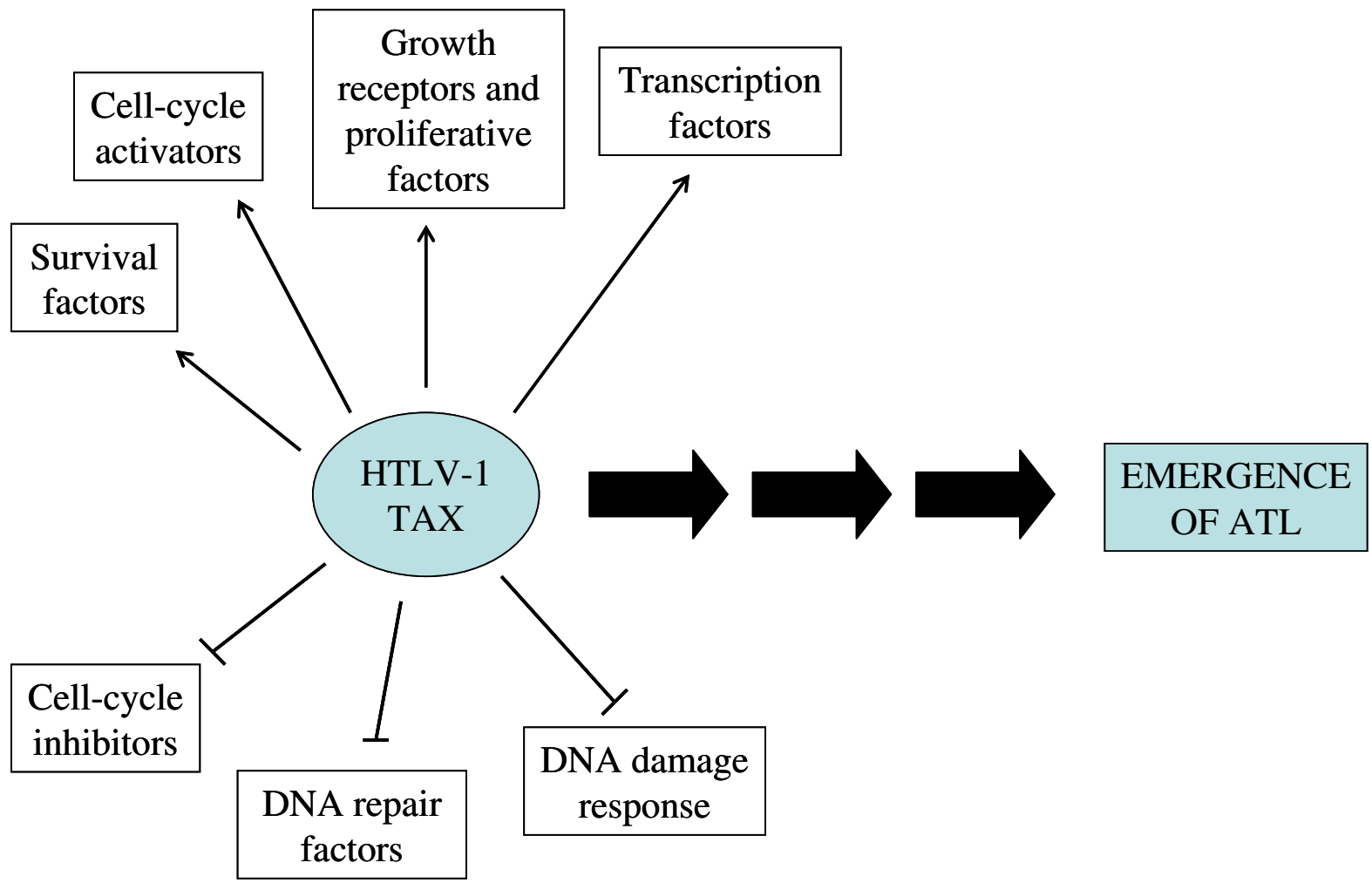
(Silic-Benussi et al., 2010). Additionally, it binds to farnesyl pyrophosphate synthase (FPPS), an enzyme required for the prenylation of Ras (Lefebvre et al., 2002).

TAX

Tax was initially discovered as a *trans*-activator of HTLV-1 gene expression (Felber et al., 1985). It activates the 5' LTR by interacting with three imperfect 21-bp repeats, called Tax responsive elements (TxRE), in the U3 region of the LTR (Brady et al., 1987). Each repeat contains an imperfect cyclic AMP response element (CRE) that is recognized by members of the CRE-binding protein/activating transcription factor (CREB/ATF) family. Tax cooperates with CREB-2 to enhance transcription of the HTLV-1 promoter (Gachon et al., 2000; Reddy et al., 1997).

Tax is also a major player in mediating viral persistence and disease development. The ultimate goal of HTLV-1 infection is the emergence of ATLL, and the virus uses Tax to this end. The ability of Tax to immortalize cells, and produce tumors in transgenic mouse models (Yoshida, 2001) (Boxus et al., 2008) supports this oncogenic potential. Tax is actively involved in numerous cellular functions (Fig. 3) – it activates cell signaling pathways, reprograms the cell cycle, interferes with checkpoint control and inhibits DNA repair – which helps it exert its functions (Boxus and Willems, 2009; Matsuoka and Jeang, 2007). Of the pathways activated by Tax, its roles in activating the CREB/ATF, nuclear factor- κ B (NF- κ B), and serum response factor (SRF) pathways (Azran, Schavinsky-Khrapunsky, and Aboud, 2004) are thought to be the most critical. Additionally, Tax has the ability to repress the function of p53

Fig. 3. Cellular activities modulated by Tax. The numerous cellular processes that Tax is involved in are diagrammed here. Tax stimulates survival factors, cell-cycle activators, growth receptors and proliferative factors, and transcription factors, and inhibits cell-cycle inhibitors, DNA repair factors, and the DNA damage response. The cumulative effect of the alterations of these processes is what eventually leads to the emergence of ATLL (Matsuoka and Jeang, 2007).



(Akagi et al., 1997; Gartenhaus and Wang, 1995; Reid et al., 1993; Yamato et al., 1993), stimulate transition of G1/S (Marriott and Semmes, 2005), interact with and degrade Rb, and modulate expression of cyclin-dependent kinase inhibitors (Grassmann, Aboud, and Jeang, 2005) all of which cooperate to ultimately lead to cellular transformation.

Due to its abilities in hijacking cellular functions to its advantage, Tax elicits a strong cytotoxic T-lymphocyte (CTL) response from the host immune surveillance machinery. However, to counteract this active immune response, Tax expression is diminished in transformed cells, as evidenced by the fact that there is no Tax expression in 60% of ATLL cases (Takeda et al., 2004). Three mechanisms for this decrease in expression have been described: (1) deletion of the viral promoter in the 5' LTR (Miyazaki et al., 2007; Tamiya et al., 1996); (2) DNA methylation of the 5' LTR, which leads to inactivation of the promoter (Koiwa et al., 2002; Taniguchi et al., 2005); and (3) genetic mutations (nonsense mutations, deletions, and insertions) of the *tax* gene (Furukawa et al., 2001; Takeda et al., 2004). Analyses of the ATLL cells showed that the 3' LTR remains intact and is not methylated. In addition, the pX region of HTLV-1 is also maintained (Matsuoka and Green, 2009). Interestingly, *hbz* is expressed from the 3' LTR. Consequently, detailed analyses uncovered that all ATLL patients express the *hbz* gene, irrespective of their Tax expression levels, implicating a role for *hbz* in the development of ATLL.

HBZ

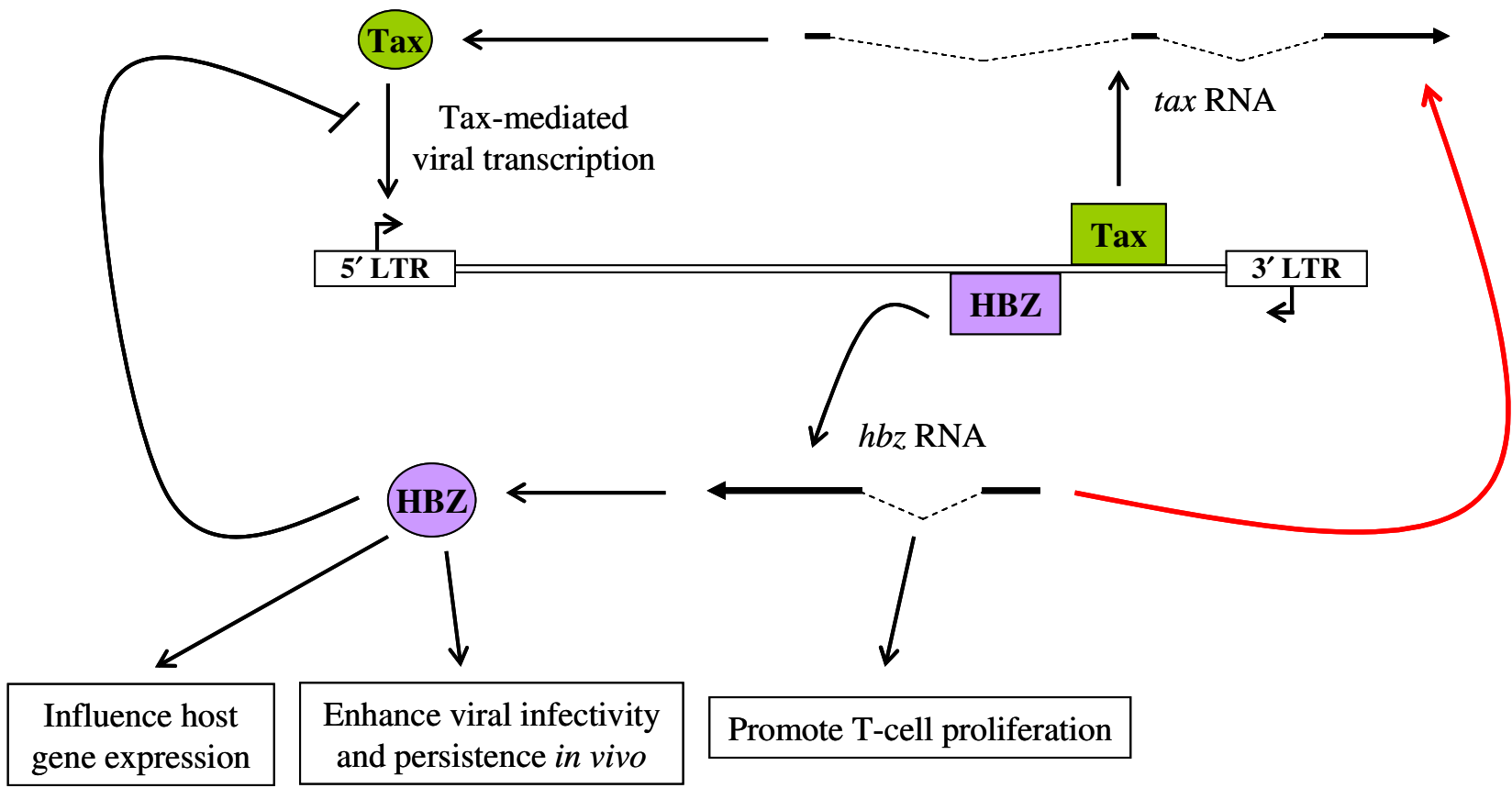
Transcription from the minus strand of the HTLV-1 genome was first reported in 1989 (Larocca et al., 1989). The authors discovered a functional promoter on the anti-sense strand and showed that minus strand RNA is transcribed in HTLV-1-infected T-cells. However, it was not until 2002 that a gene product encoded by the minus strand was characterized (Gaudray et al., 2002). The authors identified this viral protein as a binding partner to CREB-2, through a yeast two-hybrid screen. As the proteins associated through their respective basic leucine zipper (bZIP) domains, the authors designated it the HTLV-1 bZIP factor, or HBZ. Using 5' and 3' rapid amplification of cDNA ends (RACE), they identified three *hbz* transcripts, generated by alternative splicing, of which two are spliced (*hbz SP1* and *SP2*) and one is unspliced (*hbz US*) (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006). Due to the low levels of expression of *hbz SP2*, studies have focused on *hbz SP1* and *hbz US*. The promoter regions of *hbz SP1* and *hbz US* have been identified and both promoters are TATA-less (Yoshida et al., 2008). *hbz SP1* has multiple transcriptional initiation sites in the R region of the 3' LTR (Cavanagh et al., 2006; Yoshida et al., 2008), whereas *hbz US* has one initiation site, in the intronic region of *hbz SP1* (Yoshida et al., 2008). The transcription factor Sp1 has been shown to be critical for expression of *hbz SP1* (Yoshida et al., 2008), and the TRE motif in the 3' LTR has been shown to enhance transcription of the *hbz* transcripts (Landry et al., 2009; Yoshida et al., 2008). However, the enhancing activity of the TRE motif in the 5' LTR is much stronger than that seen in the 3' LTR.

The HBZ SP1 and HBZ US proteins are 206 and 209 amino-acids long, respectively. They share >95% amino acid sequence identity, and differ only at their N-termini. Both HBZ SP1 and HBZ US have been shown to down-regulate Tax-mediated LTR transcription (Arnold et al., 2006; Gaudray et al., 2002) by interacting with CREB-2, via its bZIP domain, thus preventing it from binding to the viral CRE. HBZ US has been shown to interact with other proteins, including the transcription factors JunB, c-Jun, and JunD, through its bZIP domain. It dimerizes with JunB and c-Jun, decreasing their DNA-binding activity and impairing the stability of c-Jun (Basbous et al., 2003; Matsumoto et al., 2005). In contrast, the interaction between HBZ US and JunD leads to activation of JunD-dependent transcription (Thebault et al., 2004). Interestingly, HBZ SP1 has also been shown to associate with JunD, and activate human telomerase reverse transcriptase (hTERT) (Kuhlmann et al., 2007). Since activation of telomerase is important in the development and progression of ATLL (Uchida et al., 1999), this suggests that HBZ may contribute to the development and maintenance of the leukemic process. HBZ SP1 and HBZ US suppress the classical NF- κ B pathway by interacting with p65 and inducing its degradation (Zhao et al., 2009). In addition to its bZIP domain, HBZ contains an N-terminal activation domain, two basic regions, and a DNA-binding domain. The central regions have been shown to contain three distinct nuclear localization signals, at least two of which are needed for nuclear localization (Hivin et al., 2005). HBZ US accumulates in nuclear speckles, and was found to localize to heterochromatin (Hivin et al., 2005), whereas HBZ SP1 was found to localize to nucleoli (Murata et al., 2006).

A number of studies, carried out with more physiologically relevant methods, revealed an interesting dual functionality of HBZ SP1. In one study, the authors generated HTLV-1 HBZ SP1 mutants in the context of a molecular clone and evaluated them for viral gene expression, protein production, and immortalization capacity. *In vitro*, the biological properties of the HBZ SP1 mutants were indistinguishable from wild-type HTLV-1. On the other hand, rabbits inoculated with these mutants became persistently infected. However, compared to wild-type infected rabbits, the mutants exhibited reduced proviral copy numbers and a decreased antibody response to viral gene products. These data suggest that HBZ SP1 is dispensable for viral replication and cellular immortalization *in vitro*, but enhances infectivity and persistence of HTLV-1 *in vivo* (Arnold et al., 2006). In another study, the authors used short interfering RNAs (siRNAs) to suppress *hbz* transcription, and found that this inhibited proliferation of ATLL cells. In addition, T-cell lines transfected with mutated *hbz* genes indicated that *hbz* promotes T-cell proliferation in its RNA form (Satou et al., 2006). The authors also defined a stem-loop structure in the first 210bp of *hbz* RNA, and showed that it was essential for the growth promoting activity of *hbz*. Furthermore, they generated *hbz* transgenic mice under the control of the CD4 promoter, and found that the percentage of CD4⁺ T-cells was elevated in splenocytes. These data indicate that *hbz* plays a critical role in HTLV-1-mediated oncogenesis, even in the absence of *tax*. Furthermore, it establishes a bimodal function for *hbz* (Fig. 4).

All tested ATLL and HTLV-1-infected cell-lines, infected cells freshly isolated from ATLL patients, and peripheral blood mononuclear cells (PMBCs) isolated from asymptomatic HTLV-1 carriers express *hbz* transcripts, with *hbz SP1* being the most

Fig. 4. Functions of HBZ. The various processes that HBZ is involved in are depicted here. The boxes represent where the genes are located in the HTLV-1 genome, while the circles represent the corresponding proteins. The arrows on the LTRs depict the direction of transcription. The Tax protein up-regulates viral transcription from the 5' LTR. The HBZ protein has been shown to inhibit this process, and down-regulate Tax-mediated viral transcription. Furthermore, it can influence host gene expression and enhance viral infectivity and persistence *in vivo*. The *hbz* RNA has been shown to promote T-cell proliferation (Matsuoka and Green, 2009). In work conducted for this dissertation (Chapter 3), a novel function of *hbz* was discovered. The *hbz* RNA was shown to promote *tax* expression (red arrow). See text for more details.



abundant transcript (Satou et al., 2006; Usui et al., 2008). However, prior to this finding, HTLV-1 viral genes were virtually undetectable in ATLL patients. Due to this finding, and the fact that the 3' LTR is present and unmodified in ATLL and HTLV-1-infected cells, *hbz* is considered indispensable for the development of ATLL. Furthermore, a recent study reported a correlation between the levels of *hbz* transcript and the severity of HAM/TSP, suggesting that expression of *hbz* contributes to the pathogenesis of HAM/TSP (Saito et al., 2009). Taken together, it is likely that the *hbz* gene is an important player in HTLV-1 infection, and is therefore a potential target for therapy of both ATLL and HAM/TSP.

p30

p30^{II} is a nucleolar protein shown to be important in maintaining high viral loads in rabbits inoculated with the ACH molecular clone (Bartoe et al., 2000). It is a negative regulator of viral expression as it selectively retains the doubly spliced *tax/rex* mRNA in the nucleus, preventing its export into the cytoplasm, and thereby decreasing Tax protein levels (Nicot et al., 2004). The specific mechanism by which it keeps the mRNA in the nucleus is still unknown. The current model of p30^{II} activity suggests that it may be involved in preventing immune recognition during viral replication, as it reduces expression of Tax. This is supported by *in vivo* evidence that abrogation of p30^{II} prevented the establishment of persistent infection and resulted in lower viral loads (Silverman et al., 2004).

The p30^{II} protein is also thought to function as a transcription factor as it affects the levels of transcription from CREB- and Tax-responsive elements (CREs, TxREs) (Zhang et al., 2001; Zhang et al., 2000). Additionally, Affymetrix microarray gene expression analyses of Jurkat cells expressing stably transduced p30^{II} indicated that p30^{II} repressed genes involved in cellular gene expression, and enhanced expression of regulatory factors (Michael et al., 2004). Taken together, these data suggest that p30^{II} plays an important role in HTLV-1 infection.

