DISSERTATION

CHARACTERIZATION OF HTLV-1 TAX MOLECULAR INTERACTION WITH THE KIX DOMAIN OF CBP

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JULITA A. RAMIREZ ENTITLED *CHARACTERIZATION OF HTLV-1 TAX MOLECULAR INTERACTION WITH THE KIX DOMAIN OF CBP* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of a rare, aggressive form of cancer called adult T-cell leukemia (ATL). The HTLV-1 oncoprotein Tax and the cellular transcription factor CREB bind to viral cyclic AMP response elements (vCREs) located in the viral promoter. Tax and serine 133 phosphorylated CREB (pCREB) bound to the HTLV-1 promoter facilitate viral transcription via the recruitment of the large cellular coactivators CBP/p300. PKA-phosphorylation of CREB at serine 133 facilitates transcription from cellular CREs by recruiting CBP/p300 via its KIX domain. However, it remains controversial whether CREB phosphorylation plays a role in Tax transactivation. We biochemically characterized the quaternary complex formed by Tax, CREB, the KIX domain of CBP, and the viral CRE by examining the individual molecular interactions that contribute to Tax stabilization in the complex. Our data show Ser133-phosphorylated CREB, KIX, and vCRE DNA are all required for stable Tax incorporation into the complex.

While the interaction between the phosphorylated kinase inducible domain (pKID) of pCREB and the KIX domain of CBP/p300 has been well-characterized, the molecular interactions between KIX, full-length Tax, and pCREB have not been examined. We further characterized the quaternary complex by examining

the interaction between Tax and KIX in a physiologically relevant complex containing pCREB and vCRE DNA. Our data show that Tax and pCREB simultaneously and independently bind two distinct surfaces on the KIX domain: Tax binds KIX at the previously-characterized mixed-lineage leukemia (MLL) protein interaction surface while pCREB binds KIX at the well-known pKID-KIX interface. These results provide evidence for a model in which Tax and pCREB bind distinct surfaces of KIX for effective CBP/p300 recruitment to the HTLV-1 promoter. During investigation of the dual binding site model, we found that a small peptide representing the minimal MLL activation domain competed with Tax for KIX binding. Tax may therefore compete with MLL for CBP/p300 binding, affecting transcription of MLL-responsive promoters in HTLV-1 infected cells expressing Tax. Chromosomal rearrangements causing MLL dysfunction characterize certain types of leukemia, suggesting a novel mechanism of Tax leukemogenesis in which normal MLL function is disrupted by Tax.

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CHAPTER 1
INTRODUCTION TO HTLV-1 TRANSCRIPTIONAL REGULATION

Chapter one begins with the background and significance of human T-cell leukemia virus, type 1 (HTLV-1), and the two most well-known diseases it causes: adult T-cell leukemia (ATL) and tropical spastic paraparesis. Selected details of the viral genome and life cycle are included to help orient the reader. The major protein players in this study, Tax, CREB, and CBP/p300 are then introduced, followed by a synopsis of the objectives and significance of the research findings.

1.1 HUMAN T-CELL LEUKEMIA VIRUS TYPE 1

A cluster of leukemia cases in southwest Japan in the late 1970s led investigators to identify a new type of cancer which they dubbed adult T-cell leukemia. This type of leukemia is extremely aggressive and almost always fatal. In 1980 and 1981, investigators from the USA and Japan separately identified HTLV-1 as the etiologic agent responsible for ATL.[1; 2; 3] This landmark finding established HTLV-1 as the first retrovirus directly associated with a human malignancy, and also described it as the first known human retrovirus. The virus requires cell-to-cell contact for transmission, and is primarily transmitted vertically through breast milk or *in utero* from mother to child, though sexual and parenteral transmission via injection or infusion can also occur. HTLV-1 is also responsible for an immune-mediated disease known as tropical spastic paresis/HTLV-1 associated myleopathy (TSP/HAM). Individuals infected with HTLV-1 do not clear

the virus from their bodies, and remain carriers for life. However, only a small percentage of infected individuals ever develop disease as a result of their infection.

1.1a ADULT T-CELL LEUKEMIA

ATL was first described as a distinct clinical entity in 1977 by Takatsuki *et al.* Three years later, the HTLV-1 retrovirus was identified by Poiesz *et al.* during analysis of a T-cell line from a patient that had erroneously been diagnosed with cutaneous T-cell lymphoma.[1] Hinuma *et al.* demonstrated a further connection between ATL and HTLV-1 by identifying an antibody against HTLV-1 in sera of ATL patients.[2] Yoshida *et al.* then went on to definitively demonstrate HTLV-1 is the causative agent of ATL by identifying monoclonally-integrated HTLV-1 provirus in transformed ATL cells.[3] While ATL and its causative virus have only recently been described, it is not a recently-emerged disease like HIV. Phylogenetic analysis has revealed that HTLV-1 diverged from STLV in Africa around 50,000 years ago.[4]

Although ATL is not a well-known disease, it remains an important cause of mortality in endemic regions such as the Caribbean basin, South America, West Africa, and southwest Japan. It has been recently estimated that 10-20 million people worldwide are infected with HTLV-1.[5] Most of the clinical knowledge of ATL comes from Japan, where approximately 1.2 million individuals are infected with HTLV-1, and 800-1,000 new cases of ATL are diagnosed each year.[6] Consistent with a long viral latency period, the average

ATL patient is 60 years old in Japan and 40 years old in Jamaica. Only ~6% of males and 2% of females infected with HTLV-1 go on to develop disease.[7] An individual's genetic background influences ATL development, since familial clustering of ATL cases has been documented.[8] Environmental and viral factors as well as the host immune response have also been implicated in leukemogenesis, though the precise mechanism remains unknown. However, it is likely that the viral protein Tax plays an important role in leukemogenesis via widespread disruption of cellular regulatory processes including DNA repair, apoptosis, and signal transduction.[9] Indeed, the Tax protein is required for cellular immortalization,[10] though in a seeming paradox *tax* transcripts are detected in only ~40% of all ATLs. While Tax initiates transformation of ATL cells, it is not needed to maintain the transformed state. After transformation has occurred, leukemic T-cells silence Tax transcription using a variety of mechanisms to evade recognition by the host immune system, specifically cytotoxic T-cells.

The clinical characteristics of ATL infection include a persistent lymphocytosis due to uncontrolled proliferation of immortalized T-cells with a characteristic lobulated, flower-shaped nucleus (see Figure 1.1). Infiltrates of leukemic cells cause lesions in multiple organs including the skin, lungs, liver, bone, central nervous system, and gastrointestinal tract. More than 70% of ATL patients experience hypercalcemia as a complication of their disease,[11] which contributes to the development of many unpleasant symptoms associated with disease progression. ATL patients also suffer from immunodeficiency, resulting in

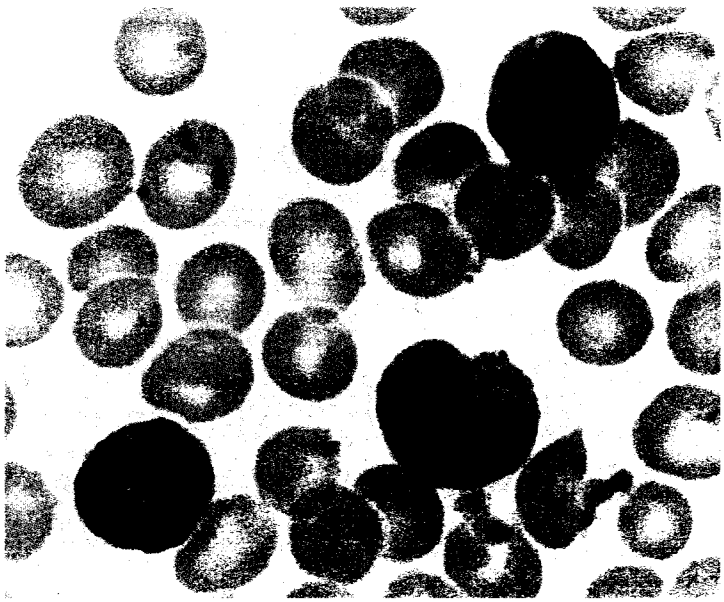


Figure 1.1 HTLV-1 infected and transformed T-cells in the peripheral blood of an ATL patient. The typical flower appearance of the hyperlobulated nuclei can be seen. From "Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL)", M. Matsuoka *Retrovirology* 2005.

many opportunistic infections. Treatment has been largely unsuccessful, since even with aggressive chemotherapy the mean survival time for ATL patients is less than one year.[12] Though stem cell transplantation regimens have shown encouraging advances in ATL treatment,[13; 14] available therapies for patients remain inadequate. Although much knowledge of the molecular biology and oncogenesis of ATL has been gained since the late 1970s, much work must still be accomplished to improve the prognosis of the disease.

1.1b TROPICAL SPASTIC PARAPARESIS

A disease known as tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) is also strongly associated with HTLV-1 infection.[15] Similar to ATL, the incidence of disease in HTLV-1-infected individuals is low: approximately 5% of individuals infected with HTLV-1 develop TSP/HAM. This disease is caused by chronic inflammation of the spinal cord resulting from a strong immune response to HTLV-1 rather than uncontrolled T-cell proliferation. TSP/HAM is a neurodegenerative disease characterized by demyelination resulting in progressive muscular weakness and paralysis, with a disease course similar to multiple sclerosis. A strong immune response triggered by various viral antigens including gag and Tax causes production of antibodies that cross-react with self proteins such as ribonuclear protein A1 and other proteins present in the human brain.[16; 17] These antibodies initiate an autoimmune response against neurons in the spinal cord. Another proposed mechanism in the pathogenesis of TSP/HAM involves the infiltration of HTLV-1 infected T-cells and anti-HTLV-1

cytotoxic T-cells into the spinal cord, possibly causing lesions through cytokine secretion.[18] The autogenous factors involved in determining whether an HTLV-1 infected individual remains asymptomatic or develops disease as a result of infection remain unknown.

1.1c VIRAL GENOME AND LIFE CYCLE

HTLV-1 is a member of the *Deltaretrovirus* genus, which includes HTLV II-IV, simian T-cell leukemia virus (STLV), and bovine leukemia virus (BLV). STLV and BLV are also associated with neoplastic diseases, while HTLV II is not. All members of the *Deltaretrovirus* genus share the pX genomic region, which encodes an imperfectly conserved protein 353 amino acids in length known as Tax. The pX region also encodes Rex, a protein involved in mRNA trafficking[19], and several accessory proteins (p12, p13, p21, p30, and HBZ). Tax is a unique transcriptional activator that exerts pleiotropic effects on cellular proliferation and participates in oncogenesis via the ATF/CREB, NF-κB, and SRF pathways.[20]

HTLV-1 can infect various cell types, including both mature CD4+ and CD8+ T-cells via interaction with the GLUT1 receptor.[21] It has also recently been suggested that surface heparan sulfate proteoglycan can serve as a receptor for HTLV-1.[22] While both GLUT1 and heparin proteoglycan are ubiquitously expressed on the surface of many cell types, most *in vivo* infection is detected in memory CD4+ T-cells.[23] This finding suggests that while HTLV-1 is capable of infecting many cell types, infection selectively increases abundance of particular cellular subpopulations.