HTLV-1 ANTIVIRAL THERAPY

Currently, no effective antiviral therapies exist to treat HTLV-1. Numerous factors have been tested and shown to be effective in inhibiting HTLV-1 replication, but none have shown sustained efficacy. HTLV-1 replication is sensitive to IFN- α , which has been shown to prevent Gag association with lipid rafts at the plasma membrane, and hence prevent viral assembly (Feng, Heyden, and Ratner, 2003). Nucleoside analogs, such as zidovudine or AZT and lamivudine, have also been tested alone or in combination with IFN- α (Gill et al., 1995; Gout et al., 1991; Sheremata et al., 1993; Taylor et al., 1999), and though the treatments have shown promise, relapses occur in the majority of individuals after discontinuation of therapy. Other nucleoside reverse transcriptase inhibitors (NRTIs) that have been shown to inhibit HTLV-1 replication, in culture, are tenofovir, abacavir, zalcitabine, and stavudine (Hill et al., 2003). Antiviral therapies effective against the human immunodeficiency virus type 1 (HIV-1) have been tested against HTLV-1. However, non-nucleoside analogs and protease inhibitors, that

are active against HIV-1, are inactive at inhibiting HTLV-1 replication (Pettit et al., 1998; Ratner, 2004). Since inhibitors targeting the HIV-1 co-receptors have recently been described (Schols, 2006), the work conducted for this dissertation (Chapter 2) becomes even more important, as it could potentially lead to antiviral therapy targets. Work currently being carried with inhibitors to HIV-1 integrase in the Ratner laboratory, by M. E. Seegulam, shows that they are effective against HTLV-1 integrase. Recently, a plant derived antiviral protein, pokeweed antiviral protein (PAP), was shown to efficiently suppress HTLV-1 gene expression (Mansouri et al., 2009). PAP depurinated HTLV-1 genomic RNA and decreased its translational efficiency. Furthermore, due to the decrease in Tax translation, less viral transcription from the viral promoter occurred. (I was co-author on this paper, and contributed to Figures 1B and 1C). Taken together, it is clear that numerous antiviral factors exist and it only remains to be seen if any can be used to effectively treat HTLV-1-associated diseases.

HTLV-1 MOLECULAR CLONE

The first functional molecular clone of HTLV-1 was generated in the Ratner laboratory (Kimata et al., 1994); since then, two additional molecular clones have been described (Derse et al., 1995; Zhao et al., 1995). Since the clone constructed by Kimata et al. (1994) contains a viral genome that is a chimera of two HTLV-1 strains, ATK and CH, it was designated ACH. Upon transfection, the ACH molecular clone produces viral particles; these particles are infectious *in vitro* and *in vivo* in rabbits, and are capable of immortalizing primary CD4⁺ T lymphocytes (Collins et al., 1996; Kimata et al., 1994).

The availability of a functional molecular clone facilitated the work conducted for this dissertation (Chapter 3), as it provided the opportunity to study the role of the HTLV-1 antisense strand in the context of the whole virus. Specifically, the ACH molecular clone was used to examine the effect of the novel viral gene, *hbz*, on other viral proteins, and its role in viral replication. This study has provided new insights into the complex interplay of viral regulatory proteins in HTLV-1 gene expression.

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Chapter 2

A Functional Retroviral cDNA Library Screen to Identify the Receptor/Attachment Factor for HTLV-1

Preface to Chapter 2

Screen 1 described in this chapter was carried out in its entirety by G.C. Screen 2

described in this chapter was carried out by the following: G.C., Dan Rauch, Intelly Lee,
and Angelique Cerceilleux.

ABSTRACT

The receptor for human T-cell leukemia virus type 1 (HTLV-1) is still unknown. Since viral envelope-receptor interactions are important in determining viral tropism, and the expression of a receptor on specific cells can determine the route of viral entry, the pattern of viral spread, and the resulting pathogenesis, it is vital to find the receptor for HTLV-1. We designed a retroviral cDNA library screen in which we introduced the cDNA from the permissive HeLa S3 cell line into the poorly susceptible NIH 3T3 cell line. The NIH 3T3 cells were selected after infection with HTLV-1 Env pseudotyped viral particles, carrying a drug resistance gene. We isolated approximately 460 cDNAs, which were sequenced and subjected to sequence analysis. Of these, 20 were selected as potential, high-priority HTLV-1 receptor candidates, based on the number of times they were identified, whether they were membrane proteins, and whether they were previously identified as HIV-dependency factors. These candidates are currently being tested to determine if any are the receptor for HTLV-1.

INTRODUCTION

Human T cell leukemia virus type 1 (HTLV-1) is a complex retrovirus belonging to the Deltaretrovirus family. It is widespread and the etiologic agent of adult T cell leukemia/lymphoma (ATLL) (Hinuma et al., 1981; Poiesz et al., 1980), a malignancy of CD4⁺ T lymphocytes, and a chronic neurological disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Bhagavati et al., 1988; Gessain et al., 1985; Osame et al., 1987). In individuals exposed to HTLV-1, those with high virus loads are at the greatest risk of developing this disease. *In vivo*, HTLV-1 primarily infects CD4⁺ T cells, though other hematopoietic cells (CD8⁺ T cells, B lymphocytes, monocytes/macrophages, dendritic cells, and megakaryocytes) and glial cells (astrocytes and microglial cells) have been shown to be infected (Eiraku et al., 1998; Grant et al., 2002; Hanon et al., 2000; Koyanagi et al., 1993; Lehky et al., 1995; Levin et al., 1997; Macatonia et al., 1992; Nagai et al., 2001). *In vitro*, however, the HTLV-1 envelope (Env) has a much broader tropism (Jassal, Pohler, and Brighty, 2001; Trejo and Ratner, 2000).

To date, no molecule has been definitively identified as the receptor utilized by HTLV-1 to enter and infect vertebrate cells. The broad *in vitro* tropism is one of the reasons the search for the receptor has been challenging. The fact that the HTLV-1 Env is highly fusogenic and cytotoxic, coupled with the technical difficulty of producing high titers of cell-free virus, has complicated efforts in identifying the receptor. Over the years, several candidate proteins have been proposed as the receptor for HTLV-1 or an adhesion molecule that promotes infection, including interleukin-2 receptor α (Lando et

al., 1983), heat shock protein Hsc70 (Sagara et al., 1998), vascular cell adhesion molecule-1 (Hildreth, Subramaniam, and Hampton, 1997), tetraspanin C33 (Imai et al., 1992), and an 80-kDa glycoprotein analog of intercellular adhesion molecule-3 (Agadjanyan et al., 1994). However, the *in vitro* tropism of HTLV-1 Env pseudotyped HIV-1, and the binding pattern of soluble HTLV-1 Env did not support the role of these proteins as receptors for the virus (Jassal et al., 2001; Jassal, Pohler, and Brighty, 2001; Trejo and Ratner, 2000). Recent studies from several laboratories have identified molecules on the cell surface that may enhance HTLV-1 entry, but their roles in enhancing infection are unclear.

Manel et al. identified glucose transporter 1 (GLUT-1) as the HTLV receptor based on their observation that cells transfected with HTLV-1 or -2 Env blocked acidification of the media by altering lactate production and glucose transport (Manel et al., 2003). However, the data presented did not conclusively establish GLUT-1 as the receptor for HTLV-1, since (i) most of the data presented were based on HTLV-2, and (ii) the studies with HTLV-1 did not show a significant increase in binding and infection in the presence of GLUT-1. Furthermore, infection studies carried out in our lab using GLUT-1 as the receptor, showed no more than a 40-fold increase in infection of a non-susceptible cell-line (compared to a more than 8000-fold increase in infection of a permissive cell-line). A subsequent paper similarly questioned the role of GLUT-1 as the primary binding receptor for HTLV-1 by showing that activated CD4⁺ T cells, that were susceptible to HTLV-1 infection, expressed undetectable levels of surface GLUT-1 (Takenouchi et al., 2007). However, they did report that GLUT-1 enhanced HTLV-1 cell-to-cell transmission, leading to the theory that other factors may be required, in

conjunction with GLUT-1, for efficient Env-binding and fusion. This led to a number of studies, with a diverse set of conclusions.

One paper showed that heparan sulfate proteoglycans (HSPGs) mediated attachment and entry of HTLV-1 into CD4⁺ T cells (Jones et al., 2005), while another paper showed that HTLV-1 uses HSPGs and neuropilin-1 (NRP-1), a co-receptor for certain isoforms of vascular endothelial growth factor (VEGF), for entry (Lambert et al., 2009). Two other studies presented data that suggested that HTLV-1 uses both NRP-1 and GLUT-1, individually or collectively, to infect a variety of cell types (Ghez et al., 2006; Jin et al., 2009). The most recent study analyzed potential HTLV-1 receptors (Sagara et al., 1996) and suggested that a fourth molecule, DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN), plays a critical role in HTLV-1 binding, transmission, and infection (Jain et al., 2009). It is evident from this plethora of data that there are a number of factors that work to enhance HTLV-1 binding and transmission, but it is unclear if any serve as the receptor for HTLV-1.

To approach this problem systematically, we designed a retroviral cDNA library screen that introduces cDNA from highly susceptible HeLa S3 cells into the poorly susceptible murine NIH 3T3 cells. We identified approximately 460 cDNAs from this screen, of which 20 were selected as potential, high-priority HTLV-1 receptor candidates.

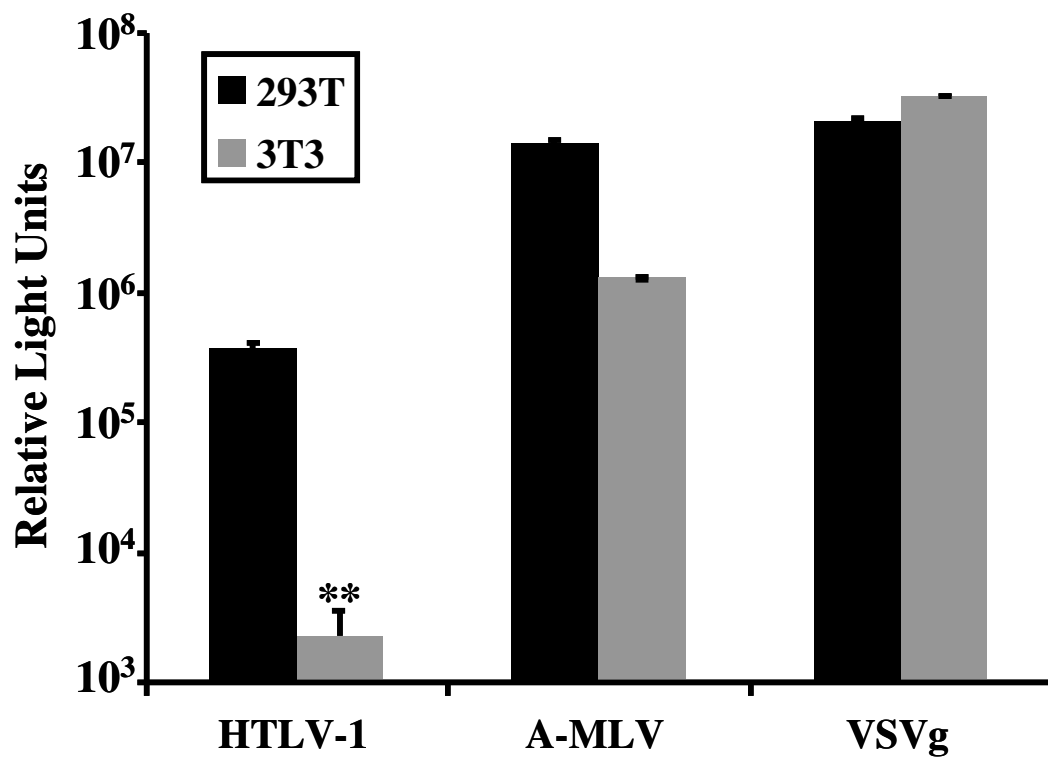
RESULTS

The retroviral cDNA library screen

We developed a retroviral cDNA library screen to detect proteins that could potentially serve as the receptor(s), attachment factor(s), or entry-enhancing molecule(s) for HTLV-1. The premise of the screen was to introduce a cDNA library from cells highly susceptible to HTLV-1 infection into poorly infectious cells, infect the resulting cDNA-expressing cells with HTLV-1 pseudotyped viral particles, select for cells that permitted infection, and isolate any cDNAs expressed in those cells. Based on previous work (Jassal, Pohler, and Brighty, 2001; Trejo and Ratner, 2000), and current studies (Fig. 1), we used the murine NIH 3T3 cells as the poorly susceptible cell line. We generated HTLV-1 Env, A-MLV Env, and VSVg pseudotyped HIV-luc particles and infected 293T and NIH 3T3 cells. Two days later we carried out luciferase assays on these cells and determined the level of entry, based on luciferase levels. As shown in Fig. 1, 293T cells were readily infected by all three envelopes, whereas the NIH 3T3 cells were permissive to the A-MLV Env and VSVg, and poorly susceptible to the HTLV-1 Env.

On our first attempt, we used a retroviral cDNA library from human brain. We isolated 240 cDNA-infected colonies and 120 background colonies. All 360 colonies were expanded and their susceptibility to HTLV-1 Env tested with HIV-luc (HTLV-1). Based on the luciferase data, we selected seven potential candidate cell-lines. The cDNAs expressed in these cell-lines were purified, sequenced, and cloned. Since each

Fig.1. NIH 3T3 cells are poorly susceptible to HTLV-1 Env infection. 293T and NIH 3T3 cells were infected with HIV-luc particles, pseudotyped with the HTLV-1 Env, the A-MLV Env, or VSVg. Forty-eight hours post-infection the cells were lysed and subjected to a luciferase assay. Background activity (non-infected cells) was subtracted. Student's *t* tests were performed to determine significant differences between samples (**, $P < 0.01$).



cell-line only expressed one cDNA, this resulted in seven candidate clones, representing the following cDNAs: hemoglobin alpha 1/2, gelsolin (clones 1 and 2), ribosomal protein L13a, structural maintenance of chromosome 5 (SMC5, clones 1 and 2), and transmembrane protein 208 (TMEM208/HSPC171). These candidate clones, a GLUT-1 cDNA clone, and the empty cloning vector, pIRES2-EGFP, were stably transfected into NIH 3T3 cells, and the resulting cell-lines infected with HIV-luc (HTLV-1) (Fig. 2). We infected the NIH3T3.GLUT-1 cells to test the effectiveness of GLUT-1 as the HTLV-1 receptor; although GLUT-1 increased entry by 40-fold, this was nowhere near the 8000-fold increase seen with infection of 293T cells (positive control), suggesting that GLUT-1 may be necessary, but is not sufficient, for HTLV-1 entry. Infection of the NIH3T3.pIRES-EGFP and NIH 3T3 cells served as negative controls. Furthermore, though we did not see a significant increase in infectivity with any of the candidates, we were able to show that a complete screen of this nature could be carried out, albeit with optimizations.

On our second attempt, we used a retroviral cDNA library from a cell-line known to be permissive to HTLV-1 infection, HeLa S3 (Jassal, Pohler, and Brighty, 2001; Li et al., 1996; Trejo and Ratner, 2000). We generated retroviral cDNA library particles (Fig. 3), infected NIH 3T3 cells, and after adding virus, selected for two weeks. In all, we counted more than 320 colonies, while there were less than five colonies on the negative plate; 114 cell-lines were successfully expanded. Since most of the colonies contained multiple plasmids, we amplified approximately 460 cDNAs from these cell lines. Each cDNA was purified and subjected to sequence analysis

Fig. 2. Candidates do not cause an increase in HTLV-1 Env infection. The candidate clones were infected with HIV-luc (HTLV-1) particles. Forty-eight hours post-infection the cells were lysed and subjected to a luciferase assay. 293T cells were infected as a positive control, while 3T3 cells were infected as one of the negative controls. Bars 3-11 represent 3T3 cells stably transfected with the following pIRES2-EGFP plasmids: -, vector alone; HA, hemoglobin alpha 1/2; G1, gelsolin (clone 1); G2, gelsolin (clone 2); L13a, ribosomal protein L13a; SM1, SMC5 (clone 1); SM2, SMC5 (clone 2); HSP, HSPC171; and GLUT-1.

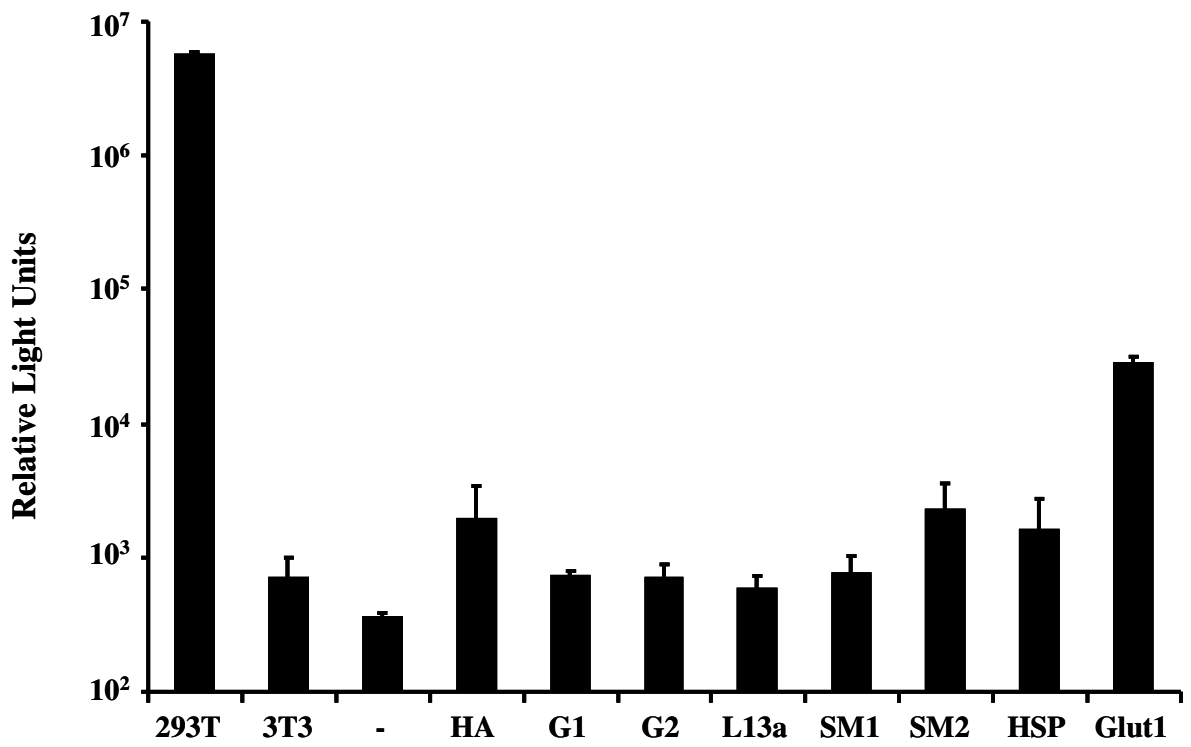
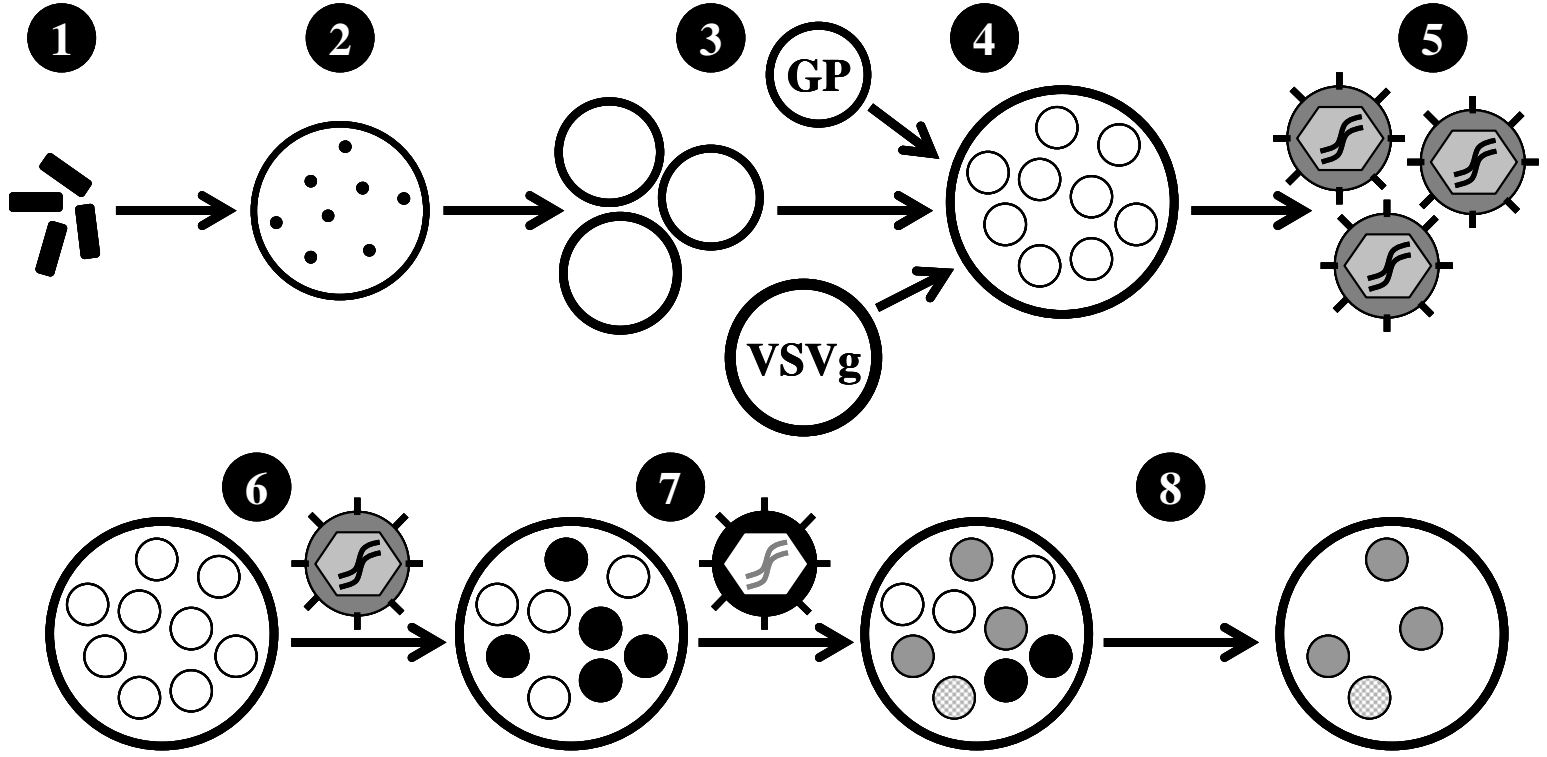


Fig. 3. Schematic representation of the retroviral cDNA library screen. (1) The HeLa S3 cDNA library *E. coli* stock was plated on 150-mm bacterial plates (2) and grown overnight. The plates were scraped and DNA from each plate was prepped (3). The DNA from each plate was combined into a master library mixture, and this DNA, along with plasmids expressing VSVg and the MLV Gag-Pol (GP), were transfected into 293T cells (4). The resulting retroviral HeLa S3 cDNA particles were harvested 72h later (5), and used to infect NIH 3T3 cells (6). The black circles represent 3T3 cells transduced with cDNA virus; the white circles represent non-transduced 3T3 cells. The next day these 3T3 cells were transduced with the HIV-neo (HTLV-1) virus (7). The black circles represent 3T3 cells transduced with cDNA that did not allow entry of the viral particles; the gray circles represent 3T3 cells transduced with cDNA that did allow entry of the viral particles; the white circles represent non-transduced 3T3 cells; the white/gray cells represent non-cDNA transduced 3T3 cells that allowed entry of the viral particles (background cells). Forty-eight hours later the 3T3 cells were placed under G418 selection. Two weeks later, the selected colonies (8) were picked off the plate and expanded.



Selecting potential candidates

We prioritized candidates that could potentially act as the receptor/attachment factor/entry-enhancing molecule for HTLV-1 based on three factors: (1) cellular localization – since we are looking for factors that can bind to and/or allow entry to the HTLV-1 Env, we selected candidates based on whether they are present at the plasma membrane and/or are known receptors; (2) frequency – pulling out a factor multiple times inherently implies its importance; hence we also focused on ‘multiple-hit’ proteins; and (3) identification as a HIV-dependency factor (HDF) – three recent studies identified over 200 host factors potentially involved in HIV-1 replication (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008); due to similarities between HIV-1 and HTLV-1 infectivity, these HDFs could also be important in HTLV-1 replication. However, some of the HDFs chosen were not the exact factors pulled out of the screens, but belonged to the same family of proteins (e.g. F-box/WD repeat-containing protein 11, FBXW11, was pulled out of one of the HDF screens, whereas we pulled out FBXW5). Based on these criteria, we selected 20 potential candidates, of which 12 are membrane proteins, including flotillin-1 (FLOT-1) and killer cell lectin-like receptor subfamily K, member 1 (KLRK1/NKG2D/CD314), 11 are multiple hits, including peroxiredoxin-1 (PrdX1) and phosphatidylinositol glycan anchor biosynthesis, class T (PIGT), and 7 are HDFs or HDF-related, including CD68 and Ras-related protein, Rab3D (Table 1).

Table 1 **List of prioritized receptor candidates**

Receptor Candidate	MP^a	MH (#)^b	HDF^c
Guanine nucleotide-binding protein subunit beta-2-like 1 (GNB2L1)		● (5)	
Peroxiredoxin-1 (PrdX1)		● (10)	
Phosphatidylinositol glycan anchor biosynthesis, class T (PIGT)		● (13)	
Killer cell lectin-like receptor subfamily K, member 1 (KLRK1)	●		
HLA-A, -B, and -C (Major Histocompatibility Complex I)	●		●
HLA-DRB1 (Major Histocompatibility Complex II)	●		
Flotillin-1 (FLOT-1)	●	● (2)	
CD68	●	● (3)	●
Solute carrier family 16, member 3 (SLC16A3)	●		
Solute carrier family 38, member 5 (SLC38A5)	●	● (2)	●
Ras-related protein Rab3D		● (2)	●
Ligatin (LGTN)	●		
Transmembrane BAX inhibitor motif containing 6 (TMBIM6)	●	● (3)	
Transmembrane emp24 domain-containing protein 10 (TMED10)		● (2)	●
Transmembrane 4 L6 family member 1 (TM4SF1)	●		
Protease, serine 21 (PRSS21)	●		
Transmembrane and coiled-coil domain-containing 1 (TMCO1)		● (3)	
Yip1 domain family member 3 (YIPF3)	●		
Ankyrin repeat domain protein 16 (ANKRD16)			●
F-box/WD repeat containing protein 5 (FBXW5)		● (2)	●

^a MP - membrane protein

^b MH (#) - multiple hit (number of hits)

^c HDF - HIV-dependency factors identified in Brass et al. (2008), König et al. (2008), and Zhou et al. (2008)

DISCUSSION

Viral tropism is an important factor to consider with viral entry/infection. It refers to the specificity of a virus for cell types that are capable of producing viral progeny or maintaining this production potential with dormant viruses (Manel et al., 2005). This tropism is determined largely by the binding of viral envelope glycoproteins to specific cell surface receptor(s). However, viral and/or cellular factors and post-binding events that influence the completion of the viral replication cycle can impact tropism significantly. Classic examples of this were revealed with the T-cell tropic strain of the mammalian feline leukemia retrovirus (FeLV-T) (Anderson et al., 2000), and HIV-1 (Bieniasz et al., 1998). For HTLV-1 infection, it seems likely that its preferential detection in CD4⁺ T cells *in vivo* is not due solely to Env-receptor interactions, but rather to a series of post-entry selection steps (Manel et al., 2005).

In the past, three experimental approaches have been used to uncover the HTLV-1 receptor (Manel et al., 2005): (1) infection with retroviral or non-retroviral particles pseudotyped with HTLV-1 Env (Clapham, Nagy, and Weiss, 1984; Manel et al., 2003; Sommerfelt and Weiss, 1990; Sommerfelt et al., 1988; Sutton and Littman, 1996; Trejo and Ratner, 2000); (2) HTLV-1 Env mediated cell-to-cell fusion (Denesvre et al., 1995; Hildreth, Subramaniam, and Hampton, 1997; Hoshino et al., 1983; Tajima, Tashiro, and Camerini, 1997); and (3) binding of HTLV-1 Env to permissive cells (Gavalchin et al., 1993; Jassal, Pohler, and Brighty, 2001; Kim et al., 2004; Manel et al., 2003). Although these approaches have yielded a vast array of data, and significantly increased our knowledge of HTLV-1 entry/infection, they have been unable to definitively pinpoint the

receptor. Our approach was designed to systematically scan the genome of a permissive cell-line and identify factors that enhance entry mediated by the HTLV-1 Env. We isolated a total of 460 cDNAs, of which 20 were of particular interest as they fulfilled our prioritization criteria. These 20 candidates fall under three broad categories – (1) receptor or receptor-like factors found at the plasma membrane, (2) non-receptor-like factors found at the plasma membrane, or (3) factors not found at the plasma membrane.

In the first category we would include factors such as KLRK-1/NKG2D, the human leukocyte antigen-A, -B, -C, and -DR beta 1 (HLA-A, -B, -C, -DRB1), CD68, and the two solute carriers, solute carrier family 16, member 3 and solute carrier family 38, member 5 (SLC16A3, SLC38A5). All are expressed at the plasma membrane of a subset of hematopoietic cells (Banerjee, Feuer, and Barker, 2007; Bauer et al., 1999; Lanier, 2005; Micklem et al., 1989; Parwaresch et al., 1986; Raulet, 2003). In addition, a number of these factors (HLA-A, -B, -C, CD68, and SLC38A5) were identified as HDFs/HDF-related, and CD68 and SLC38A5 were isolated multiple times in our screen, lending further support to their possible role in enhancing HTLV-1 infectivity.

In the second category we would include factors such as FLOT-1, ligatin (LGTN), transmembrane 4 L6 family member 1 (TM4SF1), protease, serine 21 (PRSS21), and Yip1 domain family member 3 (YIPF3). These factors are all localized at the plasma membrane (de Gassart et al., 2003; Dermine et al., 2001; Jakoi and Marchase, 1979; Jakoi, Zampighi, and Robertson, 1976; Lekishvili et al., 2008; Prost et al., 2002; Stuermer et al., 2001), but are not known to have classic receptor-like functions. Due to their localization, it is possible that these molecules somehow act as attachment factors and/or are involved in receptor signaling. FLOT-1, which was isolated multiple times in

our screen, has been shown to be associated with lipid rafts (Lang et al., 1998; Schulte et al., 1997; Stuermer et al., 2001), TM4SF1 is in the family of tetraspanins, which have been shown to be loosely localized to lipid rafts (Berdichevski, 2001), and the murine form of PRSS21, TESP5, has been shown to be associated with lipid rafts (Honda et al., 2002). Lipid rafts are microdomains of the plasma membrane, rich in cholesterol and glycosphingolipids (Brown and London, 2000). Since cholesterol has been shown to be important in the uptake of a number of viruses, including HTLV-1 and HIV-1 (Carter et al., 2009; Wielgosz et al., 2005), lipid rafts have become an important factor in viral entry. Furthermore, FLOT-1 and LGTN have both been shown to lack transmembrane domains; instead, they are anchored to the plasma membrane by post-translational fatty acylation modifications, specifically palmitoylation (Gkantiragas et al., 2001; Jakoi et al., 1987; Morrow et al., 2002). It is interesting to note that many palmitoylated proteins are associated with lipid rafts, and this association has been shown to be important in T cell signaling (Resh, 1999).

The factors in the third category are not known to reside at the plasma membrane, but could possibly be involved in enhancing HTLV-1 entry and/or infectivity indirectly. Of these, guanine nucleotide-binding protein subunit beta-2-like 1 (GNB2L1), PrdX1, PIGT, Rab3D, transmembrane BAX inhibitor motif containing 6 (TMBIM6), transmembrane emp24 domain-containing protein 10 (TMED10), transmembrane and coiled-coil domain-containing 1 (TMCO1), and FBXW5 were identified multiple times in our screen, while Rab3D, TMED10, ankyrin repeat domain protein 16 (ANKRD16), and FBXW5 were identified as HDFs/HDF-related. Though they are not directly linked to the plasma membrane, Rab3D and TMED10 are known to translocate to the plasma

membrane (Blum et al., 1996; Millar et al., 2002; Tuvim et al., 1999), while GNB2L1 has been shown to loosely co-localize with clathrin (Gallina, Rossi, and Milanesi, 2001). PrdX1 and FBXW5 have been shown to be important in receptor signaling and signal transduction (Kang et al., 1998; Kang et al., 2005; Minoda et al., 2009), and PIGT, which is a type I transmembrane protein, has been shown to be critical for the formation of the glycosylphosphatidylinositol (GPI) transamidase complex and essential for attachment of GPI to proteins (Ohishi, Inoue, and Kinoshita, 2001).

Due to our prioritization scheme and categorization of the 20 factors, we are confident that we have a strong subset of candidate proteins. However, due to the design of our screen, a few possible caveats must be addressed. Firstly, since we infected the NIH 3T3 cells with the retroviral cDNA library at an MOI of 1.5, we would expect, and did find, multiple cDNAs in all the clones isolated after the selection process. Due to this fact, there is a possibility that the entry of the HTLV-1 Env was due to the expression of multiple cDNAs in the clones, rather than just one cDNA. Secondly, since we are pseudotyping a HIV-based vector with the HTLV-1 Env, it is possible that some of the cDNAs we isolated were identified because they alleviated a post-entry block to HIV, rather than enhanced entry of HTLV-1 Env. Thirdly, if HTLV-1 interacts with multiple distinct receptors/attachment factors, our screen may not pick up factors involved in enhancing entry, since the other, possibly crucial molecules might not be expressed. Despite these possible drawbacks, we have designed a unique way of searching for the HTLV-1 receptor(s)/attachment factor(s), and through a focused search uncovered many potential new candidates.

FUTURE DIRECTIONS

The next step in our screen is to clone and re-test the 20 candidate factors we selected. The clones will initially be stably transfected into NIH 3T3 cells. These stable candidate cell-lines will be infected with HTLV-1 Env pseudotyped HIV-luc particles, and luciferase assays carried out to determine the level of entry, based on luciferase activity. Any candidate cDNAs that increase luciferase activity significantly above background levels, to be determined empirically, will then be further tested by fusion assays, to determine if they cause an increase in HTLV-1 Env fusion, and knockdown studies, to determine if expression of the candidate in permissive cell-lines is necessary for HTLV-1 Env infection. It will also be interesting to determine whether any identified candidate factor(s) interacts with any of the factors currently thought to increase HTLV-1 entry, i.e. GLUT-1, NRP-1, and/or HSPGs.

MATERIALS AND METHODS

Retroviral cDNA library screens

For the first screen, we purchased the ViraPort VSV-G Retroviral Supernatant Human Substantia Nigra cDNA library from Stratagene, which had a titer of 2×10^6 infectious particles/mL. We infected one plate with 2×10^6 NIH 3T3 cells (MOI = 1.0), while another, with a duplicate number of cells, was not infected (this was designated the 'negative' plate, and was used to calculate our background). Twenty-four hours later, the

cDNA particles were washed off the NIH 3T3 cells, and both these cells and the cells on the 'negative' plate were infected with HIV-neo (HTLV-1) particles (MOI = 5.0). These HTLV-1 Env pseudotyped particles (selection virus) were generated by transfecting 293T cells with the pHTE-1 and pNL4-3SV40Neo⁺Env⁻ plasmids. Forty-eight hours later, the selection virus was washed off the plates, and fresh media supplemented with G418 was added to the cells. Fresh G418-supplemented media was added every three days, for the next three weeks, by which time individual colonies had formed. We picked colonies from both the cDNA-infected and negative plates into 24-well plates, from where they were expanded. We simultaneously plated cells for infection with HIV-luc (HTLV-1). Plasmid DNA was isolated from the candidate cell-lines and subjected to PCR (using primers provided with the ViraPort library) to amplify the cDNAs they harbored. These cDNAs were sequenced and then identified by running the sequences through the Basic Local Alignment Search Tool (BLAST).

For the second screen, we purchased the ViraPort® XR HeLa S3 cDNA library (*E. coli* glycerol stock) from Stratagene, in which the cDNA plasmid library is cloned into pFB, a replication-defective retroviral (MMLV-based) vector. After titering the *E. coli* glycerol stock (2×10^6 cfu/mL), we plated one hundred 150-mm LB-ampicillin agar plates (2×10^4 cfu/plate), and incubated them at 30°C for 24 hours (Fig. 3). We harvested the colonies by scraping each plate into a small pool of LB medium. Each of these 100 pools was subjected to alkaline lysis using the PureYield™ Plasmid Midiprep System (Promega), to generate cDNA library plasmid DNA. We created a master pool of cDNA plasmids by combining DNA from each of the 100 individual pools. We transfected 293T cells with the master pool, a VSVg expression plasmid, and a MLV

Gag-Pol expression plasmid, and 72 hours later harvested the retroviral cDNA library particles, representing the 2×10^6 distinct cDNAs. These virus particles were used to infect 3×10^6 NIH 3T3 cells (MOI = 1.5). Another plate with a duplicate number of NIH 3T3 cells was not infected with the cDNA particles; this was designated the 'negative' plate, and was used to calculate our background. Twenty-four hours later, the cDNA particles were washed off the NIH 3T3 cells, and both these cells and the cells on the 'negative' plate were infected with HIV-neo (HTLV-1) particles (MOI > 10). Forty-eight hours later, the selection virus was washed off the plates, and fresh media supplemented with G418 was added to the cells. Fresh G418-supplemented media was added every three days, for the next two weeks, by which time individual colonies had formed. We picked these colonies from the plate, and into 24-well plates, from where they were expanded. Plasmid DNA was isolated from these colonies and subjected to PCR to amplify the cDNAs they harbored. These cDNAs were sequenced and then identified by running the sequences through the Basic Local Alignment Search Tool (BLAST).

Cells

293T and NIH 3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FetalClone III (FCIII) serum, 2mM L-glutamine, 1mM sodium pyruvate, penicillin (100 U/mL), and streptomycin (100 μ g/mL). After addition of the HTLV-1 Env pseudotyped selection virus and after transfection with the candidate clones, NIH 3T3 cells were selected in complete DMEM with 850 μ g/mL G418 (Mediatech) for two-three weeks.