The HTLV-1 virion contains two copies of a single-stranded positive sense RNA genome, 9 kB in size.[24; 25; 26] In addition to the unique viral proteins encoded in the pX region of the genome, the HTLV-1 genome also encodes the capsid (CA), nucleocapsid (NC), and matrix (MA) retroviral structural proteins in the *gag* region. The *env* genomic region encodes the transmembrane (TM/gp 21) and surface (SU/gp 46) glycoproteins, while the *pol* region encodes reverse transcriptase (RT, converts viral RNA to DNA), integrase (IN, integrates viral DNA into the host cell genome), and protease, which cleaves viral polyproteins. Two identical long terminal repeats (LTRs) flank the transcribed region of the provirus at the 5' and 3' ends. These LTR elements contain promoter sequences important for regulation of viral transcription.[24; 27; 28] Each LTR is composed of an R, or repeat region, as well as U3 and U5 regions. Three semiconserved 21 base pair repeats called viral cyclic AMP response elements (vCREs) are located within the U3 region of each LTR at approximately -250, -200, and -100 base pairs from the transcription initiation site. Each vCRE contains a core off-consensus octanucleotide CRE element with GC-rich flanks to which Tax binds.[29; 30] These sites are critical for transcription of viral genes, as mutagenesis studies have shown that at least two vCREs are necessary for activated viral transcription.[28] A schematic of the HTLV-1 provirus is shown in Figure 1.2.

HTLV-1 requires cell-cell contact and formation of a virological synapse for effective transmission. This is demonstrated by the fact that cell-free HTLV-1 virions are not detected in the sera of infected individuals, and *in vitro* infectivity by virions in tissue culture is much lower than that achieved by cell-to-cell

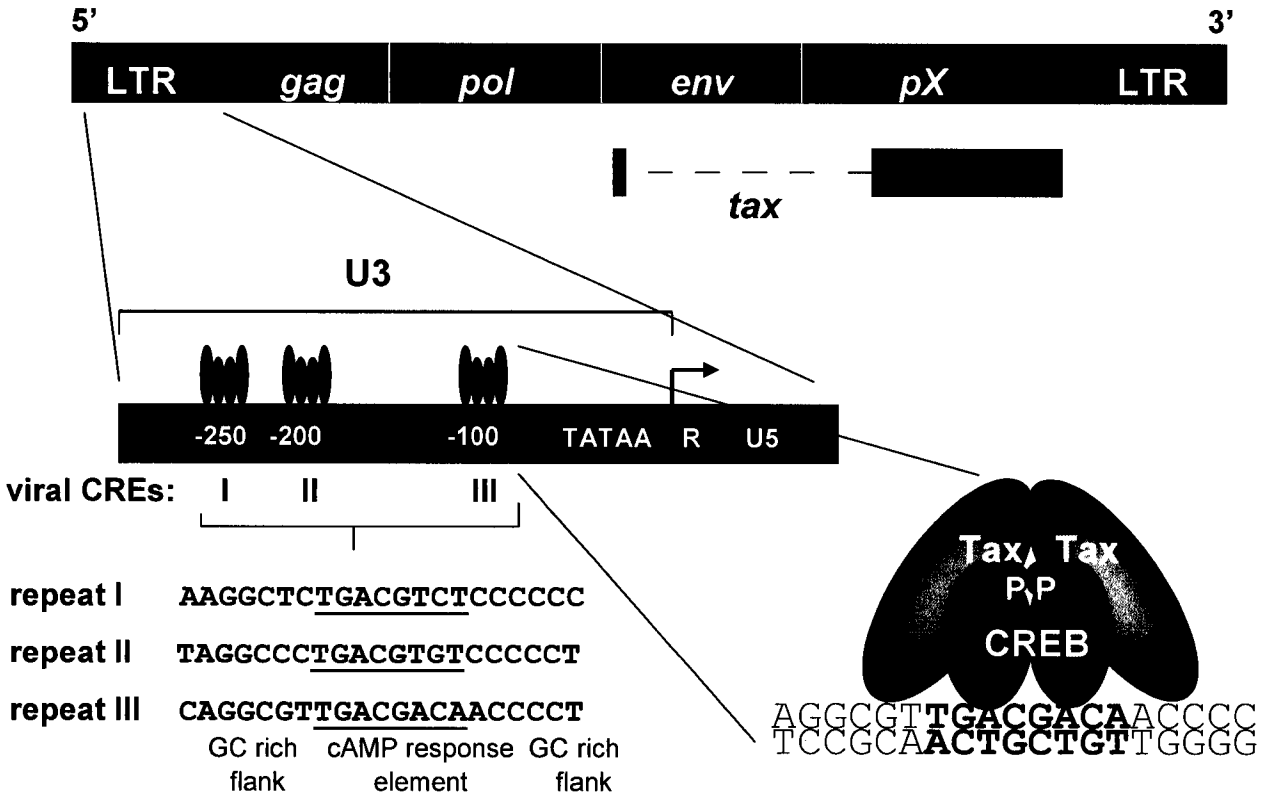


Figure 1.2 Schematic of the HTLV-1 provirus and promoter with viral CREs shown binding pCREB and Tax.

transmission. After formation of a virological synapse, the HTLV-1 gag complex and viral genomic RNAs accumulate at the synapse and migrate into the uninfected cell. Synapse formation is triggered by the Tax protein as well as cell-cell contact, as Tax has been shown to promote microtubule organizing center (MTOC) formation and ICAM1 engagement.[31] The interaction of ICAM1 and lymphocyte function-associated antigen 1 (LFA1) is important for HTLV-1 infection.[32] As LFA1 is expressed on T lymphocytes, this mechanism is consistent with preferential infection of T lymphocytes by HTLV-1 *in vivo*. After infection of a human T-cell, the HTLV-1 RNA genome is reverse transcribed to DNA in the cytoplasm. The viral DNA is then randomly integrated into the host cell genome by integrase. After integration a long latency period occurs during which the viral genome lies dormant within the cell. During this time the virus is propagated primarily through mitotic division of infected cells. An unknown stimulus, possibly associated with the development of ATL or TSP/HAM, triggers transcription of the provirus after a variable period of time.

1.2 TAX-MEDIATED ACTIVATION OF HTLV-1 TRANSCRIPTION

HTLV-1 randomly integrates into the chromosomal DNA of the host cell. Therefore, the mechanism of leukemogenesis employed by HTLV-1 does not typically involve disruption of cellular proto-oncogenes. In addition, the viral oncoprotein Tax does not share significant amino acid sequence homology with any known cellular protein. Several *in vitro* and *in vivo* studies demonstrate that Tax is directly responsible for ATL development, since introduction of the Tax

gene is sufficient for cellular transformation and development of a variety of solid tumors and leukemia in mice.[33; 34; 35]

The Tax protein is necessary for HTLV-1 transactivation and subsequent expression of viral genes,[36; 37; 38; 39] though it does not perform this function by itself. Tax participates in both protein-DNA and protein-protein interactions with cellular transcription factors bound to the HTLV-1 LTR. These Tax-containing nucleoprotein complexes provide the framework for high-level expression of the HTLV-1 genome.

1.2 a TAX-CREB INTERACTIONS

The core octanucleotide CREs present within the three viral CRE elements in the HTLV-1 LTR bind proteins in the ATF/CREB family of cellular transcription factors.[40; 41; 42] The ATF/CREB family of transcription factors is one of the largest eukaryotic protein families, including 53 different proteins in humans.[43] All ATF/CREB family members share a basic leucine zipper (bZIP) motif that binds DNA through sequence-specific interaction with the major groove. The bZIP motif is a dimeric parallel coiled-coil domain capable of forming either homodimers or heterodimers with other bZIP proteins. The ability of bZIP proteins to dimerize with each other results in a large number of possible transcription factor combinations, each with the potential to differentially regulate transcription.

Of this large family, the role of CREB in HTLV-1 transcriptional activation has been studied in greatest detail. CREB is a 43 kilodalton transcription factor

that regulates approximately 20% of all mammalian protein-coding genes.[44] Its functions include glucose metabolism, cellular survival and proliferation, learning, and memory. More recently CREB has also been shown to play a role in human leukemias.[45; 46] Although CREB binds CRE DNA enhancer elements in promoters from a wide array of genes, the role it plays in the activation of genes involved in the cAMP signaling pathway has been studied the most extensively. In addition to the bZIP domain, CREB contains several other domains. These include the kinase-inducible domain (KID) involved in transcriptional regulation, and the glutamine-rich Q2 domain involved in basal transcriptional activity through interaction with the general transcription factor TAFII130/135. A schematic illustrating the functional domains of CREB is shown in Figure 1.3. CREB transcriptional activity is regulated in part by phosphorylation of the KID at multiple sites by many different kinases.[47]

During HTLV-1 transcriptional activation, CREB dimers bind an octanucleotide off-consensus CRE site. The affinity of CREB for the viral CRE is weaker than for the consensus cellular CRE due to a single base pair difference within one of the half CREs comprising the viral CRE core.[48] Tax mediates strong CREB binding to the viral CRE by increasing the affinity of CREB for the viral CRE through direct protein-protein interactions.[49; 50; 51] Tax enhances CREB binding to the viral CRE by stimulating CREB dimerization.[48; 52; 53]

Since Tax does not stably interact with CREB bound at cellular CREs,[50; 54] the protein-protein interactions between Tax and CREB are necessary but not sufficient for the formation of the ternary complex on the HTLV-1 promoter. The

Figure 1.3. Schematic of CREB functional domains. The basic leucine zipper (bZIP) domain binds DNA and Tax. The glutamine-rich domain (Q2) mediates basal transcriptional activity through interaction with TAFII130. The kinase-inducible domain (KID) mediates signal-induced activation, including phosphorylation by PKA, AKT, PKC, MEK/ERK, and CaMK kinases, among others. These enzymes phosphorylate CREB at serine 133, hereafter referred to as pCREB.

ternary complex is instead stabilized through direct interactions between Tax and GC-rich DNA sequences flanking the CRE core. These GC-rich flanks are critical for Tax function both *in vitro* and *in vivo*.[29; 42; 55]

1.2b TAX-DNA INTERACTIONS

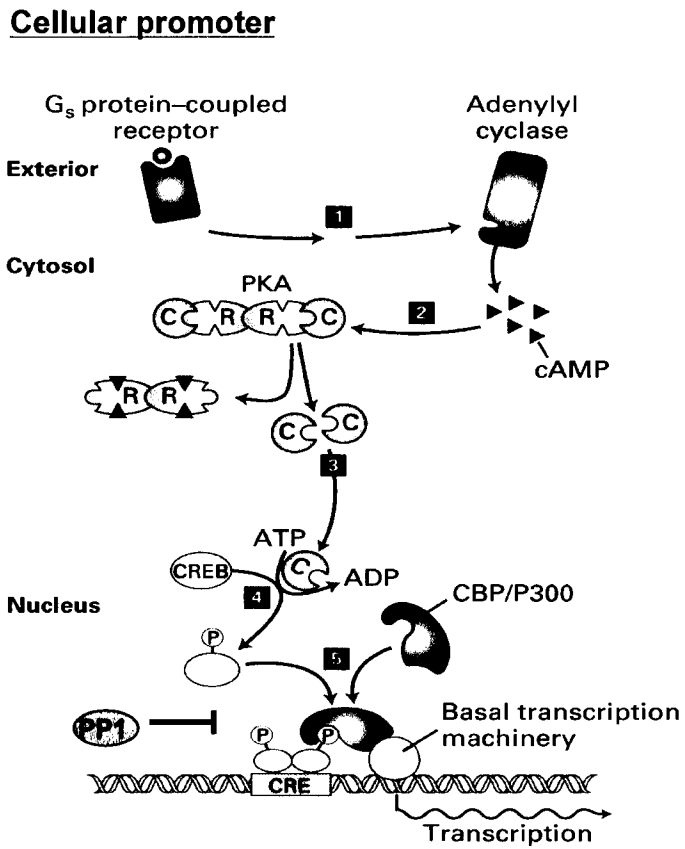
The interactions between Tax and the viral CRE DNA make an important contribution to formation of the high-affinity ternary complex incorporating Tax, CREB, and vCRE DNA. Tax directly binds the GC-rich flanks present in the viral CREs, but does not bind to the core CRE element.[29; 56; 57] Unlike CREB, Tax binds DNA in the minor groove as synthetic drugs designed for sequence-specific minor groove binding are able to inhibit the Tax-DNA interaction.[30] Since Tax contacts both the upstream and downstream flanks of the viral CRE, it likely binds the vCRE as a dimer.[29; 30; 56] In addition, Tax dimerization is important for Tax-dependent complex formation and transcriptional activation.[58; 59; 60] However, other evidence has indicated that Tax only requires one of the two GC-rich flanks of the viral CRE.[29; 30; 61] Until structural data are available, it remains unknown whether Tax contacts DNA as a monomer or dimer in complex with CREB.