Plasmids, viruses, transfections, infections and luciferase assays

Five million 293T cells were transfected with the HeLa S3 cDNA library plasmids (described above), pHCMVg (VSVg expression plasmid), and pVPack-GP (MLV Gag-Pol expression plasmid, Stratagene) to generate the retroviral HeLa S3 cDNA virus. Five million 293T cells were transfected with pHTE-1 (HTLV-1 Env expression plasmid) and pNL4-3SV40Neo⁺Env⁻ (envelope-deficient HIV plasmid, carrying the neo resistance gene under the control of the SV40 promoter) to generate the HTLV-1 Env pseudotyped selection virus, HIV-neo (HTLV-1). Five million 293T cells were transfected with pHTE, SV-A-MLV-env (amphotropic MLV Env expression plasmid), or pHCMVg and pNL4-3SV40Luc⁺Env⁻ (envelope-deficient HIV plasmid, carrying the luciferase reporter gene under the control of the SV40 promoter) to generate the pseudotyped reporter viruses, HIV-luc (HTLV-1), HIV-luc (A-MLV), and HIV-luc (VSVg), respectively. The pHCMVg, pHTE-1, pNL4-3SV40Luc⁺Env⁻, and SV-A-MLV-env plasmids have been described previously (Landau, Page, and Littman, 1991; Trejo and Ratner, 2000). The pNL4-3SV40Neo⁺Env⁻ plasmid was generated by MM Wielgosz in our laboratory. To generate the candidate clones, the candidate cDNAs were cloned into the EcoRI and SalI sites of the pIRES2-EGFP plasmid (Clontech). Two million NIH 3T3 cells were stably transfected with the candidate clones. All transfections were carried out using *TransIT*[®]-LT1 Transfection Reagent (Mirus) as per the manufacturer's recommendations. One million cDNA-infected NIH 3T3 cells or candidate-clone-transfected NIH 3T3 cells were infected with HIV-luc (HTLV-1). Forty-eight hours post-infection, the cell pellets were

lysed in lysis buffer, and subjected to a luciferase assay (Luciferase Assay System, Promega).

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Chapter 3

The HTLV-1 *hbz* Antisense Gene Indirectly Promotes *tax* Expression via Down-regulation of *p30^{II}* mRNA

Preface to Chapter 3

Chapter 3 has been submitted as a manuscript to *Virology* by G.C. All experiments and data were performed and collected by G.C.

TITLE

The HTLV-1 *hbz* Antisense Gene Indirectly Promotes *tax* Expression via Down-regulation of *p30^{II}* mRNA

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Key Words: *hbz*, *tax*, HTLV-1, *p30^{II}*, antisense

Running Title: HTLV-1 *hbz* promotes *tax* Expression

Abstract

Human T-cell leukemia virus type 1 (HTLV-1) basic leucine zipper factor (HBZ) is transcribed from the antisense genomic DNA strand and functions differently in its RNA and protein forms. To distinguish between the roles of *hbz* mRNA and HBZ protein, we generated mutants in a proviral clone that specifically disrupt the *hbz* gene product. A proviral clone with a splice acceptor mutation that disrupts expression of the predominant *hbz* mRNA resulted in lower levels of *tax* mRNA. Heterologous *hbz* expression restored Tax activity in cells expressing this mutant clone. In contrast, proviral mutants that disrupt HBZ protein did not affect levels of *tax* mRNA. Expression of *hbz* resulted in lower levels of *p30^{II}* mRNA. Mutation of *p30^{II}* overcame the effects of the splice acceptor mutation of *hbz*, and restored *tax* expression. Thus, there is a complex interplay of viral regulatory proteins controlling levels of HTLV-1 gene expression.

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus belonging to the delta retrovirus family. It is the etiologic agent of adult T cell leukemia lymphoma (ATLL) (Hinuma et al., 1981; Poiesz et al., 1980), a malignancy of CD4⁺ T lymphocytes, and a chronic neurological disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). In addition to the *gag*, *env*, and *pol* genes encoding structural and enzymatic proteins common to all retroviruses, the HTLV-1 genome includes several accessory proteins, which facilitate virus transmission *in vivo* by establishing persistent infection (Collins et al., 1998; Hiraragi et al., 2006; Nicot et al., 2004; Silverman et al., 2004; Ye et al., 2003). The *tax* gene product is a potent activator of viral transcription (Boxus and Willems, 2009; Felber et al., 1985; Yin and Gaynor, 1996). In addition, through its involvement in a number of cellular transcription pathways, Tax stimulates the proliferation, survival, and transformation of HTLV-1-infected T cells (Boxus and Willems, 2009; Matsuoka and Jeang, 2007; Peloponese, Kinjo, and Jeang, 2007). Another regulatory protein, p30^{II}, down-regulates Tax expression by interaction with, and nuclear retention of *tax* mRNA (Nicot et al., 2004). Other HTLV-1 regulatory proteins include Rex, p12^I, p27^I, and p13^{II} (Fig. 1) (Albrecht and Lairmore, 2002).

Recently, another HTLV-1 regulatory gene was identified on the antisense genomic DNA strand; since its protein product includes a basic region and a leucine zipper, it was designated HTLV-1 bZIP factor, or HBZ (Cavanagh et al., 2006; Gaudray et al., 2002; Larocca et al., 1989). The *hbz* mRNA is expressed in primary ATLL cells, despite repression of other viral transcripts (Matsuoka and Green, 2009; Satou et al.,

2006). Multiple *hbz* mRNA initiation sites have been identified in the 3'-LTR (Cavanagh et al., 2006; Yoshida et al., 2008), which result in three transcripts; two are spliced (*hbz SP1* and *SP2*) while the other is unspliced (*hbz US*) (Fig. 1) (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006). The *SP1* spliced variant is the most abundant form in ATLL cell lines and infected lymphocytes from ATLL patients (Satou et al., 2006; Usui et al., 2008). The promoters for *hbz SP1* and *hbz US* have been identified, and studies suggest that the levels of antisense transcription are 20-50 fold lower (Arnold et al., 2006; Larocca et al., 1989) than sense transcription (Yoshida et al., 2008). The *hbz SP1* and *hbz US* mRNAs encode protein products that differ only in their N-terminal seven amino acids, whereas the *SP2* transcript does not code for a protein product, and its function is undefined. The *hbz* transcripts include sequences complementary to the other HTLV-1 transcripts encoded from the sense genomic DNA strand.

HBZ has been reported to be a negative modulator of Tax. Upon exogenous over-expression, HBZ binds to and inhibits CREB-2, an essential transcription factor for Tax-mediated *trans*-activation of the viral promoter (Basbous et al., 2003; Gaudray et al., 2002). However, HBZ expression does not affect the ability of HTLV-1 to immortalize T lymphocytes in culture (Arnold et al., 2006); in fact, HBZ enhances virus infectivity and persistence *in vivo*. Another study identified a distinct activity of *hbz* mRNA, and demonstrated enhanced T cell proliferation in culture and transgenic mice (Satou et al., 2006). They identified a domain of *hbz* mRNA, localized to the first stem-loop (SL), that mediated this activity. These data led to the hypothesis that the *hbz* gene has dual functionality: *hbz* mRNA promotes T-cell proliferation, while HBZ protein suppresses Tax-mediated viral transcription.

In order to delineate and differentiate the activities of *hbz* mRNA and HBZ protein, we used an infectious molecular proviral clone of HTLV-1 (Kimata et al., 1994) to assess mutations that specifically affect the structure and/or expression of the *hbz* gene product. This led to the unexpected identification of a third activity of *hbz* that promotes *tax* mRNA expression. These results have important implications for understanding the regulation of HTLV-1 gene expression.

Results

Construction and characterization of pACH.HBZ mutant proviral clones

To investigate the role of HBZ in the context of an HTLV-1 provirus, we generated four HBZ mutant proviral clones (Fig. 2A). For this purpose, we used a well characterized infectious molecular clone, designated pACH (Kimata et al., 1994). pACH.HBZ- Δ SA has a mutation in the splice acceptor site of the *hbz* gene that prevents the proper splicing of the *SP1* and *SP2* *hbz* mRNAs (Fig. 1). pACH.HBZ-KO is a knockout mutant that terminates the predominant HBZ protein derived from the SP1 variant at amino acid seven, as well as the HBZ protein derived from the US variant at amino acid 10 (Fig. 2A). pACH.HBZ-TRUN is a truncation mutant that deletes the leucine zipper portion of the bZIP domain of variants SP1 and US, through insertion of a termination codon in place of codon 158 (SP1)/codon 161 (US) within the 206 (SP1)/209 (US) amino acid open reading frame. The leucine zipper portion of HBZ is required for the association of HBZ with CREB-2, JunB, and c-Jun (Basbous et al., 2003; Gaudray et al., 2002). pACH.HBZ-SL has a mutation that disrupts the stem-loop at the 5' end of the *hbz* mRNA that is only found in the *SP1* variant (Satou et al., 2006). Although mutants similar to pACH.HBZ-KO and pACH.HBZ-TRUN were constructed and tested by other investigators (Arnold et al., 2006), our studies were aimed at distinguishing the role of *hbz* mRNA versus HBZ protein at physiological levels.

We first determined the levels of *hbz* *SP1* RNA expressed from the proviral mutant plasmids (Fig. 2B). 293T cells were transiently transfected with each of the various mutants, and total RNA isolated from these cells was subjected to real-time RT-PCR, using primers that spanned the *hbz* splice junction (Fig. 1). The results from an

average of at least three independent experiments are shown in Fig. 2B. Mutating the splice acceptor site of the *hbz* gene significantly reduced the steady state levels of *hbz SPI* RNA by greater than 90% ($P = 0.03$). As expected, mutants pACH.HBZ-KO and pACH.HBZ-TRUN, which were constructed to truncate the HBZ protein without affecting *hbz* mRNA, were found to have no significant effects on the levels of *hbz* RNA (pACH.HBZ-KO: $P = 0.57$; pACH.HBZ-TRUN: $P = 0.15$). Disrupting the stem-loop structure of *hbz* mRNA did not affect the stability of the *hbz* mRNA, since steady state levels were equivalent to those of cells expressing pACH.HBZ-WT ($P = 0.35$). We also measured levels of total *hbz* RNA in cells transfected with the pACH.HBZ-WT and pACH.HBZ- Δ SA plasmids (Fig. 2B, inset), and found that the levels were decreased by 69% in the presence of the splice acceptor mutation. These levels of total *hbz* most likely correspond to the *hbz US* transcript, as this level is consistent with previously reported levels of *hbz US* (Usui et al., 2008)

In order to examine the expression of HBZ protein from the various mutants, we generated HBZ wild-type (pHBZ) and mutant (pHBZ-KO, pHBZ-TRUN, and pHBZ-SL) expression plasmids, inserting a triple flag tag at the C-terminus of each predicted protein product. 293T cells were transiently transfected with these plasmids, and Western blot analysis was carried out (Fig. 2C). As expected, no HBZ expression was seen from cells transfected with the HBZ-KO mutant. The HBZ-TRUN mutant produced a truncated HBZ-flag protein of 27 kDa. Cells expressing wild-type HBZ and the HBZ-SL mutant both produced HBZ-flag proteins of 34 kDa. Levels of expression of WT, TRUN, and SL HBZ proteins were equivalent.

To confirm that pACH.HBZ- Δ SA does not produce an unspliced gene product, we generated an HBZ- Δ SA expression plasmid (pNHBZ- Δ SA) that contains both the exons of *hbz SPI* and the sequence corresponding to the intronic region between them. 293T cells were transiently transfected with this plasmid and a HBZ wild-type expression plasmid, pNHBZ, and Western blot analysis was carried out with anti-HBZ antiserum (Fig. 2D). We did not see any HBZ expression from pNHBZ- Δ SA, confirming the lack of an unspliced gene product. Additionally, we did not see a difference in unspliced mRNA levels between cells transfected with the wild-type and Δ SA mutant proviral plasmids (data not shown).

Loss of *hbz* leads to reduction in *tax* expression

The pACH.HBZ mutants were used to determine the effects of *hbz* mRNA and HBZ protein on expression of other HTLV-1 genes. Total RNA isolated from 293T cells transiently transfected with the HBZ proviral mutants was subjected to real-time RT-PCR to measure the levels of *tax* transcript. The primers used to amplify *tax* spanned the second splice junction (Fig. 1). The results from an average of four independent experiments are shown in Fig. 3A. There was a 70% decrease in the steady state level of *tax* mRNA expressed from the pACH.HBZ- Δ SA mutant, as compared to the level of *tax* mRNA expressed by pACH.HBZ transfected cells. Jurkat cells transfected with the pACH.HBZ-WT plasmid expressed 0.12 ± 0.07 pg Tax mRNA, while those transfected with the pACH.HBZ- Δ SA mutant plasmid expressed 0.0 ± 0.02 pg Tax mRNA ($P = 0.03$) (data not shown). No significant changes were seen in *tax* mRNA expressed from the other pACH mutants (Fig. 3A). The levels of *tax* mRNA, relative to that of

pACH.HBZ-WT expressing cells, with pACH.HBZ-KO, pACH.HBZ-TRUN, and pACH.HBZ-SL were 87%, 104%, and 73%, respectively ($P = 0.78, 0.96, \text{ and } 0.51$, respectively).

Levels of viral proteins were also assessed, and normalized to levels of actin, used as a loading control (Fig. 3B). There was a 64% decrease in the amount of Tax protein expressed from the pACH.HBZ- Δ SA mutant, compared to Tax protein expressed from pACH.HBZ transfected cells. No significant decrease in Tax protein was seen with the other mutants.

Since Tax is responsible for the *trans*-activation of viral transcription, the levels of the viral structural protein, Gag were measured in the cellular lysate and virus particles in the culture supernatant. As expected, levels of Gag p24 and p19 were also decreased in cells transfected with pACH.HBZ- Δ SA compared to pACH.HBZ-WT (Fig. 3B, lane 3), as well as in virus particles released into the supernatant from those cells (Fig. 3C, lane 3). These results suggest that the effect of *hbz* mRNA on the levels of *tax* mRNA also influences the levels of Gag expression in transfected cells and the levels of released virus particles.

***Trans*-complementation of *hbz* increases Tax activity in pACH.HBZ- Δ SA cells to wild-type levels**

To ensure that the effects on steady state levels of Tax in pACH.HBZ- Δ SA transfected cells were due to loss of *hbz* mRNA, we measured levels of Tax-mediated LTR gene expression in the presence of increasing amounts of *hbz*. Co-transfection of pACH.HBZ-WT or pACH.HBZ- Δ SA, as a source of Tax, together with an LTR-

luciferase reporter plasmid, provided a sensitive quantitative measure of Tax activity. It has previously been shown that Tax expressed from the pACH plasmid is able to *trans*-activate the HTLV-1 LTR (Robek and Ratner, 1999). In this study, the specific role of Tax in transcriptional transactivation was confirmed using the M47 Tax mutation in pACH, a mutation which prevents Tax from upregulating transcription through CREB/ATF (Smith and Greene, 1990). This mutant had 50-fold lower levels of HTLV-1 LTR activation, using the LTR-luciferase reporter plasmid, compared to wild-type pACH.

In the trans-complementation experiment with *hbz*, increasing quantities of a plasmid were utilized in which *hbz* was expressed under the regulation of a CMV promoter, pHBZ (Fig. 4A). In cells transfected with pACH.HBZ- Δ SA, together with increasing amounts of the *hbz* expression plasmid, the levels of LTR-directed gene expression increased. It should be noted that in the presence of 4 μ g pHBZ, the level of Tax activity in pACH.HBZ- Δ SA transfected cells increased to levels seen in pACH.HBZ-WT transfected cells. The levels of Tax activity also increased in cells transfected with pACH.HBZ-WT in the presence of pHBZ. However, there was no significant increase in Tax activity with increasing amounts of pHBZ in cells transfected with a Tax expression plasmid (CMV-Tax). One explanation for this result is that another viral gene, expressed from the proviral plasmid, is required for the effect of *hbz* on *tax* expression.

As in the previous experiment (Fig. 3), we examined the levels of Gag protein in the cellular lysate and viral supernatant, as a further measure of Tax activity. As expected, the levels of Gag p24 in the lysate increased in the presence of exogenous *hbz*

(Fig. 4B, lanes 4 and 6, compared to lanes 3 and 5, respectively), and significantly more viral particles were released into the supernatant (Fig. 4C, lanes 4 and 6, compared to lanes 3 and 5, respectively).

***hbz* represses *p30^{II}* to indirectly promote *tax* expression**

The HTLV-1 accessory protein $p30^{II}$ is a nuclear protein that retains the doubly spliced *tax* mRNA in the nucleus, leading to a reduction in the amount of Tax protein (Nicot et al., 2004). Since $p30^{II}$ is a negative regulator of *tax*, we hypothesized that *hbz* may negatively regulate $p30^{II}$ in order to promote *tax* mRNA expression. Since *hbz* mRNA is transcribed from the (-) strand of the HTLV-1 provirus, it is a natural antisense RNA to genes expressed from the (+) proviral DNA strand. Since the majority of the *hbz* transcript overlaps the *p30^{II}* transcript, we hypothesized that the *hbz* mRNA binds to the *p30^{II}* mRNA to promote its degradation or inhibit its translation.

To test our hypothesis, we co-transfected 293T cells with pMHP30^{II}_{HAI1}, a $p30^{II}$ expression plasmid and pHBZ, and carried out real-time RT-PCR on total RNA from the cells (Fig. 5A). In the presence of increasing amounts of HBZ, we saw a significant decrease in the levels of *p30^{II}* mRNA. To determine whether this effect was dependent on HBZ protein or *hbz* mRNA, we generated a protein mutant of pHBZ, pmHBZ, mutating the initiator ATG to TTG. In the presence of increasing amounts of mutant pHBZ, we also found a significant decrease in the levels of *p30^{II}* mRNA, suggesting that the *hbz* mRNA, not the HBZ protein, is responsible for inhibiting levels of *p30^{II}* mRNA. The $p30^{II}$ protein levels are also reduced in the presence of the wild-type and mutant pHBZ (Fig. 5B), confirming that *hbz* mRNA inhibits synthesis of $p30^{II}$ protein.

To investigate the role of p30^{II}, we generated two p30^{II} mutant proviral clones. Both pACHΔp30 and pACH.HBZ-ΔSAΔp30 include a termination codon in place of amino acid eight of the p30^{II} open reading frame, in the context of the *hbz* wild-type and *hbz* splice-deficient expression plasmids, respectively (Fig. 1). 293T cells were transiently transfected with these plasmids and the cellular lysate and supernatant evaluated for levels of Gag (p24 and p19) protein. In the absence of p30^{II}, there was a 1.95-fold increase in the levels of Gag protein in the cellular lysate (Fig. 5C, lane 3, compared to lane 2) and a four-fold increase over wild-type in the viral supernatant (Fig. 5D, lane 3, compared to lane 2), which corresponds, as expected, to an increase in the level of Tax protein. As seen previously (Fig. 3), loss of *hbz* leads to a decrease in the amount of Gag protein in the cellular lysate and supernatant (Fig. 5C and 5D, lane 4). However, the loss of *hbz* was overcome by deleting p30^{II}, as evidenced by an increase in the levels of Gag protein in the cellular lysate (Fig. 5C, lane 5) and viral supernatant (Fig. 5D, lane 5) from cells transfected with pACH.HBZ-ΔSAΔp30. Taken together, these results suggest that *hbz* promotes expression of *tax* indirectly through p30^{II}.

Discussion

Since the discovery of the HTLV-1 antisense *hbz* gene (Gaudray et al., 2002), numerous studies have been carried out to establish its role in HTLV-1 replication and pathogenesis. Though these studies have been informative and have established a foundation for *hbz* in the HTLV-1 field, the function of *hbz in vivo* is still unclear. HBZ was identified as a binding partner to CREB-2; it binds CREB-2 through its basic leucine zipper (bZIP) domain (Gaudray et al., 2002). Since CREB-2 has been shown to

cooperate with Tax in *trans*-activating the HTLV-1 LTR (Ching et al., 2004), studies have shown that exogenously over-expressed HBZ protein down-regulated Tax-mediated viral transcription by binding CREB-2 (Gaudray et al., 2002; Lemasson et al., 2007). Furthermore, Tax has been shown to activate a number of cellular pathways, such as the activator protein-1 (AP-1) pathway (Fujii et al., 1991; Fujii, Sassone-Corsi, and Verma, 1988; Hooper et al., 1991). Studies with exogenous HBZ showed that, through its bZIP domain, HBZ could bind to members of the AP-1 pathway (cJun and JunB) and down-regulate Tax-mediated AP-1 transcription (Basbous et al., 2003; Clerc et al., 2009; Hivin et al., 2007; Matsumoto et al., 2005). However, since these studies employed over-expression of HBZ in the absence of other viral proteins, a more physiologically relevant study was carried out using HBZ expressed from a molecular clone of HTLV-I (Arnold et al., 2006). Data from this study showed that HBZ protein enhanced viral infectivity and persistence *in vivo*. In addition to this function of HBZ protein, the *hbz* mRNA has been shown to promote T-cell proliferation (Arnold et al., 2008; Satou et al., 2006).

Since previous studies suggested that *hbz* has dual functionality, we examined the effects of various proviral *hbz* mRNA and protein mutants on viral gene expression. We showed that a loss of *hbz* mRNA leads to a significant reduction in the levels of *tax* mRNA (Fig. 3). This reduction of *tax* mRNA leads, as expected, to lower Tax protein levels, and a reduction in the levels of Gag protein and virus particle production. These data are in contrast to previous work that showed that *hbz*-specific short hairpin (sh)-RNAs, which down-regulated the levels of *hbz* in an HTLV-1 T-cell line, did not affect levels of *gag/pol* and *tax/rex* mRNAs or p19 Gag and Tax protein (Arnold et al., 2008). A possible reason for this discrepancy is discussed below.

To confirm that the reduction in Tax levels was a consequence of the loss of *hbz* mRNA, a complementation study was carried out in which increasing amounts of exogenous *hbz* were expressed. In contrast to previous work (Arnold et al., 2006), we found that when Tax was expressed from a proviral plasmid, the levels of Tax activity increased with increasing amounts of pHBZ (Fig. 4). Of particular note is that, in the presence of 4ug of pHBZ, Tax activity in cells transfected with the splice-deficient *hbz* mutant increased to wild-type levels.

Another interesting finding was that when Tax was expressed from an expression plasmid rather than a proviral expression plasmid, there was no increase in Tax activity in the presence of pHBZ (Fig. 4). We concluded that a viral gene, expressed from the proviral plasmid, was required for *hbz* to have its effect on *tax*. Possible explanations for this result are: (1) *hbz* represses an inhibitor of *tax* or (2) *hbz* promotes an activator of *tax*. For two reasons, the first hypothesis is more attractive. First, since *hbz* is transcribed from the (-) strand of the HTLV-1 proviral genome, it is a natural antisense RNA to other viral transcripts; it could form a double stranded (ds)-RNA structure with other viral transcripts, which may be degraded by cellular nucleases or merely inhibit their translation. Second, it is notable that another HTLV-1 gene encoding p30^{II}, has a repressive function on *tax* (Nicot et al., 2004), similar to that manifested by loss of *hbz*. The p30^{II} protein retains doubly spliced *tax* mRNA in the nucleus, thereby decreasing the levels of Tax protein. It is notable that the *hbz* transcript overlaps those encoding pX open reading frames I and II, which encode p12^I and p30^{II} and p13^{II}, respectively (Fig. 1). Of the three accessory proteins encoded by these regions of the viral genome, only p30^{II} has been shown to directly regulate *tax* expression (Albrecht and Lairmore, 2002).

Hence, we hypothesized that *hbz* inhibits *p30^{II}* mRNA, and the reduction in p30^{II} protein expression leads to an increase in the levels of Tax protein. Moreover, steady state levels of *p30^{II}* mRNA are low compared to the level of *hbz* mRNA, in contrast to levels of *tax* and *gag* mRNAs, thus *p30^{II}* mRNA would be predicted to be exquisitely sensitive to inhibition by a complementary RNA sequence.

The discrepancy between the currently reported studies and those reported previously based on studies of the effects of shRNAs to *hbz* (Arnold et al., 2008), can be explained by the fact that the expression of p30^{II} may have been suppressed in the transformed cell lines used in the previous study. Furthermore, it is unclear whether the shRNAs were able to reduce the levels of *hbz* RNA effectively enough to repress its effect on *p30^{II}*. Another difference between the current study with an infectious proviral clone and the previous study using transformed cell lines may be related to differences in levels of *hbz* mRNA and its mechanism of gene expression. It is unclear whether *hbz* expression in transformed cell lines is derived predominantly from intact proviruses or deleted proviruses (Matsuoka and Green, 2009). Expression of *hbz* mRNA from a deleted provirus in ATLL cell lines may repress *tax* expression from intact proviruses in the same cell, which may resemble the results in the current study in which *trans*-complementation effects of *hbz* were examined (Fig. 4). Although (+) strand transcriptional occlusion of the *hbz* promoters in an intact provirus may inhibit its expression, we found only four-fold lower levels of *hbz* mRNA generated from the full provirus compared to that generated from the 3' half of the provirus, under our transient transfection conditions (data not shown). Thus, promoter occlusion is not likely a major

restrictive mechanism for *hbz* expression except when the 5' LTR promoter is highly upregulated (Greger et al., 1998; Palmer et al., 2009).

To examine the effect of *hbz* on *p30^{II}*, we measured levels of *p30^{II}* mRNA in the presence of increasing amounts of wild-type HBZ and an HBZ protein mutant. We found that levels of *p30^{II}* mRNA were significantly reduced in either case, implicating *hbz* mRNA for this function (Fig. 5). Furthermore, we generated two *p30^{II}* mutants in the context of the *hbz* wild-type and *hbz* splice-deficient mutant plasmids, to examine the effect of *p30^{II}* on Tax activity. We observed that in the absence of *p30^{II}*, there was an increase in the levels of Gag proteins, and virus particle production (Fig. 5). Further studies focusing on the relationship between *hbz* and *p30^{II}* mRNA will further define the detailed molecular mechanism for this interaction.

The current studies implicate *hbz* mRNA rather than HBZ protein in promoting Tax expression and activity. Mutations that disrupt the coding capacity of *hbz* but do not affect *hbz* mRNA, pACH.HBZ-KO and pACH.HBZ-TRUN, did not perturb Tax expression (Figs. 2 and 3). It has been proposed that a stem-loop structure at the 5'-end of *hbz* mRNA is important for its growth-promoting activity (Satou et al., 2006). It is conceivable that this stem-loop RNA sequence may bind specific cellular proteins or is processed into microRNA that could inhibit other viral genes. However, in the current studies, pACH.HBZ-SL exhibited similar activity to pACH.HBZ-WT with respect to Tax expression (Fig. 3). This suggests that the effects of *hbz* mRNA in promoting Tax expression are not specifically localized to the 5' stem-loop structure.

An interesting recent observation is that the spliced *hbz* transcript (*hbz SPI*) and the pX region are present in all ATLL cell lines, regardless of levels of *tax* expression

(Matsuoka and Green, 2009; Satou et al., 2006). Since it is known that Tax elicits a strong cytotoxic T-lymphocyte (CTL) response *in vivo*, it is conceivable that the virus has evolved intricate mechanisms to regulate the levels of Tax, in order to maximize its spread. It is possible that *hbz* and p30^{II} work together to provide this tight regulation early in infection. A possible model of how the *hbz* mRNA controls *tax* expression, via p30^{II}, is shown in Fig. 6. This model proposes that *hbz* mRNA down-regulates *p30^{II}* mRNA and inhibits synthesis of the p30^{II} protein (Fig. 6, step 1). Thus, in the presence of *hbz* mRNA, less p30^{II} protein is present in the nucleus (step 2) to retard *tax* mRNA nucleo-cytoplasmic transport (step 3). Consequentially, *tax* mRNA is more readily transported to the cytoplasm, and translated (step 4). Therefore, in the presence of *hbz* mRNA, there is an increased level of Tax protein and thus, increased levels of other viral gene products. These hypotheses, testable in animal models and clinical studies, may provide new insights into HTLV-1 pathogenesis.

Materials and Methods

Cells

293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FetalClone III (FCIII) serum (Hyclone), 2mM glutamine, 1mM sodium pyruvate, penicillin (100 U/mL), and streptomycin (100µg/mL).

Plasmids

The HTLV-1 molecular proviral clone pACH was used in this study (Kimata et al., 1994). Site directed mutagenesis was performed by a PCR overlap extension method (Higuchi, Krummel, and Saiki, 1988). pACH.HBZ-ΔSA was generated by introducing a T to G silent point mutation (nt. 7268), which abolishes the splice acceptor site of the *hbz* gene. pACH.HBZ-KO was generated by introducing a G to A point mutation (nt. 7258), which terminates the HBZ protein at amino acid seven (HBZ SP1 open reading frame). This mutation also results in an arginine to glutamine change in the p30^{II} accessory protein. However, this does not affect the activity of p30^{II} (Arnold et al., 2006). pACH.HBZ-TRUN was generated by introducing a C to A point mutation (nt. 6807), which terminates the HBZ protein at amino acid 158, and truncates the protein by deleting its leucine zipper domain. pACH.HBZ-SL was generated by mutating the sequence ⁸⁶⁷⁰GGC⁸⁶⁷² to TTT. This change was based on a previously described mutant, which mutates the stem-loop structure in the *hbz* gene (Satou et al., 2006). pACHΔp30 and pACH.HBZ-ΔSAΔp30 were generated by introducing a C to G point mutation (nt. 6850) in pACH and pACH.HBZ-ΔSA, respectively. This mutation terminates the p30^{II}

protein at amino acid eight. The sequence numbers are based on that of pACH. To ensure that the mutations had no effect on the integrity of the proviral plasmids, we digested the plasmids with the PvuII restriction enzyme, to check for any gross deletions in the plasmids, and sequence analysis of the plasmids was performed. The HBZ expression vectors, pHBZ1 and pHBZ2, were generated by cloning the *hbz SPI* cDNA into the SnaBI and XbaI and EcoRI and XbaI sites of p3xFLAG-CMVTM-14 (Sigma-Aldrich), respectively. The mutant HBZ expression plasmids, pHBZ-KO, pHBZ-TRUN, and pHBZ-SL, were generated in a similar manner to pHBZ1, by cloning the mutant *hbz SPI* cDNAs. The modified HBZ expression vector, pNHBZ, was generated by cloning the *hbz SPI* cDNA into the EcoRI and BamHI sites of p3xFLAG-CMVTM-10 (Sigma-Aldrich). The pNHBZ-ΔSA plasmid was generated by amplifying the sequence corresponding to the two *hbz SPI* exons and the intronic region from pACH, and cloning into the same plasmid as pNHBZ. The pmHBZ plasmid was generated by mutating the initiating ATG in pHBZ2 to TTG. The pMHP30^{II}_{HAI} plasmid was a generous gift from Dr. C. Nicot (Nicot et al., 2004). The LTR-luciferase (LTR-luc) reporter plasmid and the CMV-Tax plasmid have been described previously (Rauch et al., 2009; Smith and Greene, 1990).

Transfections and luciferase assay

To test the various mutants, 5×10^6 293T cells were transfected with 10ug of pACH, a mutant proviral plasmid, or an empty plasmid, in the presence or absence of 5ug of pHBZ1. At 96h post-transfection, the cells were washed from the plate and pelleted. The supernatants were filtered through 0.45μm filters (Corning) and subjected to

ultracentrifugation in a Beckman-Coulter L7 Ultracentrifuge. These lysates were used for Western blot analysis. The cell pellets were washed with PBS; half of the cells were used for real-time RT-PCR, while the other half was used for Western blot analysis. 293T (3×10^6) cells were transfected with 5ug of pHBZ1 or pNHBZ, a mutant HBZ expression plasmid, or an empty plasmid. At 48h post-transfection, the cells pellets were lysed and used for Western blot analysis. 293T (3×10^6) cells were transfected with 3ug of pMHp30^{II}_{HA1}, and either 0.5ug, 2ug, or 8ug of pHBZ2 or pmHBZ, or 5ug of pHBZ2 or pmHBZ. DNA levels were kept constant with empty vector. At 48h post-transfection the cell pellets were lysed, and used for real-time RT-PCR, or Western blot analysis. To test for Tax activity, 2.5×10^5 293T cells were transfected with 1ng LTR-luc, 1ug of pACH/mutant, pCMV-Tax, and 0, 2, 3, or 4ug of pHBZ. Empty vector was included to equalize the amount of DNA added, where necessary. At 48h post-transfection, the cell pellets were resuspended in lysis buffer and subjected to a luciferase assay (Luciferase Assay System, Promega). All transfections were carried out using *TransIT-LT1* Transfection Reagent (Mirus) according to the manufacturer's recommendations. Five million Jurkat cells were transfected with 15ug of pACH.HBZ-WT or pACH.HBZ-ΔSA proviral plasmids, using polyethylenimine (PEI) transfection reagent (Sigma-Aldrich).

Quantitative real-time RT-PCR

Total RNA was extracted from transfected 293T cells using the RNeasy kit (Qiagen). One microgram of RNA was subjected to reverse transcription using the SuperScript™ III First-Strand Synthesis System (Invitrogen). Gene-specific reverse primers were used to generate cDNAs; the primers used were as follows: Tax (GctaxR) –

⁷⁶⁴⁹CCATTTCCGGAAGGGGGAGTATTTGC⁷⁶²⁵, *hbz SP1* and total *hbz* (GChbzoR) –
⁶⁸⁵⁷TTGTCTCCACTTGCGCTCACGGCG⁶⁸⁸⁰, and p30 (GChbzoF) –
ATGGTAACTTTGTATCTGTAGGGC. Reactions were performed both in the
presence and absence of reverse transcriptase to control for DNA contamination. The
cDNAs (3µL) were subjected to real-time PCR using a 2X iQ SYBR Green Supermix
(Bio-Rad) and gene-specific primer pairs (500nM each), as follows: Tax (GctaxF) –
⁵¹¹⁷AGCTGCATGCCCAAGACCCGTCGGA⁵¹⁴¹ and GctaxR, HBZ SP1 (GChbznF) –
⁸⁷⁹⁹TCTAAGGGAGCGCCGGACAAAG⁸⁷⁷⁸ and GChbzoR, total HBZ (GChbztotalF) –
AAACGCATCGTGATCGGCAGC and GChbzoR, and p30 – GChbzoR and GChbzoF.
The amount of each transcript present was determined based on a specific standard curve
generated from log₁₀ dilutions of plasmids containing cloned copies of the particular
amplified sequences. Samples and standards were run in triplicate and the final values
were averaged, after background values were subtracted.

Western blot analysis

Cell lysates were prepared using lysis buffer (50mM Tris-Cl, pH 6.8, 150mM NaCl, 1mM EDTA, 1% Triton X-100) plus protease inhibitors. Lysate (200µg) was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF; Millipore) membranes, blocked with 5% dry milk in TBS-Tween (10mM Tris-Cl, pH 7.5, 150mM NaCl, 0.05% Tween-20) and probed overnight with 1:100 HTLV-1 positive patient serum (p19, p24), 1:25 anti-Tax antibody (supernatant from hybridoma cell line 168A51-42; NIH AIDS Research & Reference Reagent Program) (Langton et al., 1988), 1:1000 anti-Flag antibody (Sigma-

Aldrich), 1:500 anti-HBZ antiserum (a generous gift from Dr. P. L. Green), or 1:250 anti-HA antibody (Abgent) or for 30 minutes with a horseradish peroxidase (HRP)-conjugated anti-actin antibody (1:2000; Santa Cruz Biotechnology, Inc.). After washing with TBS-Tween, the appropriate HRP-labeled secondary antibody was added (1:3000 anti-human antibody [Amersham], 1:5000 anti-mouse antibody [Amersham], or 1:2000 anti-rabbit antibody [Pierce]). The blots were developed using SuperSignal West Femto substrate (Pierce) and the proteins were visualized with an Alpha Innotech imager (Model: ChemiImager).

Acknowledgements

The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: 168A51-42 from Dr. Beatrice Langton. We thank Dr. C. Nicot for the pMHP30^{II}_{HA1} plasmid, and Dr. P. L. Green for the HBZ antiserum. We thank Dr. D. Rauch for critique of the manuscript and experimental advice. Supported by NIH grants CA94056, CA63417, and CA10073.

Fig. 1. Schematic representation of the HTLV-1 genome. The viral mRNAs, their direction of transcription and location of the open reading frames (black boxes) are shown. *hbz SP1* and *SP2* are two spliced variants of *hbz* mRNA, while *hbz US* is the unspliced transcript. The dotted lines represent the introns removed after mRNA splicing. The arrows represent the primers used to amplify the viral mRNAs for the RT-PCR assays. The white cross in p30 represents the location of the p30 stop codon, introduced to generate the Δ p30 mutants. The black line above *hbz SP1* represents the location of the stem-loop structure in *hbz*.