1.2c THE ROLE OF CREB PHOSPHORYLATION IN TAX TRANSACTIVATION

CREB binding *in vivo* is regulated in a cell-specific manner, demonstrating promoter-specific occupancy depending on cell type.[62; 63] CREB promoter occupancy depends on the presence and methylation state of consensus CREs

near the promoter.[44] As implied by its name, CREB was originally characterized based on its response to a cAMP stimulus.[64] Protein kinase A (PKA) is activated by increases in intracellular cAMP and phosphorylates CREB at serine 133, activating transcription of CREB-regulated genes through recruitment of the CBP/p300 coactivators. A subsequent dephosphorylation by one of several cellular phosphatases returns CREB to its pre-activation transcriptional level. A diagram of this signaling pathway is shown in Figure 1.4. Other kinases such as Akt, PKC, MEK/ERK, and CaMK also phosphorylate CREB serine 133 in response to diverse signaling pathways including growth factor, cytokine, and steroid hormone stimuli.[47] However, CREB phosphorylation alone is not a reliable predictor of target gene activation; additional CREB regulatory partners are required for recruitment of the transcriptional apparatus to the promoter.[44]

The role of CREB phosphorylation in Tax transactivation has been controversial for decades. Previous studies have shown that CREB Ser133 phosphorylation is not necessary for effective Tax transactivation,[65] while other studies have found that PKA stimulation increases Tax transactivation and prevents apoptosis.[66; 67; 68; 69; 70] Two studies by Trevisan *et al.* support a direct role for Tax in enhancement of CREB phosphorylation *in vivo*.[71; 72] These investigators found that Tax transfection enhanced CREB phosphorylation *in vivo*, and propose that Tax might in part function by affecting the phosphorylation state of CREB. They hypothesize that Tax might do so by stabilizing the CREB/CBP complex and thus prolonging the phosphorylation state of CREB or alternatively that it might inactivate specific phosphatases or activate specific



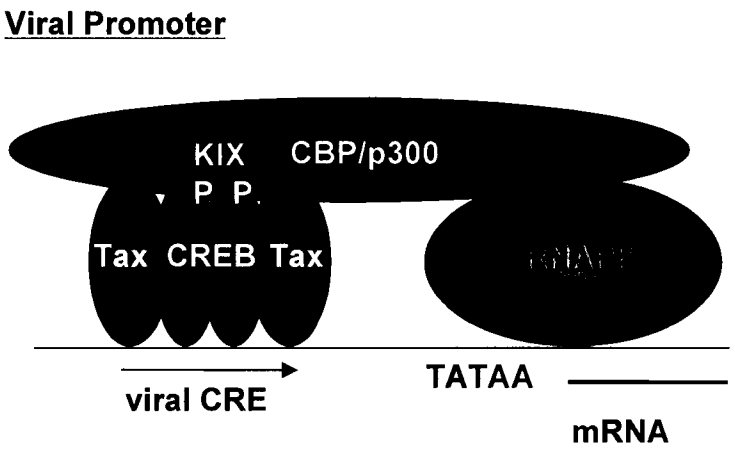


Figure 1.4 Transcriptional activation through CREB. Tax and pCREB cooperate to recruit CBP/p300, which then recruits the RNA pol II complex and other transcription factors to initiate transcription. Although PKA phosphorylation of CREB is shown, many other kinases also phosphorylate CREB.

From Lodish *et al.*, *Molecular Cell Biology* (2000)

kinases. Despite many previous studies addressing this question, the significance of CREB phosphorylation for Tax transactivation in HTLV-1 infected human T-cells remains unknown.

1.3 THE CBP/p300 COACTIVATORS

Formation of the high-affinity ternary complex composed of Tax, CREB, and viral CRE DNA is insufficient for mediating high levels of HTLV-1 transcription. Tax-mediated transactivation of the viral genome also requires the cellular coactivators CBP/p300. CBP and p300 are high molecular weight paralogous eukaryotic proteins incorporating many diverse functional domains. p300 was originally identified by co-immunoprecipitation with the adenoviral oncoprotein E1A.[73; 74] As implied by its name, CBP was later identified through its interaction with CREB.[75] Due to their similarity, CBP and p300 are widely referred to interchangeably in the literature even though they are not completely functionally identical. Although both proteins were independently identified, it soon became apparent that they share a high degree of amino acid sequence homology, are both highly evolutionarily conserved, and share a number of functional domains. CBP and p300 knockout mice display distinctly different phenotypes that vary depending on the relative dose of each protein.[76]

CBP/p300 play very important roles in regulating embryonic development, cellular proliferation, differentiation, DNA damage repair, and apoptosis.[77; 78; 79; 80] They are tremendously important for transcriptional control, as they likely regulate transcription of every gene in all metazoans. In addition, mutations of

CBP/p300 are present in certain subtypes of leukemia[76; 81; 82] and Rubenstein-Taybi syndrome. Rubenstein-Taybi syndrome is a CBP/p300 haploinsufficiency disorder in which affected individuals have developmental disorders and a high risk of tumor development.[83; 84; 85]

CBP/p300 regulate transcription through interaction with a wide array of DNA-bound transcriptional activators rather than directly binding DNA enhancer elements within promoters.[74; 86; 87] These multifunctional coactivators participate in activation of the HTLV-1 promoter through interactions with both Tax and CREB.

1.3a CBP/p300 INTERACTIONS WITH TAX AND CREB

Tax directly interacts with several domains of CBP/p300 including C/H1,[88] CR2,[89] and KIX. However, the interaction between Tax and the KIX domain of CBP/p300 has been the most extensively studied.[65; 90; 91; 92] A schematic of various CBP functional domains is shown in Figure 1.5. The KIX domain (amino acids 588-683 of CBP and 566-663 of p300) is composed of three α-helices that form a compact hydrophobic core.[93] KIX binds to the Tax/CREB/viral CRE DNA ternary complex and stabilizes it. Several lines of evidence indicate that a direct Tax-KIX interaction plays an important role in CBP/p300 binding to the ternary complex. Tax has been found to specifically bind KIX in solution,[90; 94] and two Tax point mutants (K88A and V89A) have been reported defective for KIX binding as well as transactivation both *in vitro* and *in vivo*.[92; 95] Tax also strongly interacts with the adjacent C/H1 domain of CBP/p300 (aa 302-451),[88] suggesting

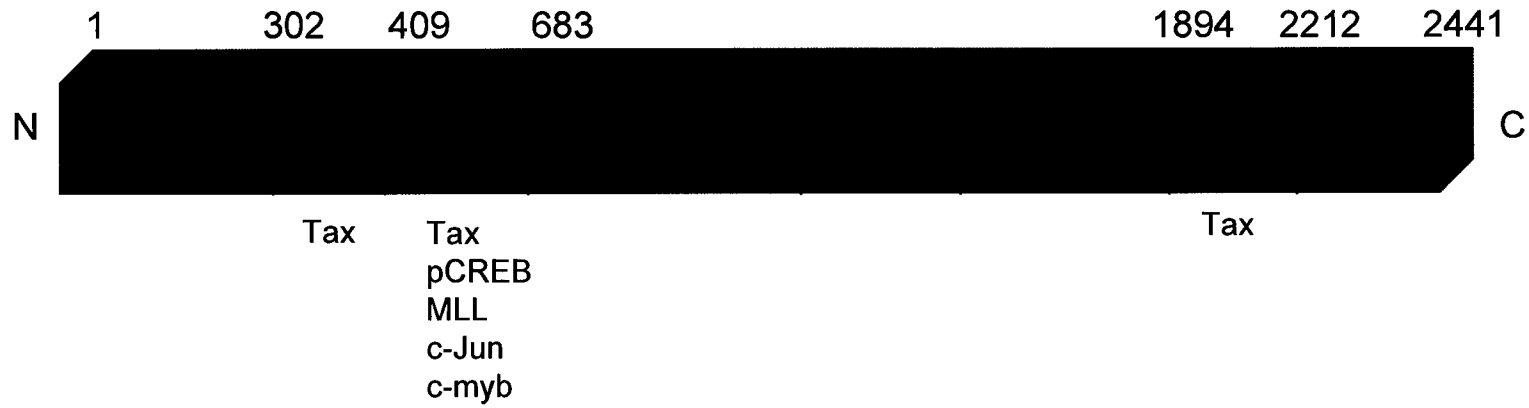


Figure 1.5 Schematic of CBP functional domains. Transcription factors mentioned in the text that bind each domain are indicated. C/H1 = cysteine/histidine-rich domain, one of three zinc finger motifs (C/H2 and C/H3 not shown). HAT = histone acetyltransferase domain. CR2 = carboxy-terminal region 2, a glutamine-rich region. Amino acid residue numbers map to mouse CBP.

an extended region consisting of the contiguous C/H1 and KIX domains interacts with Tax.

Several studies support a two-binding site model for transcription factor binding to KIX. The structure of a ternary complex containing KIX, a domain of the c-Myb transcription factor, and the minimal activation domain of mixed-lineage leukemia (MLL) protein has been determined[96] suggesting the pCREB/Tax/KIX ternary complex may be analogous to the c-Myb/MLL/KIX ternary complex. In addition, the c-Jun transcription factor has been shown to occupy the MLL binding site on KIX.[97] Tax was found to compete with c-Jun for KIX binding,[98] providing further support for a two-site binding model in which Tax and pCREB simultaneously bind separate surfaces of KIX.

While activation of cellular CREB-responsive genes by CBP/p300 is dependent on CREB phosphorylation, it remains controversial whether CREB phosphorylation augments the Tax-KIX interaction to mediate CBP/p300 binding to ternary complexes at the HTLV-1 promoter. In addition to the previously-cited studies showing cAMP stimulation activates transcription from the HTLV-1 LTR, KIX was found to bind the ser-133 phosphorylated CREB (pCREB)/Tax complex with more than 10-fold higher affinity than to the unphosphorylated CREB/Tax complex.[91] Both the CREB-KIX and Tax-KIX interactions likely contribute to the binding of CBP/p300 to the viral LTR, and play a vital role in Tax transactivation of the HTLV-1 genome.

1.4 THESIS RESEARCH OBJECTIVES AND SIGNIFICANCE

The objective of my thesis research was to characterize the molecular interactions between the various components of the Tax/pCREB/KIX/vCRE DNA quaternary complex. My aim was to examine the individual effects of ser-133 CREB phosphorylation, vCRE DNA, and KIX on Tax incorporation into the complex. In addition, I wanted to focus on the interaction between Tax and KIX and definitively show that full-length Tax and pCREB simultaneously and independently bind to separate sites on KIX. I used *in vitro* assays to achieve these objectives, including pull-down assays and electrophoretic mobility shift assays (EMSAs). My strategy was to design and purify KIX mutants that would bind pCREB but not Tax, and to show that a KIX mutant defective for pCREB binding could still bind Tax. I hypothesized that Tax and MLL bind to the same surface of KIX, and used a peptide corresponding to a small part of the MLL activation domain for competition studies with Tax. The data presented herein demonstrate that *i*) ser-133 phosphorylated CREB, the KIX domain of CBP, and vCRE DNA are all necessary for stable Tax incorporation into the quaternary complex *in vitro*, *ii)* Tax and pCREB make separate and independent contacts with KIX *in vitro*, and *iii)* the MLL activation domain inhibits Tax binding to KIX. This study defines the role of the individual components of the quaternary complex in maintaining complex stability, and provides strong evidence that Tax and MLL share the same binding surface on KIX. Tax competition with MLL for CBP/p300 binding may adversely affect transcription of MLL-responsive promoters in Tax-expressing HTLV-1 infected

cells. Since chromosomal rearrangements causing MLL dysfunction are known to characterize certain types of leukemia, this finding suggests a novel mechanism of Tax leukemogenesis in which normal MLL function is disrupted by Tax.

CHAPTER 2
MOLECULAR CHARACTERIZATION OF THE TAX-CONTAINING HTLV-1 ENHANCER COMPLEX REVEALS A PROMINENT ROLE FOR CREB PHOSPHORYLATION IN TAX TRANSACTIVATION

Chapter two describes a study of the role played by Ser133 phosphorylated CREB in Tax-mediated transactivation using a variety of *in vitro* and *in vivo* assays. I performed the experiments shown in figures 2.1D, 2.2B, 2.3B, and 2.3D. Young-Mi Kim performed the experiments shown in figures 2.4A-C, and Jeanne E. Mick performed the experiments shown in figures 2.1B, 2.2C, 2.3A, and 2.3C. Holli A. Giebler contributed the experiment shown in figure 2.2A, while Jian-Ping Yan contributed experiments shown in figures 2.1A and 2.1C. This work has been published in the *Journal of Biological Chemistry*, and is presented here exactly as it appeared in the journal. The citation for the publication is:

Kim, Y.M., Ramirez, J.A., Mick J.E., Giebler H.A., Yan, J.P., & Nyborg, J.K. (2007). Molecular characterization of the Tax-containing HTLV-1 enhancer complex reveals a prominent role for CREB phosphorylation in Tax transactivation. *J. Biol. Chem.* in press, April 2007.

2.1 ABSTRACT

Transcriptional activation of human T-cell leukemia virus type 1 (HTLV-1) is mediated by the viral oncoprotein Tax, which utilizes cellular transcriptional machinery to perform this function. The viral promoter carries three cyclic AMP response elements (CREs), which are recognized by the cellular transcription factor CREB. Tax binds to GC-rich sequences that immediately flank the CREs. The coactivator CBP/p300 binds to this promoter-bound ternary complex, which promotes the initiation of HTLV-1 transcription. PKA-phosphorylation of CREB at serine 133 facilitates transcription from cellular CREs by recruiting CBP/p300 via its KIX domain. However, it remains controversial whether CREB phosphorylation plays a role in Tax transactivation. In this study, we biochemically characterized the quaternary complex formed by Tax, CREB, KIX, and the viral CRE by examining the individual molecular interactions that contribute to Tax stabilization in the complex. Our data shows KIX, Ser^{133}-phosphorylated CREB, and vCRE DNA are all required for stable Tax incorporation into the complex *in vitro*. Consonant with a fundamental role for CREB phosphorylation in Tax recruitment to the complex, we found that CREB is highly phosphorylated in a panel of HTLV-1 infected human T-cell lines. Significantly, we show that Tax is directly responsible for promoting elevated levels of CREB phosphorylation. Together, these data support a model in which Tax promotes CREB phosphorylation *in vivo* to ensure availability for Tax transactivation. Since pCREB has been implicated in leukemogenesis,

enhancement of CREB phosphorylation by the virus may play a role in the etiology of adult T-cell leukemia.

2.2 INTRODUCTION

Infection with human T-cell leukemia virus, type 1 (HTLV-1) can cause a rare and ultimately fatal cancer known as adult T-cell leukemia (ATL). Although the vast majority of individuals infected with the retrovirus remain asymptomatic for life, two to five percent go on to develop this aggressive leukemia.[99] Expression of the HTLV-1-encoded Tax protein is strongly linked to the development of ATL. Tax is a potent transcription factor that strongly activates transcription, and thus replication, of the HTLV-1 genome. Tax stimulates viral transcription by binding the minor groove of the GC-rich DNA sequences that flank the CRE elements within three conserved 21-bp enhancers. [29; 30; 56; 57] These enhancers, called viral cyclic AMP response elements (vCREs), are located within the promoter of the provirus. Tax also interacts with the cellular transcription factor CREB, which binds the off-consensus CRE octanucleotide centered within each vCRE. These interactions enable formation of a ternary complex that is critical for activation of viral transcription by Tax.

The Tax, CREB, vCRE complex recruits the multifunctional cellular coactivator CREB binding protein CBP, and its paralogue p300, to the HTLV-1 promoter, forming a quaternary complex that enables strong transcriptional activation through the intrinsic properties of these coactivators. [65; 91; 94; 100; 101] CBP/p300 are necessary for mediating activated transcription by a large number of transcription factors, though they do not directly bind DNA. CBP was originally

discovered and named for its interaction with protein kinase A (PKA)-phosphorylated CREB (pCREB).[75] Serine 133 (Ser^{133})-phosphorylated CREB binds to the KIX domain of CBP (~aa 585-680), which is composed of three α-helices that form a compact hydrophobic core.[93] A shallow, hydrophobic groove on the surface of the core serves as the binding site for the kinase inducible domain of phosphorylated CREB. Tax also binds KIX, both free in solution and when assembled in the ternary complex. [65; 91; 92; 94]

The role of CREB phosphorylation in Tax transactivation remains controversial after over a decade of study. Previous studies have shown that CREB phosphorylation was not necessary for optimal Tax transactivation, while other studies have found that PKA stimulation increases Tax transactivation.[67; 69; 102] This question has been difficult to address *in vivo* due to the pleiotropic effects of CREB phosphorylation and the wide variety of cell lines studied. A basal level of CREB phosphorylation exists even in unstimulated and serum-starved conditions, and phosphorylation-defective CREB mutants may exert global effects on transcription with obvious ramifications for Tax transactivation. The precise role of CREB phosphorylation in Tax transactivation remains elusive.

In this report, we sought to better characterize the detailed molecular interactions that contribute to the formation and stabilization of the Tax-containing quaternary complex. We demonstrate that strong Tax binding to the KIX domain of CPB/p300 requires viral CRE DNA and phosphorylated CREB. Additionally, we find that KIX greatly stabilizes Tax binding to the CREB/vCRE DNA complex. Further, we show that the incorporation of phosphorylated CREB

is required for formation of a stable complex containing Tax, KIX, and vCRE DNA. Our data support a concerted mechanism of complex formation in which Tax, pCREB, and the KIX domain of CBP/p300 are all required for optimal binding at the HTLV-1 promoter.

Based on these *in vitro* results showing the importance of pCREB for Tax stability in the complex, we investigated levels of CREB phosphorylation in HTLV-1 infected cells. We observed higher levels of intracellular pCREB in a panel of HTLV-1 infected versus uninfected T-cell lines. Significantly, we found that Tax expression directly enhanced CREB phosphorylation. These observations suggest that Tax promotes CREB phosphorylation *in vivo* to ensure sufficient pCREB availability for promoter-bound complex formation and robust Tax transactivation.

2.3 RESULTS

2.3a THE VIRAL CRE AND PCREB ENHANCE TAX BINDING TO THE KIX DOMAIN OF CBP

Transcriptional activation of HTLV-1 requires, in part, the formation of a viral CRE-bound complex composed of Tax, CREB, and CBP/p300. However, a careful biochemical characterization of the precise molecular interactions that contribute to complex stability has not been performed. To carry out this study, we first examined whether Tax binding to the KIX domain of CBP is affected by vCRE DNA and/or pCREB. Purified GST-$KIX_{588-683}$ was bound to glutathione-

agarose beads and used in a GST pull-down assay together with full-length, purified, recombinant Tax and increasing amounts of purified, recombinant, PKA-phosphorylated human CREB A (pCREB). Binding reactions were performed in the absence or presence of double–stranded oligonucleotides carrying the viral CRE or cellular CRE DNA. The cellular CRE possesses a higher affinity CREB binding site than the vCRE and lacks the GC-rich flanking sequences required for Tax binding. The cellular CRE thus serves as a negative control for complex formation with Tax. The amount of Tax bound to GST-KIX was determined by Western blot analysis. Figure 2.1A shows that Tax was poorly recruited to GST-KIX in the absence of DNA (lanes 2-5). The cellular CRE DNA only modestly enhanced Tax binding, consistent with the fact that this sequence lacks the GC-rich flanks (Fig. 2.1A, lanes 10-13). The addition of viral CRE DNA resulted in a dramatic increase in the amount of Tax associated with GST-KIX (Fig. 2.1A, lanes 6-9). Importantly, we found that pCREB is also required for Tax recruitment to GST-KIX, as only small amounts of Tax bound to KIX in the absence of pCREB (Fig. 2.1A, lanes 2,6,10). Titration of pCREB in the presence of the vCRE DNA yielded significantly more Tax binding to the complex (Fig. 2.1A, lanes 6-9). In binding reactions that contained both vCRE DNA and pCREB, we observed up to a one hundred-fold increase in Tax association with GST-KIX. In the presence of vCRE DNA, Tax and pCREB binding precisely correlated, further underscoring the importance of pCREB in the Tax-KIX interaction (Fig. 2.1B). Together, these experiments support previous studies demonstrating the importance of the GC-rich flanking sequences in the Tax-DNA interaction.[29; 30] We extend these

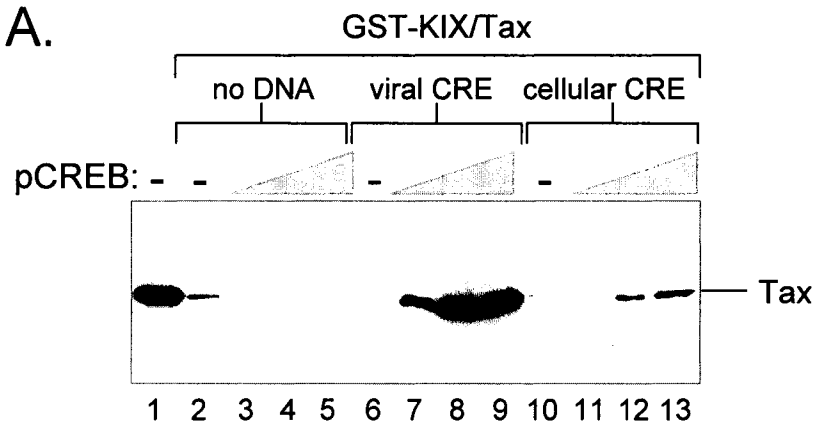


Figure 2.1. The viral CRE and pCREB enhance Tax binding to the KIX domain of CBP. GST pull-down assay. **A.** *Tax binding to KIX is strongly enhanced by vCRE DNA and pCREB.* Tax (25 nM) was incubated with GST-KIX $aa_{588-683}$ (25 nM) in the absence (lanes 2-5) or presence of vCRE (lanes 6-9) or consensus CRE (lanes 10-13) DNA (500 nM). Phosphorylated CREB (pCREB) was added in increasing amounts (2.5, 25, and 250 nM), as indicated. Samples were washed and bound proteins were resolved by 12% SDS-PAGE and analyzed by Western blot analysis. Tax input (20%) is shown in lane 1. Tax binding was detected using an anti-His_6 antibody. Experiment performed by J.-P. Yan.