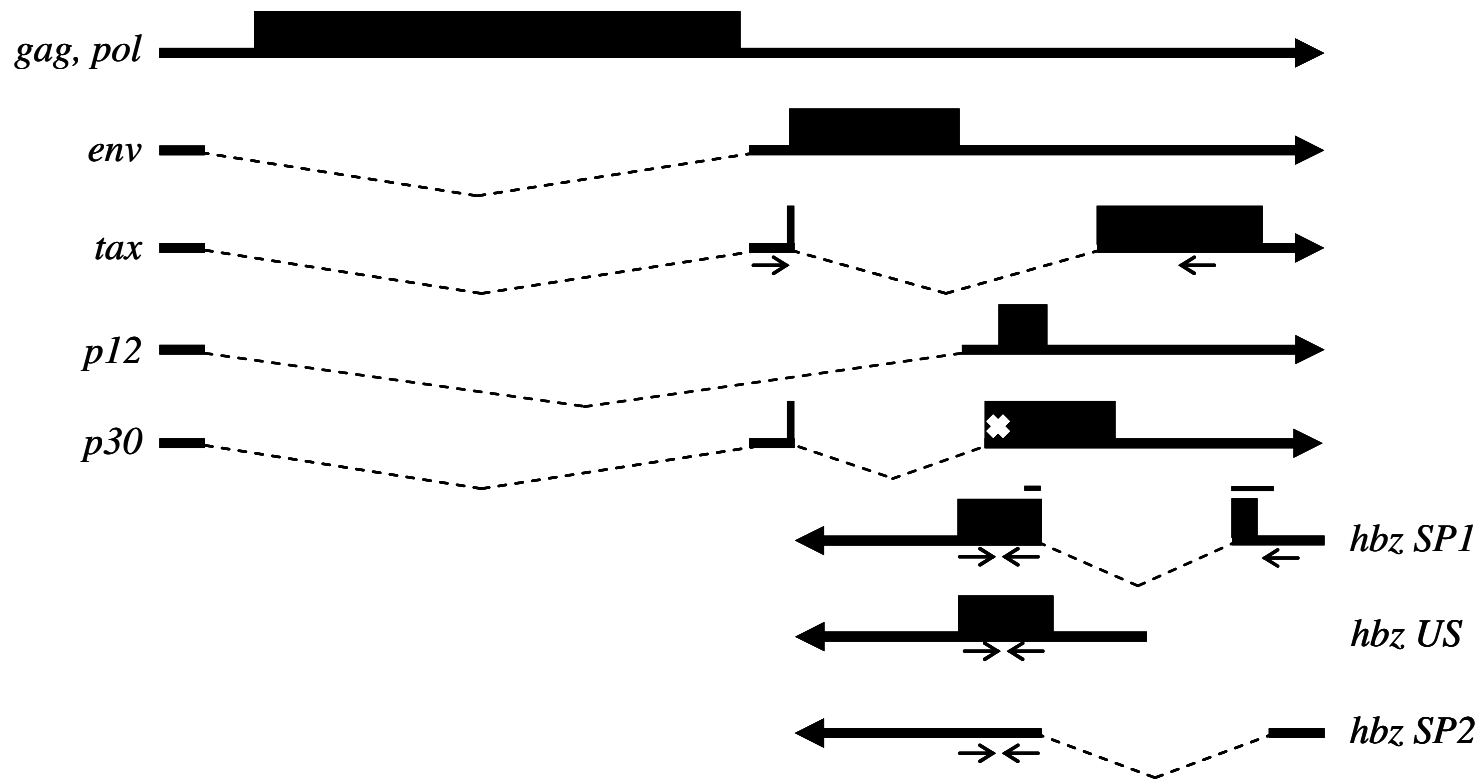
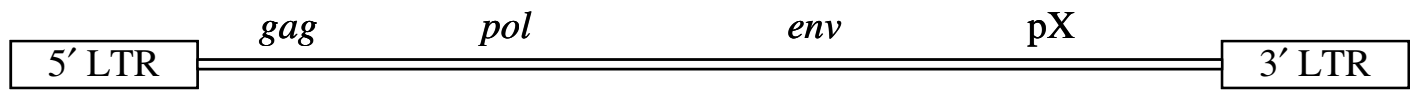
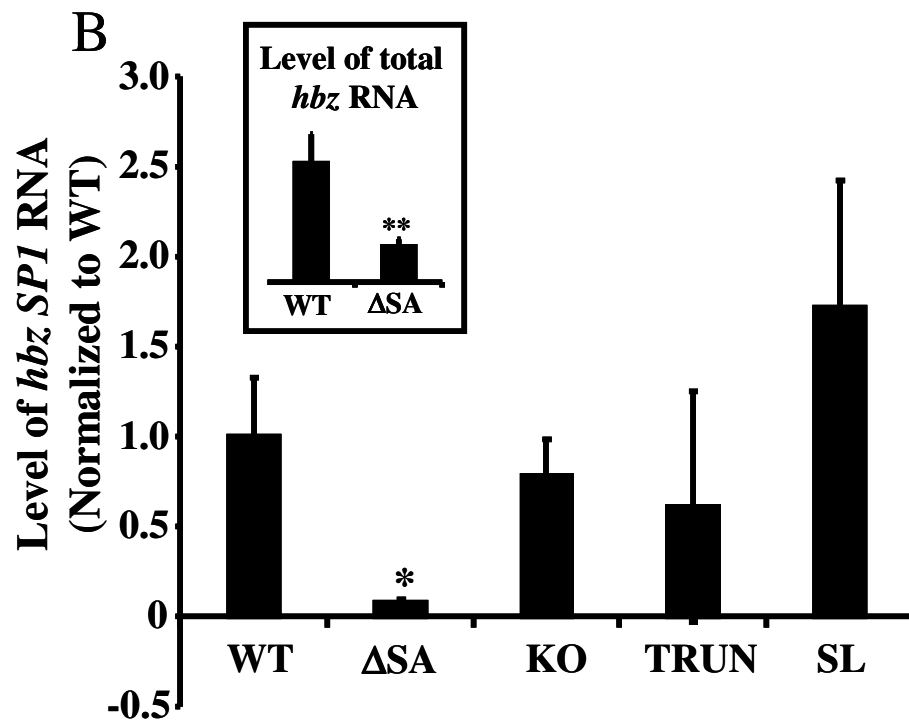
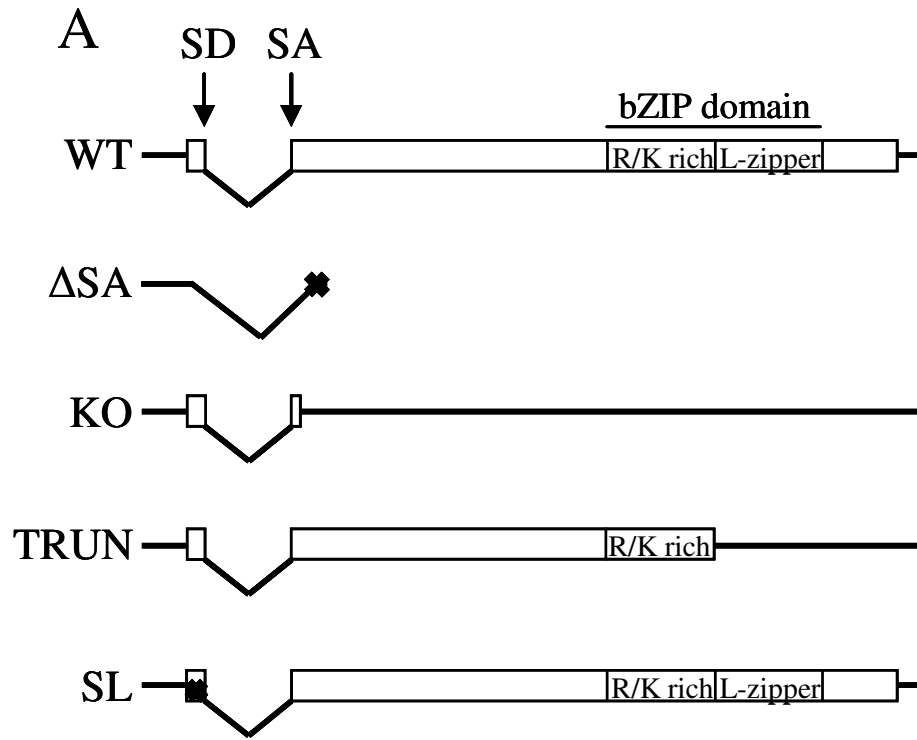


Fig. 2. Analysis of the *hbz* mRNA transcript and HBZ protein. (A) Schematic representation of wild-type HBZ (WT), with its splice donor (SD) and acceptor (SA) sites and bZIP domain indicated. The four HBZ mutants generated for this study are also depicted. These mutants were made in the context of the pACH proviral clone. Δ SA, splice-deficient mutant; KO, knockout mutant; TRUN, truncation mutant; SL, stem-loop mutant. (B) 293T cells were transfected with the pACH.HBZ-WT or mutant plasmids. Total RNA was isolated 96h post-transfection, and the *hbz SPI* mRNA amplified by real-time RT-PCR using primers shown in Fig. 1. Background values were subtracted and data normalized to WT. GAPDH was amplified as an internal control, and did not differ significantly between samples. These results represent an average of at least three independent experiments. Inset, total *hbz* mRNA was amplified by real-time RT-PCR using the primers within exon 2 of the *hbz* gene shown in Fig. 1. Background values were subtracted and data normalized to WT (Δ SA value is 0.31). Student's two-tailed *t* tests were performed to determine significant differences between samples (*, $P < 0.05$; **, $P < 0.01$). (C) 293T cells were transfected with pHBZ1 (pHBZ) or mutant HBZ expression plasmids. Western blot analysis was carried out to show HBZ-flag expression from these plasmids, using anti-Flag antibody. The size of the proteins are indicated to the right of the blot. Detection of actin was carried out as a loading control. (D) 293T cells were transfected with pNHBZ or pNHBZ- Δ SA expression plasmids. Western blot analysis was carried out to show HBZ expression (arrow) from these plasmids, using anti-HBZ antibody. Detection of actin was carried out as a loading control. The background bands (bkgd. bands) are indicated to the left of the blot. **, the intensity of this band (as compared to the others) is probably due to protein degradation. It cannot

represent an HBZ protein translated using an internal ATG, since there is no start site that would produce a protein of that size.



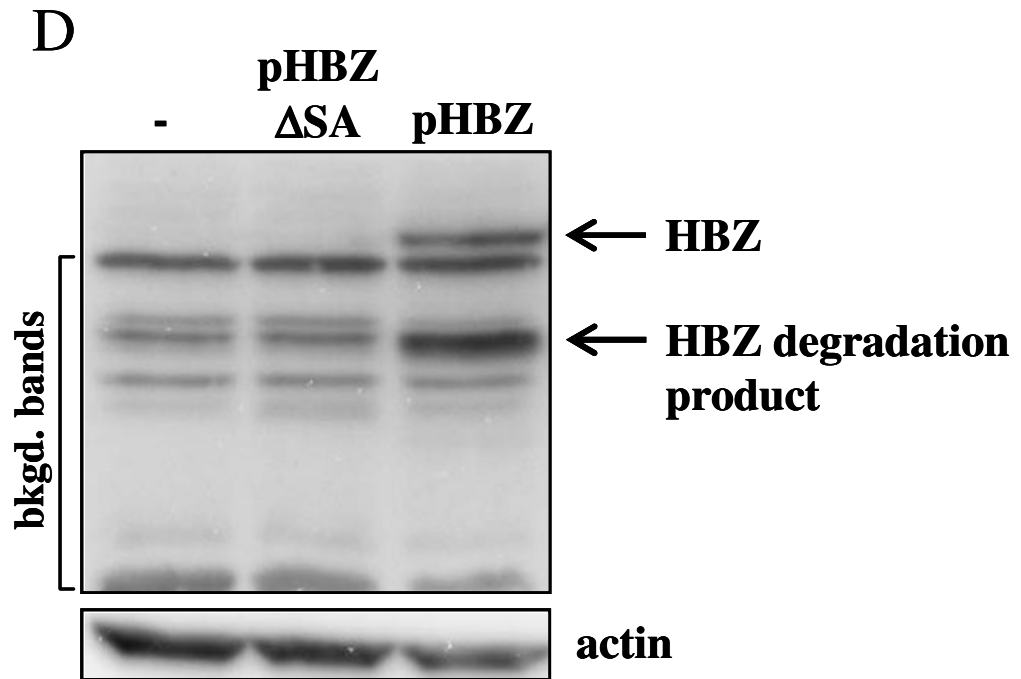
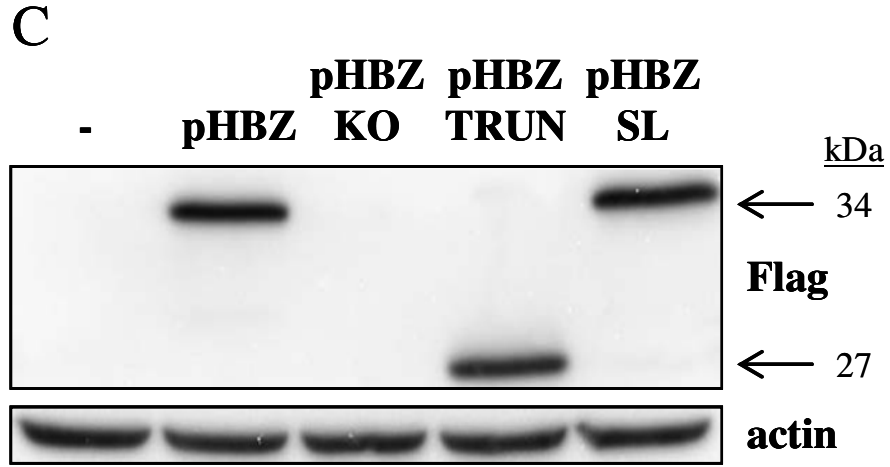


Fig. 3. HTLV-1 gene expression in the absence of *hbz* mRNA or HBZ protein. (A) 293T cells were transfected with the pACH.HBZ-WT or mutant plasmids. Total RNA was isolated 96h post-transfection, and the *tax* mRNA amplified by real-time RT-PCR, using primers shown in Fig. 1. GAPDH was amplified as an internal control, and did not exhibit significant differences between samples. These results represent an average of four independent experiments. Student's two-tailed *t* tests were performed to determine significant differences between samples (*, $P < 0.05$). (B and C) Western blot analysis was carried out to measure levels of Tax and Gag (p19 and p24) proteins in the (B) lysates and (C) viral supernatant. Detection of actin was carried out as a loading control. The numbers above the blots represent the lane numbers, while the numbers below the blots represent the densitometry signal of the bands. A representative experiment is shown from a total of three experiments.

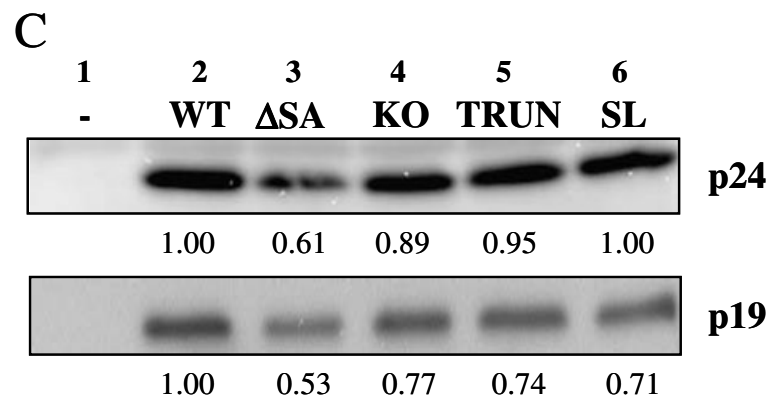
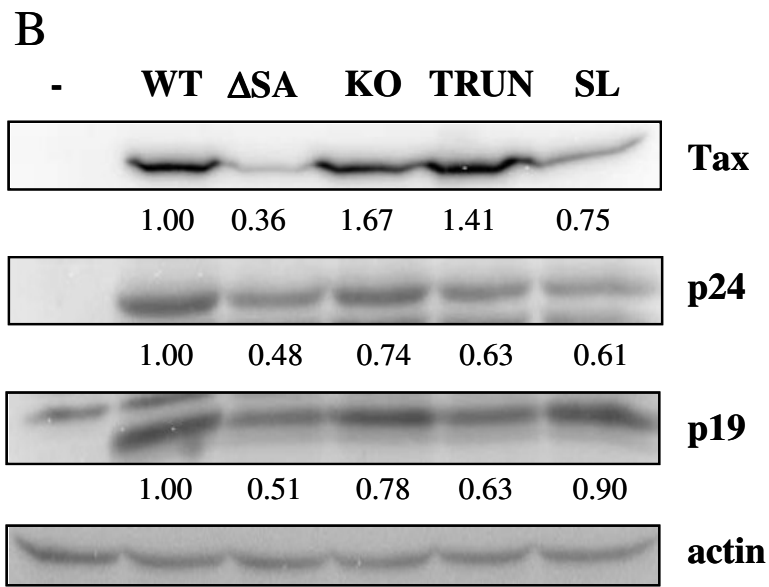
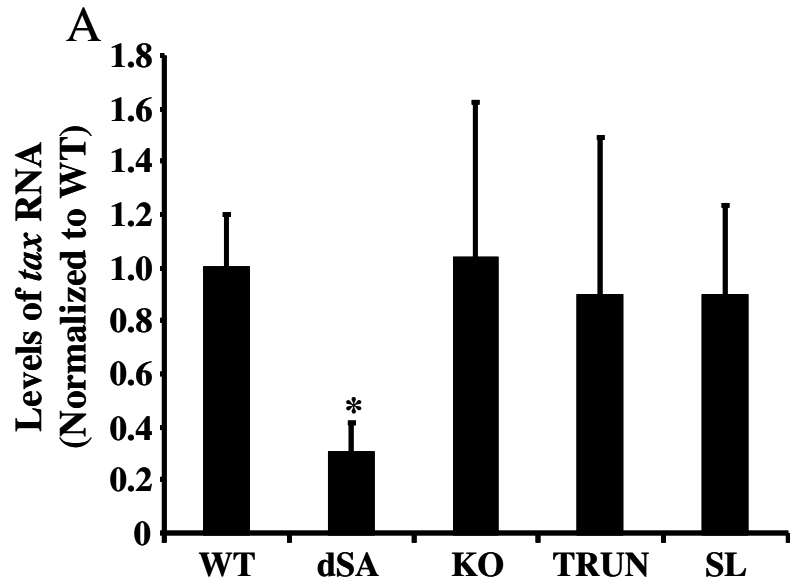


Fig. 4. **Trans-complementation with heterologous *hbz* rescues the defect seen with the mutation of *hbz* in the pACH infectious clone.** (A) 293T cells were co-transfected with LTR-luc, and with the pACH.HBZ-WT or pACH.HBZ- Δ SA plasmids, or CMV-Tax, in the presence of increasing amounts of pHBZ. Luciferase assays were carried out 48h post-transfection. Student's two-tailed *t* tests were performed to determine significant differences between samples (*, $P < 0.05$; **, $P < 0.01$). A representative experiment is shown from three total experiments. (B and C) 293T cells were transfected with the pACH.HBZ-WT or pACH.HBZ- Δ SA plasmids in the presence or absence of pHBZ. At 96h post-transfection Western blot analysis was carried out to measure levels of Gag (p19 and p24) proteins in the (B) lysates and (C) viral supernatant. Detection of actin was carried out as a loading control. The numbers above the blots represent the lane numbers, while the numbers below the blots represent the densitometry signal of the bands. Student's two-tailed *t* tests were performed to determine significant differences between samples (*, $P < 0.05$).