B.

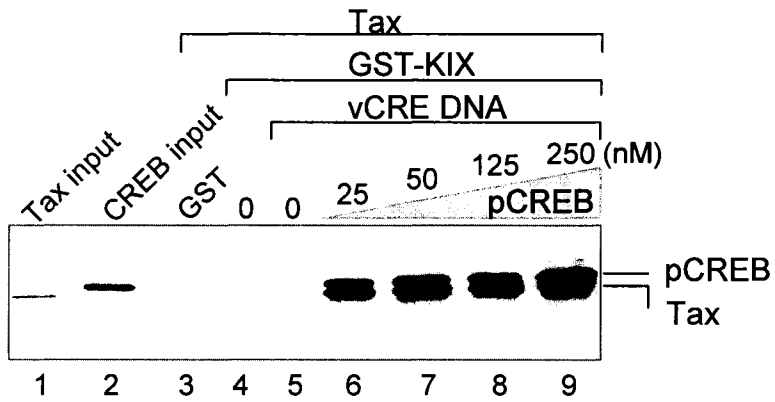


Figure 2.1B. *Tax and pCREB binding to KIX precisely correlates in the presence of DNA.* GST pull-down assay. Tax (45 nM), vCRE DNA (250 nM), and the indicated amount of pCREB were bound to GST- KIX (250 nM), and assayed as described in Figure 2.1A. As a negative control, Tax binding to GST (250 nM) was also tested (lane 3). Input Tax and pCREB (0.6 pmol each) are shown in lanes 1 and 2, respectively. Western blot was performed using a mixture of antibodies against CREB and His_6. Experiment performed by J. E. Mick.

observations to show that the vCRE DNA, together with pCREB, synergistically enhance Tax association with KIX. We performed the reciprocal experiment to that shown in Figure 2.1A to test whether DNA and Tax enhanced pCREB association with GST-KIX. Figure 2.1C shows that Tax modestly enhanced pCREB binding (~three-fold), but only in the presence of the vCRE DNA (lanes 5-8). These data show that pCREB has a much greater effect on Tax binding in the quaternary complex than Tax has on pCREB. Figure 2.1D shows that Tax directly interacts with KIX without DNA or pCREB, as previously shown.[92; 94; 103; 104; 105] However, this interaction is dramatically weaker than the Tax/KIX interaction in the presence of pCREB and DNA: 25 nM Tax was used in the binding reaction shown in figure 2.1A, whereas 1 μM Tax was used in the binding reaction shown in figure 2.1D.

2.3b STRONG TAX BINDING TO THE VIRAL CRE REQUIRES THE KIX DOMAIN OF CBP

To investigate the effect of KIX on Tax binding, we performed DNA pull-down assays using complimentary oligonucleotides in which a biotin group was added to the 5' end of the upper strand, enabling immobilization on streptavidin-agarose beads. The binding site, called vCRE', carried the full vCRE sequence with a single base pair change to convert the off-consensus CRE to a consensus sequence. We have shown Tax recruitment to vCRE' and vCRE is identical, however, CREB binds the vCRE' with a slightly higher affinity than to the natural vCRE. We first used the DNA pull-down assay to evaluate the ability of KIX to

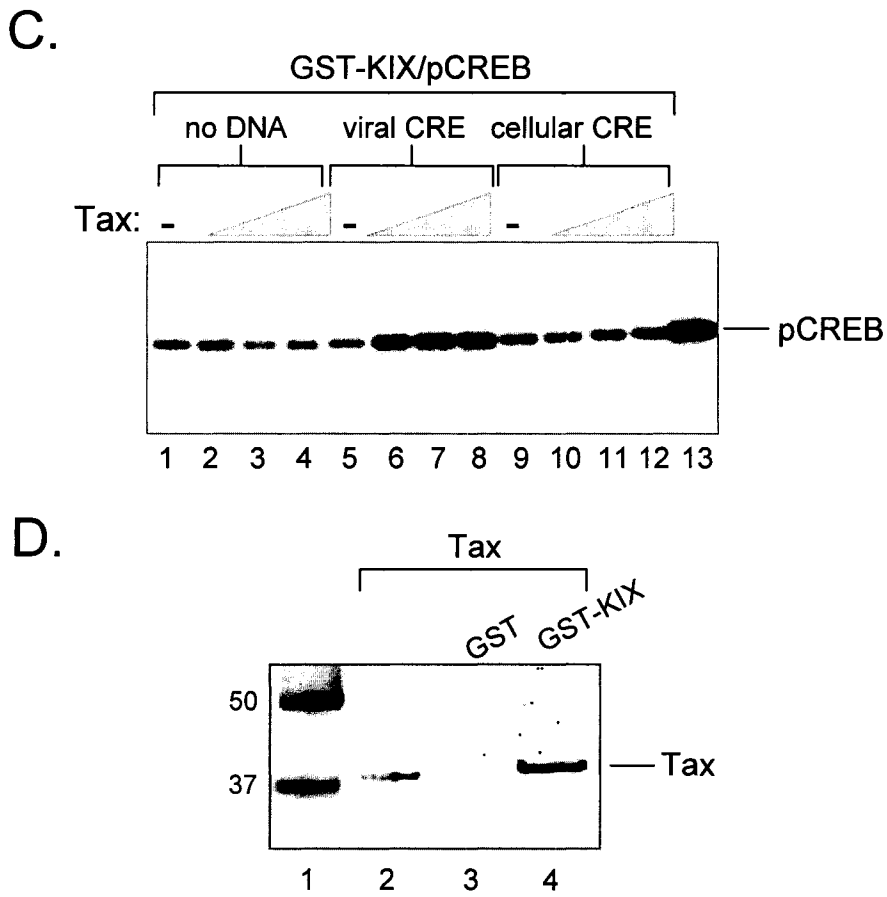


Figure 2.1C. *pCREB binding to KIX is modestly enhanced by Tax and vCRE DNA.* The experiment shown here is the reciprocal of the experiment shown in Figure 2.1A. pCREB (25 nM) was bound to GST-KIX $aa_{588-683}$ (25 nM) in the absence (lanes 1-4) or presence of vCRE (lanes 5-8) or consensus CRE DNA (lanes 9-12) (500 nM). Tax was added in increasing amounts (25, 63, and 125 nM) as indicated. pCREB input (20%) is shown in lane 13. Samples were washed and bound proteins were resolved by 12% SDS-PAGE. pCREB was detected by Western blot analysis. Experiment performed by J.-P. Yan.

D. *Tax directly interacts with KIX.* Tax (1 μM) was incubated with GST-KIX $aa_{588-683}$ (100 nM) (lane 4) or GST (100 nM) (lane 3) as a negative control. Input Tax (1 pmol) is shown in lane 2.Tax was detected by Western blot analysis using an anti-His_6 antibody.

stabilize Tax in the pCREB/DNA complex. We compared GST- $KIX_{588-683}$ with full-length p300 to assess the physiological relevance of our results. We observed a similar enhancement in Tax binding in the presence of KIX and p300 (Fig. 2.2A, compare lane 2 with lanes 4-6). We obtained similar results using purified, full-length CBP (data not shown). These data indicate that the KIX domain, and the full-length coactivators, play a role in the stabilization of Tax in the quaternary complex. We next evaluated the effect of KIX on Tax binding by performing a titration of $KIX_{588-683}$ into DNA pull-down reactions containing Tax, pCREB, and vCRE'. We found that Tax binding to the vCRE' DNA in complex with pCREB was strongly dependent on the presence of KIX in the reaction, giving an approximate seventy-fold increase in Tax binding relative to the absence of KIX (Fig. 2.2B, upper panel, lanes 3-6). Tax and KIX binding precisely correlated in a dose-dependent fashion (Fig. 2.2B, lower panel). A biotinylated half-CRE site was used as a negative control for Tax binding (Fig. 2.2B, lane 2). Electrophoretic mobility shift assays (EMSAs) with ^{32}P-labeled vCRE DNA were then used to more quantitatively assess the effect of KIX on Tax stability in the complex. We compared the apparent affinity of Tax for pCREB/DNA versus pCREB/KIX/DNA (Fig. 2.2C). The apparent affinity of Tax for each complex was established by determining the Tax concentration at the mid-point of the binding transition from the binary to the ternary, and the ternary to quaternary complex. We found that the apparent affinity of Tax for the pCREB/DNA complex was sixty-fold higher in the presence of KIX (Fig. 2.2C). The binding of Tax reveals the presence of KIX in the complex, as it shifts to a

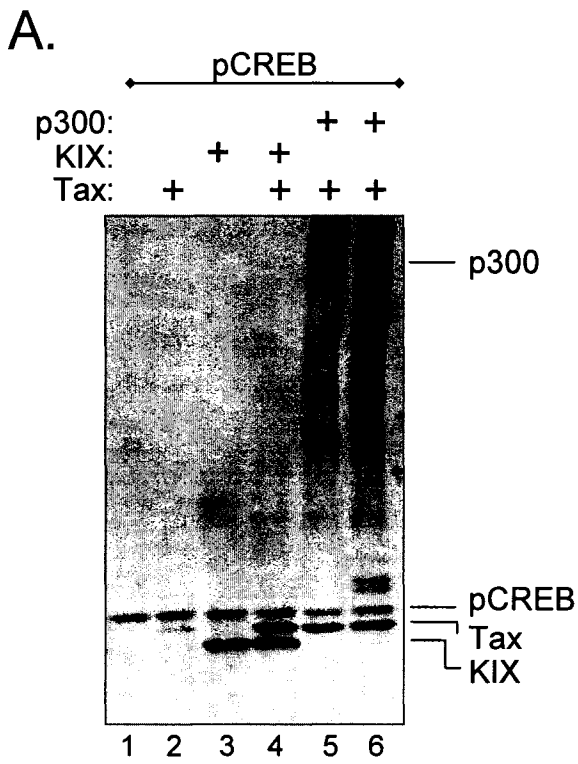


Figure 2.2. Strong Tax binding to the viral CRE requires the KIX domain of CBP. A. *The KIX domain and full-length p300 stabilize Tax binding.* Immobilized vCRE' DNA (5 nM) was incubated with pCREB (25 nM), Tax (25 nM) and either GST-KIX (25 nM) or full-length p300 (25 nM), as indicated. Lanes 5 and 6 in are duplicate reactions, except lane 5 lacks the nonspecific competitors present in all other reactions. Samples were washed, and DNA-bound proteins were separated on a 10%-20% gradient SDS polyacrylamide gel and analyzed by Western blot using a mixture of antibodies against CREB, the His_6 tag, and GST. Experiment performed by H. A. Giebler.