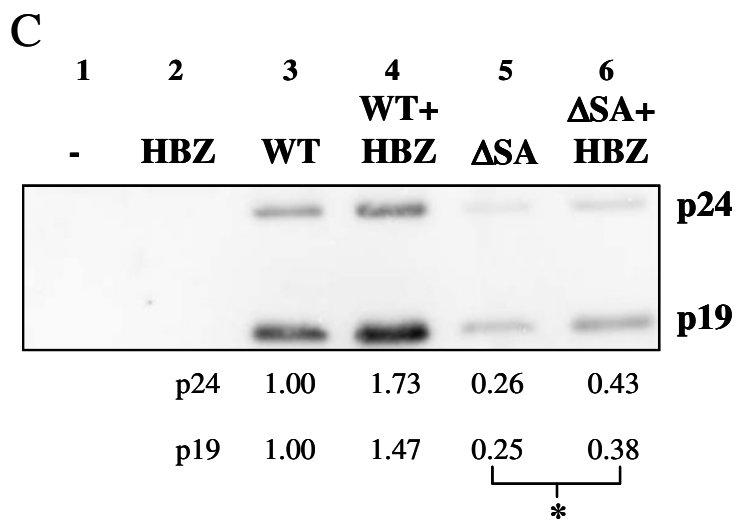
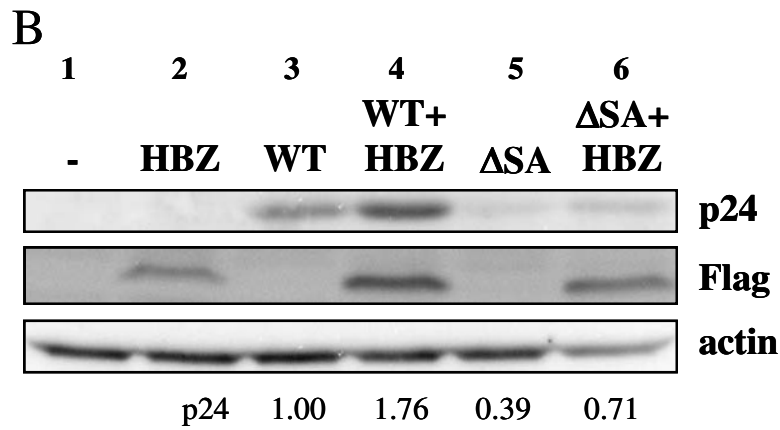
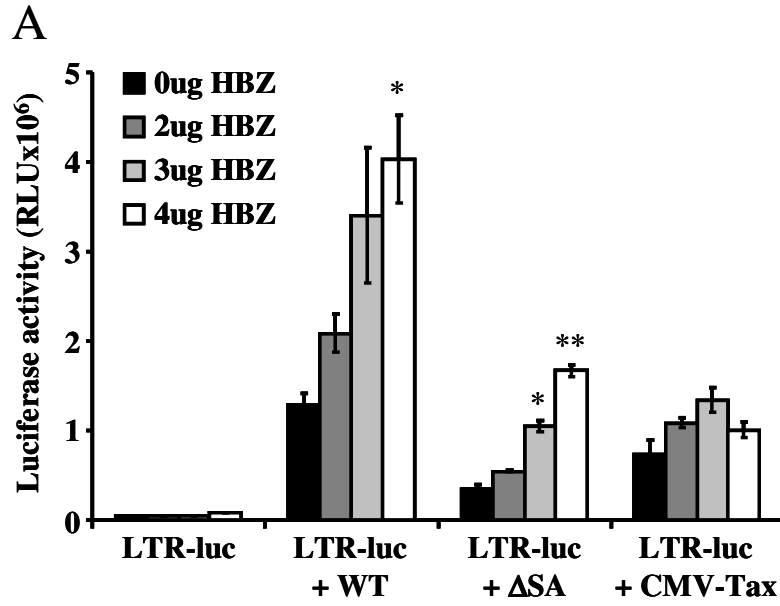
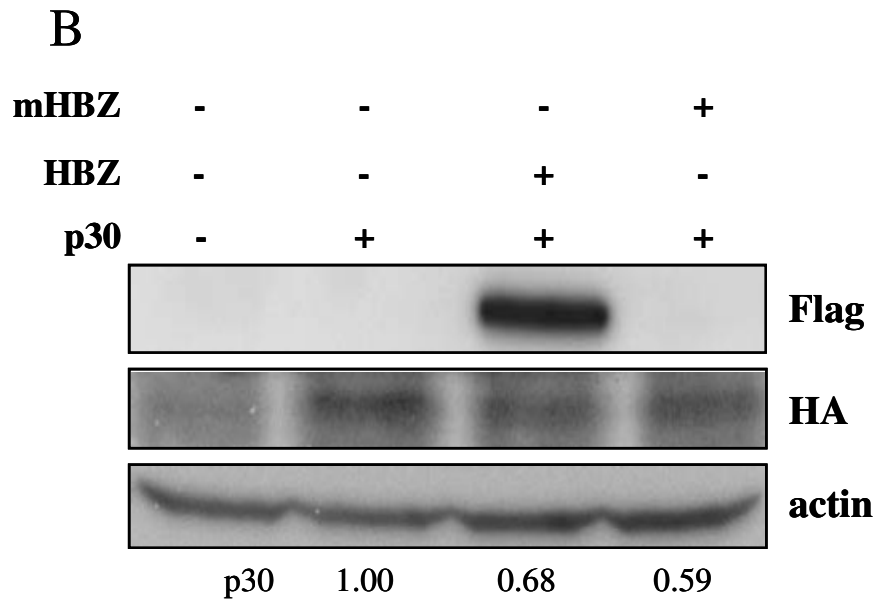
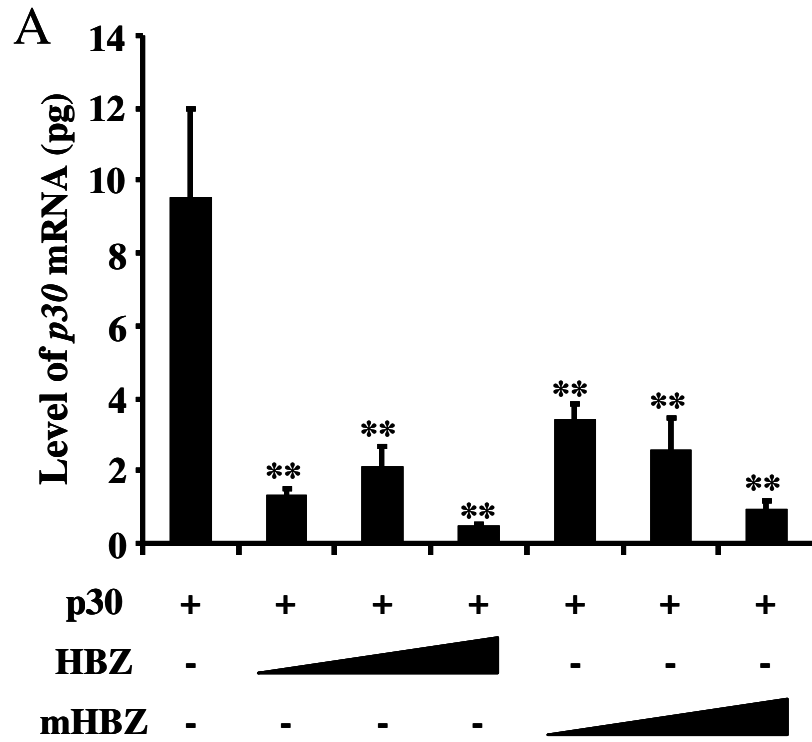
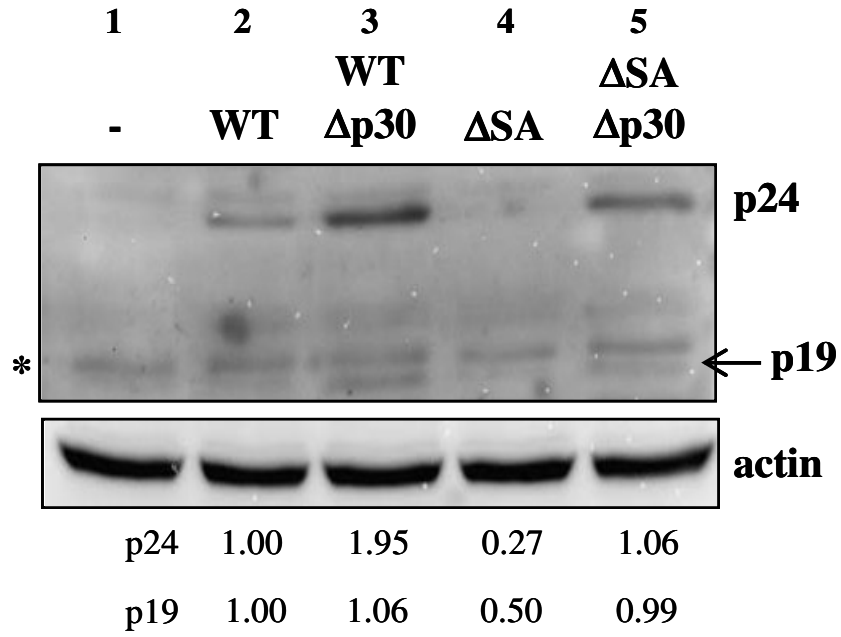


Fig. 5. Expression of *hbz* depresses p30^{II} levels, and loss of p30^{II} compensates for loss of *hbz*. (A) 293T cells were transfected with pMHp30^{II}_{HA1} (p30) and increasing amounts of either pHBZ2 (HBZ) or pmHBZ (mHBZ). Total RNA was isolated 48h post-transfection, and the *p30* mRNA amplified by real-time RT-PCR. GAPDH was amplified as an internal control, and did not exhibit significant differences between samples. A representative experiment is shown from two experiments. Student's two-tailed *t* tests were performed to determine significant differences between samples (**, *P* < 0.01). (B) 293T cells were transfected with pMHp30^{II}_{HA1} (p30) and either pHBZ2 (HBZ) or pmHBZ (mHBZ). Western blot analysis was carried out 48h post-transfection to measure levels of HBZ (Flag) and p30 (HA) protein. Detection of actin was carried out as a loading control. The numbers below the blots represent the densitometry signal of the bands. (C and D) 293T cells were transfected with the pACH.HBZ-WT or pACH.HBZ-ΔSA plasmids with or without the p30^{II} mutation, WTΔp30 and ΔSAΔp30. At 48h post-transfection, Western blot analysis was carried out on the (C) lysates and (D) viral supernatant for levels of viral Gag (p19 and p24). *, indicates background bands. Detection of actin was carried out as a loading control. The numbers above the blots represent the lane numbers, while the numbers below the blots represent the densitometry signal of the bands.



C



D

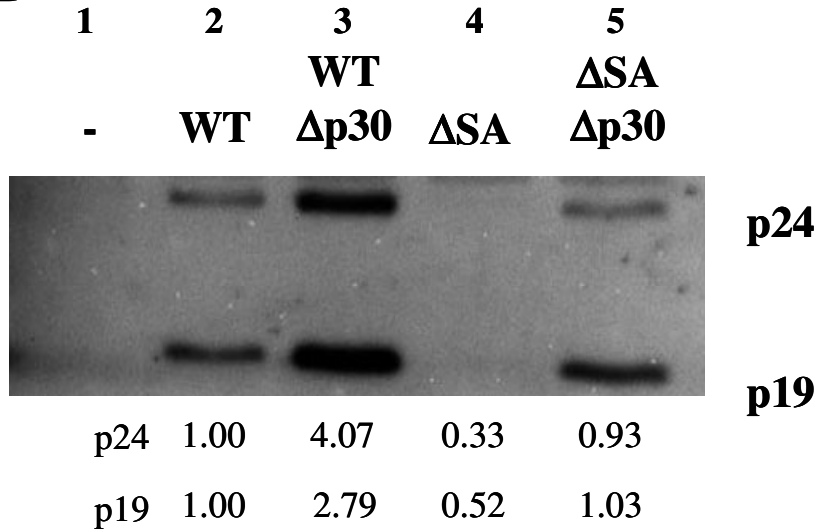
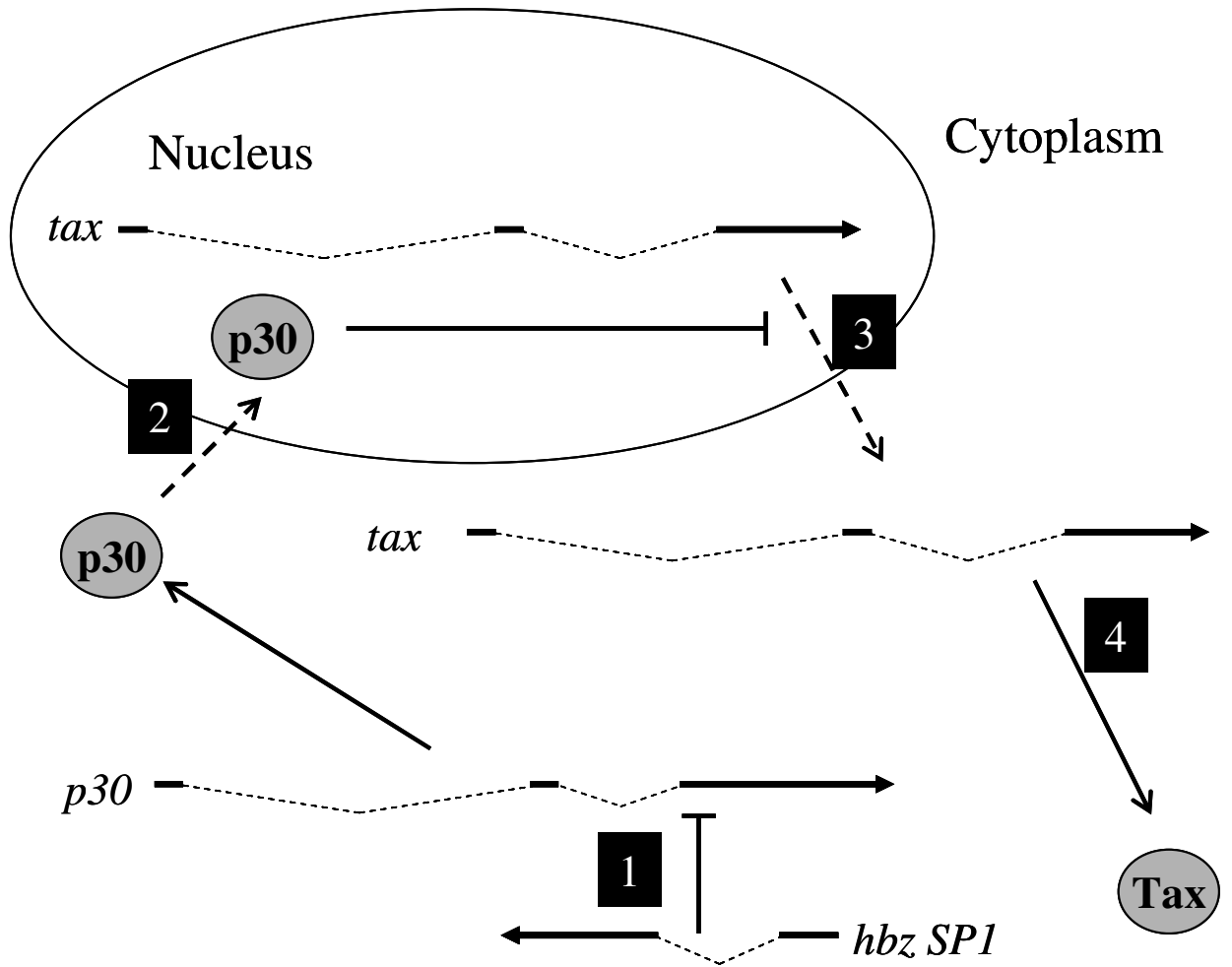


Fig. 6. Model for effect of *hbz* on *tax*. Schematic representation of a possible mechanism for *hbz*'s control of *tax*: (1) *hbz* mRNA inhibits *p30^{II}* mRNA, which (2) leads to a reduction in the amount of p30^{II} protein. Thus, less p30^{II} is translocated to the nucleus, and as a result, (3) less *tax* mRNA is retained in the nucleus. (4) This results in the synthesis of more Tax protein, leading to an increase in viral gene expression and virus production.



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Chapter 4

Discussion and Future Directions

Human T-cell leukemia virus type 1 (HTLV-1) infection depends on several virus-cell interactions to promote a productive replication. Viral tropism and gene expression are major determinants of the levels of virus replication. Viral tropism refers to cell-type specific differences in levels of virus expression in culture or *in vivo*. For most viruses, tropism is regulated at the level of cell entry or at the level of viral gene expression (Ashok and Atwood, 2006; Marshall and Major, 2010; Tan et al., 2007). The first step in establishing an infection is viral entry. For this to occur, the viral Env must bind to a cell surface receptor. However, entry into a cell does not guarantee the completion of the replication cycle. Numerous post-entry events and/or cellular factors can influence the outcome of an infection, and hence, tropism. In the case of HTLV-1, it is thought that its *in vivo* infection is probably influenced by a number of post-entry blocks, due to the discrepancy between its *in vivo* and *in vitro* tropisms, rather than exclusively by its interaction with a receptor. However, it is difficult to conclusively state what influences HTLV-1 infection, since its receptor is still unknown. A number of factors have been implicated in enhancing its entry (Jain et al., 2009; Jin et al., 2009; Jones et al., 2005; Lambert et al., 2009; Manel et al., 2003), but none to the level that would be expected for a cellular receptor.

Viral gene expression is an important factor in the outcome of an infection, since it influences the way a virus interacts with its host. In addition to structural and enzymatic genes, the HTLV-1 genome encodes several regulatory genes, including Tax, Rex, p12^I, p30^{II}, p13^{II}, and HBZ. Of these, Tax is considered to be the most important since it is a potent *trans*-acting transcriptional factor (Boxus et al., 2008), required by the virus to transform cells (Akagi, Ono, and Shimotohno, 1995; Matsuoka and Jeang, 2007).

Rex is a positive regulator of infection, which is involved in the nuclear export of unspliced or singly spliced viral RNA (Heger et al., 1999). The p12^I protein has been implicated in viral replication and T-cell activation (Albrecht and Lairmore, 2002), while p30^{II} has been shown to be a negative post-transcriptional regulator (Albrecht and Lairmore, 2002; Baydoun, Bellon, and Nicot, 2008). The p13^{II} protein has not been as well studied, but could possibly be involved in influencing the balance between viral latency and productive infection (Silic-Benussi et al., 2010). HBZ is a newly identified gene that has numerous possible functions. In its protein form, it is thought to down-regulate Tax-mediated viral transcription (Matsuoka and Green, 2009), while in its RNA form, it is thought to promote proliferation of T-cells (Arnold et al., 2008; Satou et al., 2006). Taken together, it is evident that HTLV-1 viral gene expression influences many aspects of its infection and/or replication.

In an effort to better understand the processes that regulate HTLV-1 infection, we sought to identify the viral receptor by carrying out a retroviral cDNA library screen of a permissive cell-line. Additionally, we investigated the role of the novel *hbz* gene in viral gene expression.

VIRAL TROPISM

Earlier studies aimed at receptor detection used numerous experimental approaches to do so. Several groups identified factors based on their ability to either positively or negatively affect HTLV-1 Env-induced syncytia formation (Al-Fahim et al., 1999; Daenke and Booth, 2000; Daenke, McCracken, and Booth, 1999; Hildreth, 1998;

Hildreth, Subramaniam, and Hampton, 1997; Imai et al., 1992; Niyogi and Hildreth, 2001; Pique et al., 2000; Sagara et al., 2001; Sagara et al., 1998; Uchiyama, Ishikawa, and Imura, 1996). Factors such as tetraspanins, integrins, and lipid raft-associated proteins were implicated in this manner. Several groups studied factors such as HSPGs and DC-SIGN, as they had previously been shown to bind viral envelopes of other viruses (Jones et al., 2005; Okuma et al., 2003; Pinon et al., 2003), or facilitate fusion of HTLV-1-infected T cells (Jain et al., 2009). One group looked at the functional properties of the HTLV-1 Env itself, by performing peptide mapping experiments (Sagara et al., 1996). GLUT-1 was investigated based on the observation that cultured cell lines transfected with the HTLV-1 receptor binding domain blocked acidification of the cell culture media (Manel et al., 2003). NRP-1 was studied since it was shown to be a constituent of the immune synapse and have properties consistent with those for a HTLV-1 receptor (Ghez et al., 2006). Despite the plethora of information gained from these experiments, they were all similar in the fact that they investigated factors based on their ability to somehow influence viral entry.