B.

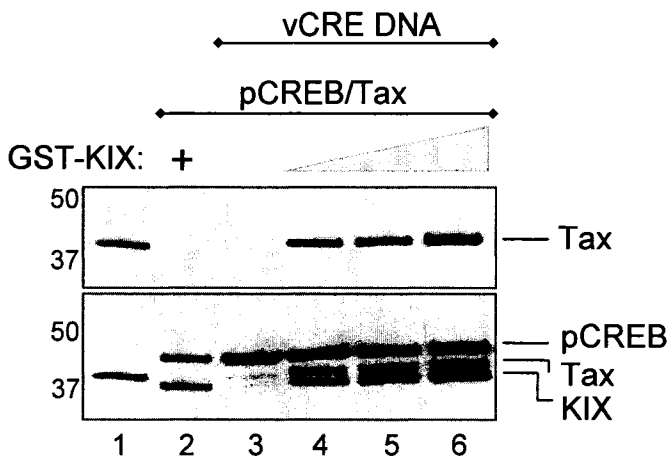


Figure 2.2B. *Tax binding precisely correlates with KIX binding to the vCRE.* Increasing amounts of GST-$KIX_{588-683}$ (2.5, 5, 12.5 nM) were added into binding reactions containing biotinylated vCRE' DNA (5 nM), pCREB (12.5 nM), and Tax (12.5 nM). A biotinylated modified half-CRE with no GC-rich flanks (lane 2) was included as a negative control for Tax; it binds pCREB with a reduced affinity compared to the full consensus CRE. Tax input (20%) is shown in lane 1. A Western blot was first performed using an anti-His_6 antibody to detect Tax (upper panel). The blot was then incubated with a cocktail of antibodies against CREB and GST (lower panel). Each DNA pull-down experiment was repeated at least three times. Molecular weight markers are indicated at left.

C.

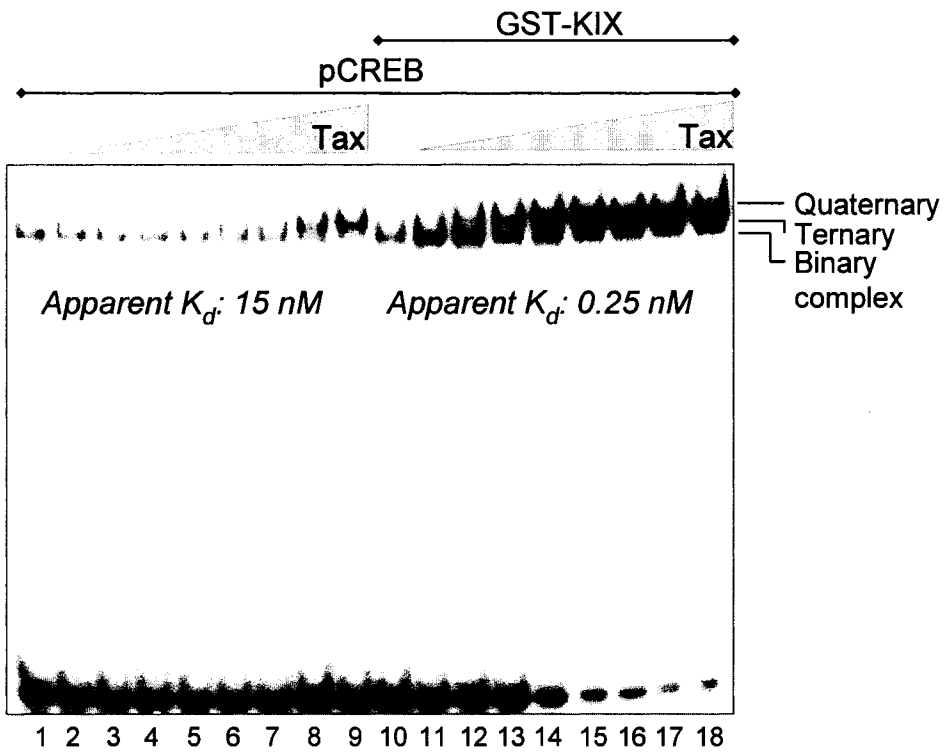


Figure 2.2C. *KIX increases the affinity of Tax for the vCRE/pCREB complex.* EMSAs were performed with γ-^{32}P-end labeled vCRE probe (0.15 nM), pCREB (1.5 nM), and increasing amounts of Tax (25 pM to 75 nM). Binding reactions were carried out in the absence (lanes 1-9) or presence (lanes 10-18) of GST-$KIX_{588-683}$ (50 nM). Each nucleoprotein complex is indicated. The concentration of Tax at the midpoint of the transition from the binary to the ternary complex (lanes 1-9), and the binary to the quaternary complex (lanes 10-18) was used to calculate the apparent *Kd* of Tax for vCRE/pCREB and vCRE/pCREB/KIX. Experiment performed by J. E. Mick.

higher position on the gel than pCREB/Tax/DNA.[91] This result clearly highlights the importance of the KIX domain in stabilization of Tax in the complex.

2.3c CREB PHOSPHORYLATION IS NECESSARY FOR STABLE TAX BINDING TO VIRAL CRE DNA

To further characterize the molecular interactions that contribute to Tax stabilization in the promoter-bound complex, we next examined the effect of unphosphorylated versus phosphorylated CREB. We performed a GST pull-down assay with immobilized GST-KIX, in the presence of vCRE DNA, and compared the ability of CREB vs. pCREB to recruit Tax to the quaternary complex. Figure 2.3A shows that pCREB was immeasurably more effective at recruiting Tax to GST-KIX in the presence of vCRE DNA (Fig. 2.3A*)*. We next used a DNA pull-down assay using the vCRE' DNA to further explore differences between CREB and pCREB in mediating KIX stabilization of Tax. As before, Tax binding was dramatically increased in the presence of pCREB and GST-KIX (Fig. 2.3B, lanes 4-6 vs. 8-10).

To further establish the role of pCREB in Tax complex formation, we compared KIX binding to ternary complexes formed with Tax and the two forms of CREB in an EMSA. The addition of Tax promoted ternary complex formation with both forms of CREB (Fig. 2.3C, lanes 2 and 7). When purified GST-$KIX_{588-683}$ was titrated into binding reactions, quaternary complexes were also observed with both CREB and pCREB (Fig. 2.3C). However, significantly less KIX was required to form the quaternary complex in the presence of pCREB, consistent

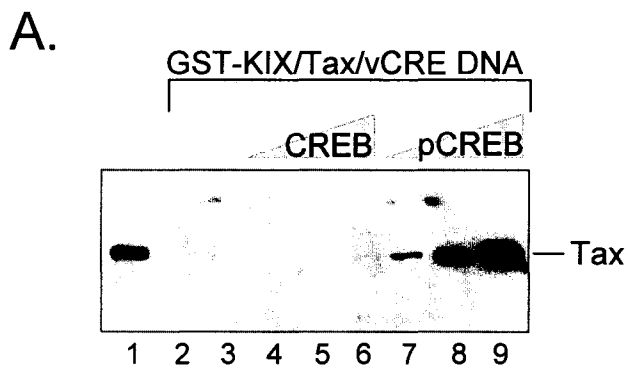


Figure 2.3. CREB phosphorylation is necessary for stable Tax binding to viral CRE DNA. A. *pCREB strongly enhances Tax binding to KIX.* Increasing amounts of CREB or pCREB (2.5, 25, 250 nM) was added to GST pull-down reactions containing Tax (25 nM), vCRE DNA (500 nM), and GST-$KIX_{588-683}$ (25 nM) immobilized on glutathione agarose. Tax input (10%) is shown in lane 1. Experiment performed by J. E. Mick.

B.

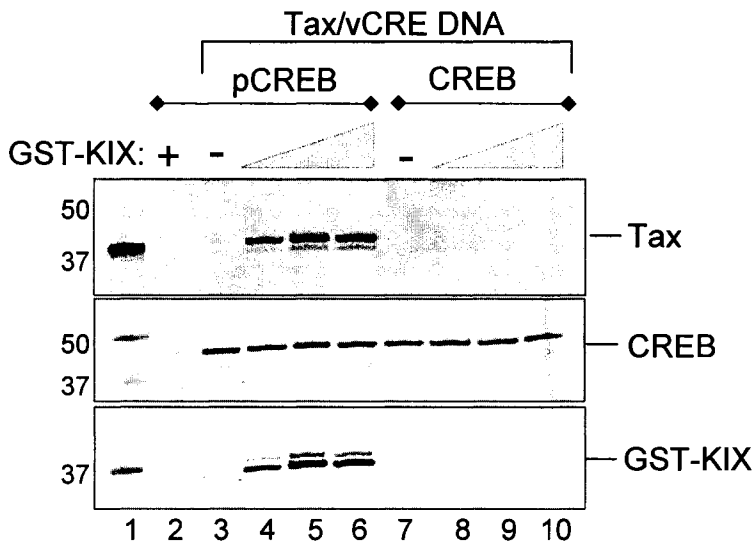


Figure 2.3B. *pCREB is necessary for Tax binding enhancement by KIX.* Immobilized vCRE' DNA (5 nM) was added to DNA pull-down reactions containing Tax (12.5 nM), pCREB or CREB (12.5 nM) and increasing amounts of GST-$KIX_{588-683}$ (2.5, 5, and 12.5 nM). As before (Fig. 2B), a reaction containing a modified CRE half site, pCREB, GST-KIX, and Tax is included as a negative control for Tax (lane 2). Samples were washed and bound proteins were resolved by 12% SDS PAGE. Consecutive Western blots were performed using antibodies against Tax (anti-His_6), CREB, and GST-KIX (anti-GST). Tax input (20%) is shown in lane 1. Molecular weight markers are indicated at left.

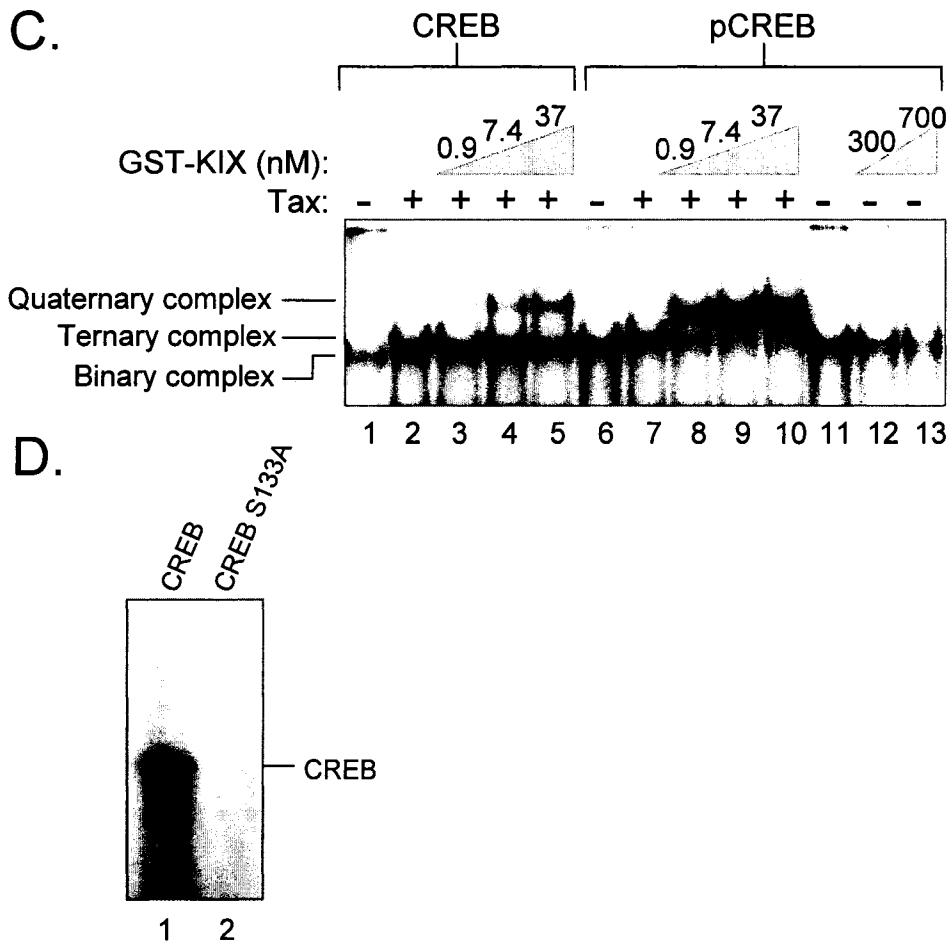


Figure 2.3C. *pCREB, Tax, and KIX are all needed for strong quaternary complex formation*. Binding reactions for EMSAs contained γ-^{32}P-end labeled viral CRE DNA probe (0.3 nM/reaction) and constant amounts of CREB (3.6 nM) or pCREB (1.8 nM), in the presence or absence of Tax (95 nM). GST-KIX$_{588-683}$ was added to the reactions in increasing amounts (0.9, 7.4, and 36.8 nM, lanes 3-5 and 8-10; or 300 and 700 nM, lanes 12 and 13). Binding reactions were analyzed on a 5% native gel. Note that the pCREB/DNA complex migrates with slightly reduced mobility relative to the CREB/DNA complex. Experiment performed by J. E. Mick. **D.** *CREB is phosphorylated by PKA only at serine 133. In vitro* phosphorylation of purified recombinant CREB and a CREB point mutant (serine 133 changed to alanine) was performed in the presence of γ-^{32}P ATP and PKA. Proteins were resolved on a 12% SDS polyacrylamide gel and analyzed by PhosphorImager.

with previously published data.[91] As a control to confirm that the CREB used in these assays was singly phosphorylated at Ser^{133}, we performed an *in vitro* kinase assay using the catalytic subunit of PKA. Figure 2.3D shows that wild-type CREB was phosphorylated whereas CREB with a serine 133 to alanine point mutation was not.

2.3d TAX ENHANCES CREB PHOSPHORYLATION *IN VIVO*

The compelling *in vitro* evidence for the importance of pCREB in Tax recruitment and quaternary complex formation led us to investigate CREB phosphorylation *in vivo*. We reasoned that pCREB levels might be elevated in HTLV-1 infected cells to ensure maximal Tax transactivation. To address this question, we examined CREB phosphorylation levels in a panel of Tax-expressing, HTLV-1 infected (SLB-1, MT-2, C8166) vs. uninfected (Jurkat, CEM, Molt-4) T-cells. Whole cell extracts were prepared from these cells following 24 hours of serum-starvation. Western blot analysis using an anti-Ser^{133} phospho-CREB-specific antibody showed significantly higher levels of CREB phosphorylation in the HTLV-1 infected cell lines compared to the uninfected cell lines (~seven-fold), while the total amount of CREB remained unchanged across samples (Fig. 2.4A). To determine whether levels of CREB phosphorylation could be increased further, we treated the panel of cell lines with forskolin, a cAMP agonist, and examined the levels of CREB phosphorylation as before. Figure 2.4B shows that as expected, forskolin treatment increased CREB phosphorylation over five-fold in the panel of uninfected T-cells. Significantly,

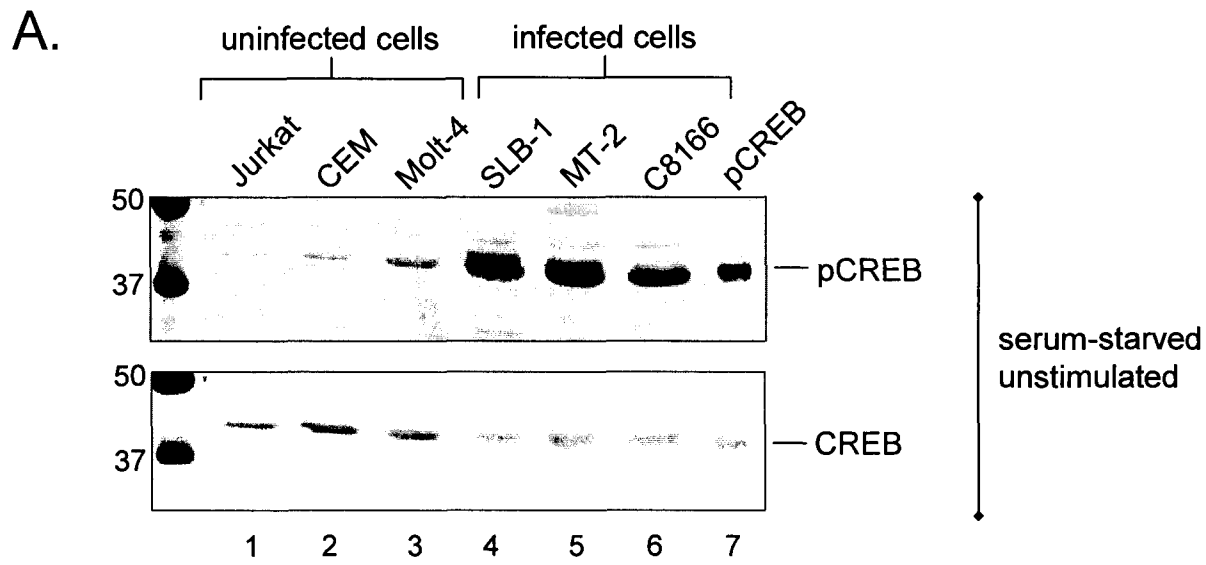


Figure 2.4. Tax enhances CREB phosphorylation *in vivo*. A. *HTLV-1 infected T-cells have increased levels of pCREB compared to uninfected cells.* Western blot analysis of whole cell extracts from three HTLV-1 infected (SLB-1, MT-2, and C8166) and uninfected (Jurkat, CEM, and Molt-4) T-cell lines. All cell lines were serum-starved (0.5% FBS) 24 hr prior to harvest. pCREB is shown in the upper panel and total CREB is shown in the lower panel. Purified, recombinant pCREB is shown in lane 7 as a positive control. Molecular weight markers are shown at left. Experiment performed by Y.-M. Kim.

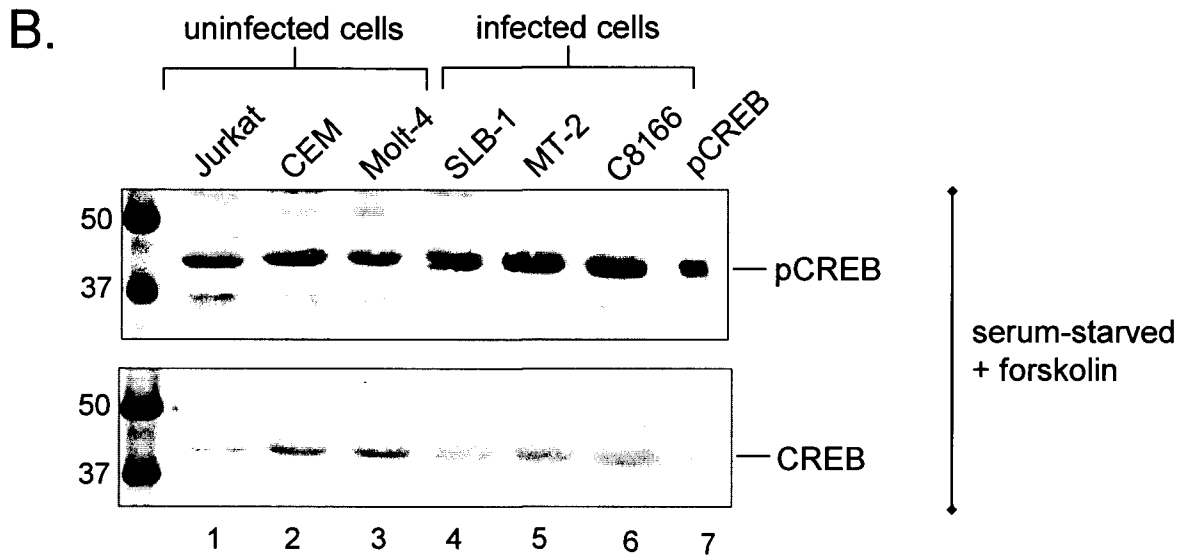


Figure 2.4B.. *Forksolin stimulation of HTLV-1 infected T-cell lines does not increase the level of CREB phosphorylation.* A Western blot of whole cell extracts was performed on the same cell lines as in Figure 2.4A, following stimulation with 10 μM forskolin for 30 min. pCREB is shown in the upper panel and total CREB is shown in the lower panel. Purified, recombinant pCREB is shown in lane 7 as a positive control. Molecular weight markers are shown at left. Experiment performed by Y.-M. Kim.

forskolin had no effect on the levels of CREB phosphorylation in the panel of infected cells. We conclude that CREB is maximally phosphorylated in these HTLV-1 infected cell lines. We next wanted to determine whether the increased CREB phosphorylation we observed in infected cells is the result of Tax expression. Human 293 cells were co-transfected with expression vectors for Tax and Gal4-CREB (Fig. 2.4C). Gal4-CREB was used to overexpress CREB since the effect of Tax on endogenous CREB was difficult to detect due to low transfection efficiency (~30%, data not shown). Ser^{133} CREB phosphorylation was enhanced in the presence of Tax, strongly suggesting that Tax is responsible for the elevated levels of CREB phosphorylation observed in the panel of HTLV-1 infected T-cells.

These results reveal that Tax expression promotes elevated pCREB levels in the cell, which is then available to facilitate Tax transactivation through the viral CREs. These *in vivo* data corroborate our *in vitro* studies and support a critical role for Ser^{133}-phosphorylated CREB in Tax transactivation. Importantly, these results suggest that an HTLV-1-specific mechanism(s) is in place to ensure constitutively high levels of pCREB in the infected T-cell.