Our approach was designed to tackle the receptor issue in a different manner and in a broader sense. We developed a retroviral cDNA library screen to detect proteins that could serve as a receptor or attachment factor for HTLV-1. The screen was based on introducing cDNA from a highly permissive cell line, which should express any potential receptor/attachment factor, into a poorly susceptible cell line, infecting the resulting cDNA-expressing cells with HTLV-1 pseudotyped viral particles, selecting for cells that permitted infection, and isolating any cDNAs expressed in those cells. We introduced a retroviral HeLa S3 cDNA library into murine NIH 3T3 cells, and isolated approximately

460 cDNAs. Of these, 20 were prioritized based on the number of times they were isolated, whether they were membrane proteins, and whether they had been implicated as HIV-dependency factors (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008) (Table 1).

The implications of finding an entry mediator(s) for HTLV-1 are tremendous, since cell surface receptors have been shown to be major determinants of cell tropism and pathology. First and foremost, a receptor determines the route of virus entry into the host. This can reveal, for example, whether a viral particle enters a cell via endocytosis or whether a signaling pathway is activated, which allows for entry. Furthermore, it reveals information about the tropism of a virus which, in the case of HTLV-1, could explain the discrepancy between its *in vivo* and *in vitro* tropisms. The receptor can also determine the pattern of viral spread. We know that HTLV-1 is most efficiently spread through cell-to-cell contacts *in vivo* (Igakura et al., 2003; Majorovits et al., 2008); finding the receptor could help better understand this process. Furthermore, though a variety of hematopoietic cells can be infected by HTLV-1 *in vivo*, CD4⁺ T-cells seem to be the largest reservoirs for the virus. If CD4⁺ T-cells express the receptor, and the receptor is involved in promoting cell-to-cell contacts between T-cells, then these cells would be efficient at promoting viral spread, which could explain the *in vivo* cell preference. One of the more important implications of determining the receptor for HTLV-1 would be its ability to act as a drug target. Drugs designed to inhibit the HTLV-1-receptor interaction at the point of virus entry or attachment would provide a way to prevent infection even before it gets started. Additionally, determining the mode of viral entry and spread also opens these processes up as potential drug targets.

Table 1 **List of prioritized receptor candidates**

Receptor Candidate	MP^a	MH (#)^b	HDF^c
Guanine nucleotide-binding protein subunit beta-2-like 1 (GNB2L1)		● (5)	
Peroxiredoxin-1 (PrdX1)		● (10)	
Phosphatidylinositol glycan anchor biosynthesis, class T (PIGT)		● (13)	
Killer cell lectin-like receptor subfamily K, member 1 (KLRK1)	●		
HLA-A, -B, and -C (Major Histocompatibility Complex I)	●		●
HLA-DRB1 (Major Histocompatibility Complex II)	●		
Flotillin-1 (FLOT-1)	●	● (2)	
CD68	●	● (3)	●
Solute carrier family 16, member 3 (SLC16A3)	●		
Solute carrier family 38, member 5 (SLC38A5)	●	● (2)	●
Ras-related protein Rab3D		● (2)	●
Ligatin (LGTN)	●		
Transmembrane BAX inhibitor motif containing 6 (TMBIM6)	●	● (3)	
Transmembrane emp24 domain-containing protein 10 (TMED10)		● (2)	●
Transmembrane 4 L6 family member 1 (TM4SF1)	●		
Protease, serine 21 (PRSS21)	●		
Transmembrane and coiled-coil domain-containing 1 (TMCO1)		● (3)	
Yip1 domain family member 3 (YIPF3)	●		
Ankyrin repeat domain protein 16 (ANKRD16)			●
F-box/WD repeat containing protein 5 (FBXW5)		● (2)	●

^a MP - membrane protein

^b MH (#) - multiple hit (number of hits)

^c HDF - HIV-dependency factors identified in Brass et al. (2008), König et al. (2008), and Zhou et al. (2008)

Future directions

The 20 candidate factors need to be evaluated to determine if any function independently as a receptor/attachment factor for HTLV-1. NIH 3T3 cells expressing these candidates should be generated and tested for entry using HTLV-1 Env pseudotyped particles. Any candidate factors conferring entry should be further evaluated by fusion assays. Previous studies have shown that immunoadhesions, fusions between a protein of interest and the Fc region of human IgG, between the HTLV-1 SU and Fc region of human IgG maintain the biochemical properties of SU, and are a useful and sensitive tool in evaluating receptor-Env interactions (Jassal, Pohler, and Brighty, 2001). Furthermore, it would be useful to determine if expression of the candidate factor in permissive cell lines is necessary for HTLV-1 Env infection. Abrogation of expression of the candidate with short interfering RNAs (siRNAs) would help in answering this question. Since GLUT-1, NRP-1, and HSPGs have been implicated in enhancing viral entry, it might be useful to determine if the candidate factor works cooperatively with one or more of these factors, to promote viral entry.

One reason for the discrepancy with *in vivo* and *in vitro* tropisms could be that *in vitro* experiments use only the HTLV-1 Env to determine viral entry, while *in vivo* infections involve the entire virus. This would suggest that the tropism of HTLV-1 is not determined solely by its Env-receptor interaction, but possibly at least partially by a post-entry selection step. To test the candidate factors against the entire virus, it would be useful to infect the NIH 3T3 cell-expressing candidates with viral particles generated using the cell-free infection assay developed by Derse et al. (2001). This system generates virus stocks by transfecting cells with three plasmids: (i) a packaging plasmid

encoding HTLV-1 structural and regulatory proteins, (ii) an HTLV-1 transfer vector containing the firefly luciferase gene, and (iii) an envelope expression plasmid (Derse et al., 2001). Single-round infections can be carried out and viral entry determined by the level of luciferase activity. Testing the 20 candidate factors by these methods should narrow down the list and potentially identify a receptor/attachment factor candidate for HTLV-1.

VIRAL GENE EXPRESSION

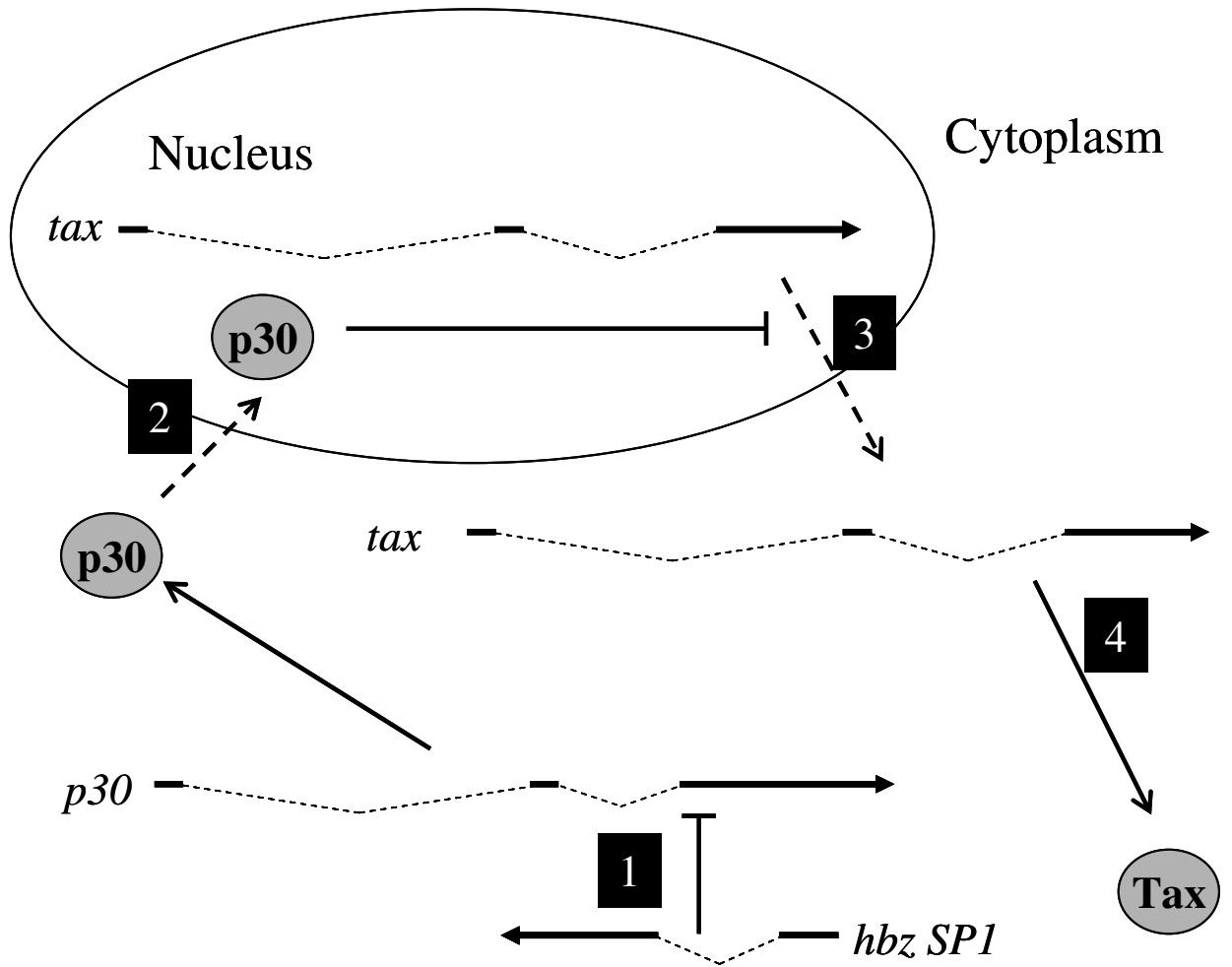
The HTLV-1 bZIP gene, *hbz*, is alternatively spliced and has three isoforms, two of which are spliced, *SP1* and *SP2*, and one which is unspliced, *US*. The *hbz SP1* and *hbz US* transcripts encode proteins, which share >95% homology. The levels of *hbz SP1* transcript are four times more abundant than levels of the *hbz US* transcript in ATLL cells (Usui et al., 2008). The *hbz* gene has dual functionality as the HBZ SP1 and HBZ US proteins are able to down-regulate Tax-mediated HTLV-1 transcription and Tax-mediated cellular transcription (Matsuoka and Green, 2009), while the *hbz SP1* mRNA has been shown to promote proliferation of T-cells (Arnold et al., 2008; Satou et al., 2006). In our efforts to better understand the role of *hbz* in HTLV-1 gene expression, we found a third function of *hbz* in which *hbz* mRNA promotes expression of *tax* mRNA, via down-regulation of *p30^h* mRNA.

We generated two RNA and two protein mutants of HBZ in the context of the ACH molecular clone. We found that in the absence of *hbz* mRNA, the levels of *tax* mRNA were also significantly decreased. This correlated to a decrease in the levels of

Tax protein, and to decreased levels of Tax activity. Furthermore, when we added exogenous *hbz*, the levels of Tax activity increased. Moreover, we noted that exogenous *hbz* only had an effect on Tax activity when Tax was expressed from the proviral clone, and not when Tax was expressed alone, without other viral proteins. This suggested that the effect of *hbz* on *tax* was not direct, and that some other viral gene(s) was involved. Since *hbz* has (-) RNA polarity, we hypothesized that it could negatively regulate one of the (+) RNA viral products, which in turn would be a negative regulator of *tax*. Hence, *hbz* could function as a repressor of a negative regulator of *tax*. The p30^{II} accessory protein is a negative regulator of *tax*, as it is able to bind *tax* mRNA and sequester it in the nucleus, preventing it from being exported into the cytoplasm and hence, being translated. We tested the effect of *hbz* on p30^{II} and found that it significantly decreased levels of *p30^{II}* mRNA, in a dose dependent manner. Furthermore, when we mutated the p30^{II} protein in the context of the ACH molecular clone which was also defective in transcribing *hbz* mRNA, we found an increase in the levels of Tax activity. Thus, we propose the following model (Fig. 1): *hbz* mRNA down-regulates *p30^{II}* mRNA, which leads to a reduction in the levels of p30^{II} protein. As a result, less p30^{II} protein is translocated to the nucleus. This leads to less nuclear retention of *tax* mRNA, and hence, more export into the cytoplasm, where it is translated. Since more Tax protein is translated, there is an increase in the levels of Tax-mediated functions.

It seems likely that HTLV-1 would control its own gene expression. Since Tax is highly immunogenic, the host mounts a strong cytotoxic T-lymphocyte (CTL) response against cells expressing Tax. Hence, this may be a mechanism whereby the virus evades immune surveillance. Most ATLL cells have been shown to have low to undetectable

Fig. 1. Model for effect of *hbz* on *tax*. Schematic representation of a possible mechanism for *hbz*'s control of *tax*: (1) *hbz* mRNA inhibits *p30^{II}* mRNA, which (2) leads to a reduction in the amount of p30^{II} protein. Thus, less p30^{II} is translocated to the nucleus, and as a result, (3) less *tax* mRNA is retained in the nucleus. (4) This results in the synthesis of more Tax protein, leading to an increase in viral gene expression and virus production.



levels of Tax. Though this phenomenon has been shown to be due to epigenetic changes of the 5' LTR, it is possible that other mechanisms could exist, such as control of gene expression. Recent studies have shown that p30^{II} is a negative regulator of HTLV-1 expression (Nicot et al., 2004), and that abrogation of p30^{II} from a molecular clone *in vivo* led to lower proviral loads and decreased viral persistence (Silverman et al., 2004). Hence, it is possible that p30^{II} is required by the virus to reduce levels of viral expression, and as a result, avoid immune recognition (Nicot et al., 2005). Due to this, the virus may have developed an additional control, to ensure that levels of Tax were not reduced too much, or too early in infection, which would prevent T-cell transformation. Since work in this dissertation has shown that *hbz* is able to promote *tax* expression by reducing the levels of *p30^{II}* mRNA, it is possible that *hbz* and p30^{II} work cooperatively to elegantly control the expression of Tax early in infection.

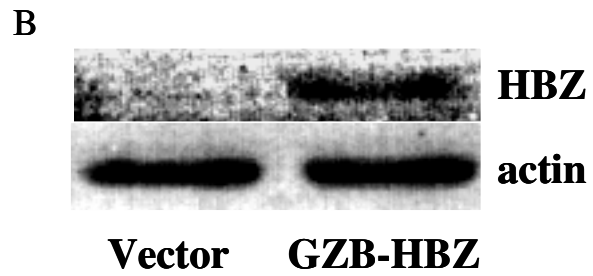
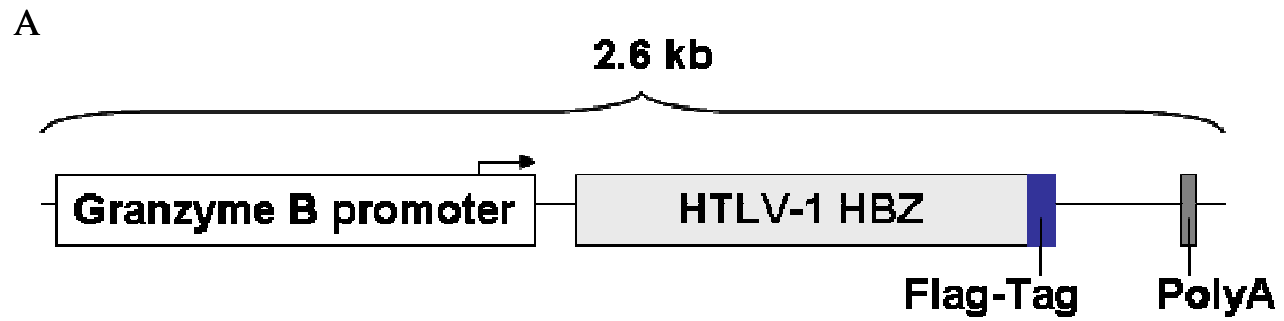
Future directions

Since *hbz* has been shown to down-regulate levels of *p30^{II}* mRNA, it would be useful to determine the mechanism of this inhibition. *hbz* is naturally an antisense RNA to genes on the positive strand of the genome, including p30^{II}. It has been evaluated for the possibility that it is processed into a microRNA (miRNA), but such a miRNA was not detected (Satou et al., 2006). Nevertheless, it would be worthwhile to independently test for the presence of miRNAs. It is possible that *hbz* and *p30^{II}* mRNAs form a double stranded (ds)-RNA structure, which could get degraded by cellular host machinery. Since ds-RNA species are processed into small interfering RNAs (siRNAs) by the

cellular protein Dicer (He and Hannon, 2004), it would be interesting to see whether Dicer is capable of degrading any *hbz/p30^H* ds-RNA species.

HBZ transgenic mice have been generated by D. Rauch and J. Harding in the Ratner laboratory. The HBZ transgene is under the control of the granzyme B promoter, which restricts HBZ expression to the mature natural killer (NK)/T-cell compartment. We have confirmed expression of HBZ from the transgene (Fig. 2). The HBZ mice do not show gross abnormalities. These mice need to be evaluated for the levels of *hbz* mRNA and HBZ protein in the different tissue compartments. In addition, the levels of NK cells and CD4⁺ T-cells should be determined, as mice generated previously showed increased levels of CD4⁺ T-cells in the spleen, in the presence of *hbz* (though the transgene in these mice was under the control of the CD4 promoter) (Satou et al., 2006). The *hbz* transgenic mice have been bred with Tax transgenic mice, also under the control of the granzyme B promoter (Grossman et al., 1995). These transgenic mice develop large granular lymphocytic leukemia. It would be interesting to see what effect, if any, *hbz* has on the tumors present in the Tax mice. Again, it would be necessary to evaluate the levels of both *hbz* and *tax* mRNA and protein in the different tissue compartments.

Fig. 2. Expression of HBZ from HBZ transgene. (A) Schematic representation of the transgene showing HBZ under the control of the granzyme B promoter. (B) 293T cells were transfected with the transgene and empty vector, and Western blot analysis with anti-HBZ antiserum and anti-actin carried out on the lysates 48 hours later.



CONCLUSIONS

The goal of this dissertation was to better understand the regulation of HTLV-1 infection and replication, as they relate to viral tropism and gene expression. As a major determinant of viral tropism is envelope-receptor binding, we designed a retroviral cDNA library screen in an effort to identify the HTLV-1 receptor/attachment factor. We have 20 potential, high-priority candidates, which we are confident will get us closer to finding the HTLV-1 receptor. In addition to being essential in regulating infection, viral gene expression is an important component of viral tropism. In an effort to dissect the role of *hbz* in gene expression, we found that there is complex interplay of viral genes, which could be important in regulating the expression of Tax, and consequently, the emergence of disease. The importance of a cellular receptor and viral genes that control Tax expression in regulating HTLV-1 infection and replication make them prime targets for the development of new therapeutics.

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Mentor: Lee Ratner, M.D., Ph.D.
Washington University School of Medicine, St. Louis, MO
Used the split-luciferase assay to determine if HIV-1 Vpx interacts with human invariant chain

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