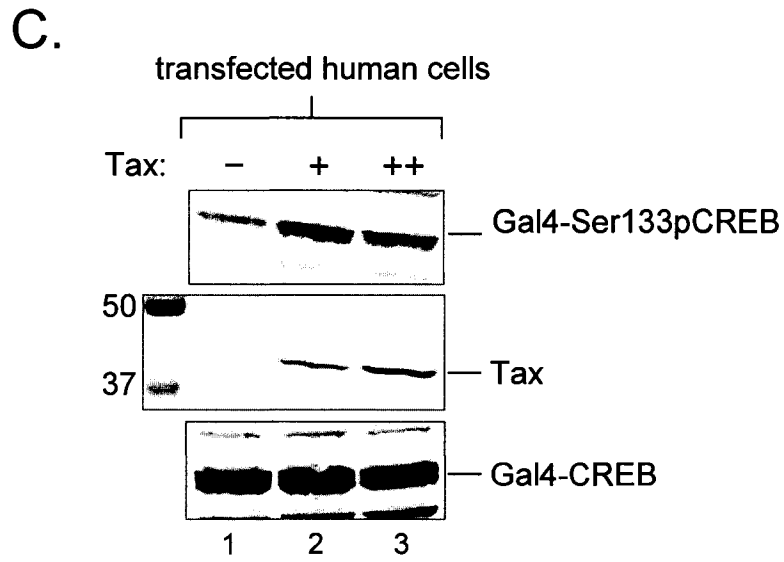


Figure 2.4C. *Tax expression increases CREB phosphorylation in vivo.* Human 293 cells were transfected with a Tax expression vector (pSG-Tax, 0.5 µg and 1 µg) or pUC19 (1 µg) as a control. After a 24 hour transfection, cells were serum-starved (0.5% FBS) for an additional 24 hours. Cells were then harvested, whole cell extracts were prepared and analyzed by Western blot. Antibodies used for detection were anti-Ser133 phosphoCREB, anti-Gal41-147, and a monoclonal anti-Tax antibody. Experiment performed by Y.-M. Kim.

2.4 DISCUSSION

In this study we characterized the detailed molecular interactions that stabilize Tax in the transcription complex containing CREB, the KIX domain of CBP/p300, and vCRE DNA. We examined the role of the individual components in Tax recruitment to the complex. We found that *i.*) the viral CRE DNA specifically and strongly enhanced Tax binding to KIX, *ii.*) pCREB specifically and strongly enhanced Tax binding to KIX, *iii.*) KIX and full-length p300 enhanced Tax binding to the pCREB/vCRE DNA complex, and *iv.*) Ser^{133}-phosphorylated CREB is required for efficient Tax recruitment into the quaternary complex. Notably, we found that intracellular CREB is maximally phosphorylated (relative to forskolin treatment) in HTLV-1 infected cell lines and that Tax expression alone is responsible for promoting high levels of pCREB *in vivo*. These findings reveal that the virus has evolved a mechanism to elevate pCREB levels in the HTLV-1 infected cell, likely as a mechanism to promote strong Tax transactivation.

Our work demonstrates that multiple protein-protein and protein-DNA contacts contribute to the stability to the Tax-containing quaternary complex. This stabilization is likely essential for the robust transactivation characteristics of HTLV-1 gene expression. Our data indicates that Tax is stabilized in the quaternary complex through interactions with pCREB, KIX, and the viral CRE DNA. Although Tax has previously been shown to bind KIX directly,[92; 94; 103; 104; 105] the experiments shown here reveal that Tax binding to KIX is dramatically enhanced (~100-fold) by pCREB and vCRE DNA. These data support a model in

which pCREB and the vCRE serve as molecular scaffolds which assemble Tax into a structure that is highly competent for interaction with KIX, and thus CBP/p300 recruitment to the HTLV-1 promoter.

Our data shed light on the controversial role CREB phosphorylation has played in Tax transactivation for many years. It is widely believed that Tax bypasses the requirement for CREB phosphorylation in the recruitment of CBP/p300, as studies have shown that KIX binds to Tax in complex with unphosphorylated CREB.[65; 91] These data suggest that CREB phosphorylation is not required for Tax recruitment of CBP/p300. However, Giebler *et al.* (1997) found that the K_d for KIX binding to the Tax/CREB complex was much higher than for the Tax/pCREB complex (25 nM vs. 1.7 nM, respectively). Furthermore, we show in figure 2*C* that the K_d for Tax binding to the KIX/CREB complex was much higher than for the KIX/pCREB complex (15 nM vs. 0.25 nM, respectively). These data indicate that pCREB is significantly more effective (~15 to 60-fold) at promoting quaternary complex formation with Tax than the unphosphorylated form of the protein.

We also demonstrate that HTLV-1 infected cell lines contain constitutively high levels of phosphorylated CREB, and that this appears to be directly due to the expression of Tax. This observation is in agreement with the idea that pCREB is essential to Tax function, and suggests that the virus has evolved a mechanism to ensure pCREB is available for efficient Tax transactivation *in vivo*. Notably, pCREB has recently been shown to be involved in cellular proliferation and has been implicated in leukemogenesis.[45; 46] Our observation that Tax

promotes elevated levels of CREB phosphorylation *in vivo* may have implications for HTLV-1-dependent malignant transformation. Tax has been shown to activate viral and cellular gene expression through both the ATF/CREB and NF-κB signaling pathways. The role of each of these pathways in Tax-mediated cellular transformation has been extensively studied, yet it remains controversial which pathway is required in this process.[106; 107; 108; 109; 110; 111] Whether Tax enhancement of CREB phosphorylation is mediated through either of these well-characterized pathways, or via a distinct mechanism of kinase activation, is not yet known. Many kinases are responsible for phosphorylation of CREB at Ser^{133} and thus our results do not necessarily implicate PKA in Tax-mediated enhancement of CREB phosphorylation.[47]

Consonant with our hypotheses that Tax promotes CREB phosphorylation *and* utilizes pCREB for transactivation, kinase inhibitors have been shown to block Tax function.[66; 69] Another study showed that Tax expression in mouse fibroblasts resulted in sustained CREB phosphorylation during serum-starvation in both the absence and presence of forskolin, in agreement with our results.[71] Trevisan *et al.* (2004) proposed that Tax may prolong the phosphorylation state of CREB via the inactivation of specific phosphatases or activation of specific kinases.[71]

In summary, our work shows CREB phosphorylation is necessary for efficient quaternary complex formation *in vitro* and implicates pCREB as an essential molecule for Tax function *in vivo*. Our findings that CREB phosphorylation is elevated in HTLV-1 infected T-cells, and that Tax expression

directly enhances CREB phosphorylation, provide further evidence for the integral role pCREB may play in Tax transactivation. We propose that HTLV-1 has evolved a mechanism to ensure high levels of CREB phosphorylation to facilitate viral replication. Our data are consistent with a model in which Tax enhancement of CREB phosphorylation is relevant in the etiology of adult T-cell leukemia.

2.5 MATERIALS AND METHODS

2.5a EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS.

Bacterially-expressed CREB A,[112] CREB S133A, Tax-His_6[113] and GST-KIX[91] proteins were purified to >95% homogeneity as previously described.[91] Full-length His_6-tagged p300 was expressed from recombinant baculovirus in Sf9 cells and purified as previously described.[114] CREB A is a naturally occurring splice variant (aa 1-327) where Ser^{119} corresponds to Ser^{133} in human CREB B (aa 1-341).[115] To avoid confusion, we will use the Ser^{133} nomenclature throughout this work. The KIX domain of CBP used in this study is 85% identical to the p300 KIX domain. Amino acid differences fall largely outside of the minimal KIX domain, which includes the region of pCREB and Tax interaction. All proteins were dialyzed against TM buffer (50 mM Tris pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA pH 8.0, 20% (vol/vol) glycerol, 0.025% (vol/vol) Tween-20, and 1 mM DTT), aliquoted and stored at –70° C. CREB was phosphorylated using the catalytic subunit of protein kinase A by incubating 1.6 μM CREB in a

reaction containing 3.3 μM ATP, 5 mM $MgCl_2$, and 55 units of PKA (Sigma) in a 25 mM potassium phosphate buffer, pH 6.6. Successful CREB phosphorylation was confirmed by the absence of γ-^{32}P-ATP incorporation following cold phosphorylation. To confirm PKA singly phosphorylates CREB at Ser^{133}, we performed a kinase assay on wild-type CREB and CREB S133A using recombinant PKA catalytic subunit (Sigma) and γ-^{32}P-ATP.

2.5b OLIGONUCLEOTIDES

The top strand sequence of the complimentary oligonucleotides used in the experiments described herein are as follows. CRE core sequences are underlined and bolded. Fig. 1 *A-C*, Fig. 2*C*, Fig. 3*A*, and Fig. 3*C*,

cellular CRE: 5'-GATCATTCCA**TGACGTCA**ATTGA-3';

vCRE: 5'-GATCAGGCGT**TGACGACA**ACCCC-3' (promoter proximal 21-bp repeat). Fig. 2*A-B*, and Fig. 3*B*:

vCRE': 5'-GAAGATCTCTCAGGCGT**TGACGTCA**ACCCCTCACAGATCTTC-3'. vCRE' carries the full vCRE sequence with a single base pair change that converts the off-consensus CRE core to a consensus CRE. It binds Tax indistinguishably from the natural vCRE.

Modified half CRE:

5'-GGGGATCT**CTCA**AATATTCTTAGGACCTTTCACCAGATCGGC-3'. The oligonucleotides were purchased from Integrated DNA Technologies (IDT). For

the DNA pull-down reactions, a biotin group was chemically added to the 5' end of the upper strand oligonucleotide (IDT).

2.5c ANTIBODIES

The antibodies used in the Western blots presented herein were as follows: anti-His (H-15), anti-CREB (C-21), anti-phospho-Ser^{133} CREB, anti-Gal4 (DBD) and anti-GST (B-14). All were purchased from Santa Cruz Biotechnologies. An anti-Tax monoclonal antibody (National Institutes of Health AIDS Research and Reference Reagent Program) was also used for detecting transfected Tax.

2.5d GST PULL-DOWN ASSAYS

GST pull-down experiments were performed as previously described.[89] The final concentrations of protein and DNA in each reaction are given in the figure legend. Bound proteins were resolved by electrophoresis on 10% or 12% SDS polyacrylamide gels and transferred to nitrocellulose for subsequent Western blot analysis.

2.5e DNA PULL-DOWN ASSAYS

DNA pull-down experiments were performed using streptavidin-coated agarose beads (Novagen). Biotinylated double-stranded oligonucleotides containing a single CRE element were bound to streptavidin-agarose beads by incubating 90 min at 25°C according to the manufacturer's directions. The

amount of DNA bound was quantified by measuring the A_{260} of the DNA-containing supernatant before and after streptavidin-agarose beads binding. DNA-bound beads were stored in a 100 mM Na_2HPO_4 pH 7.5, 0.02% sodium azide solution and washed with 0.5X TM buffer before use in assays. Purified proteins were added to aliquots of the streptavidin-agarose bead-bound DNA in 0.5X TM buffer with 0.6 ng/μL poly(dA)·poly(dT) and 39 nM BSA added as nonspecific competitors, incubated 45 min at 4°C, and washed three times to remove unbound proteins. DNA-bound proteins were separated by electrophoresis on a 10% or a 10-20% gradient SDS gel and transferred to nitrocellulose for detection by Western blot analysis.

2.5f ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

EMSAs were performed by incubation of the indicated amount of purified CREB, Tax, or GST-KIX (aa588-683) in 12.5 mM HEPES pH 7.9, 75 mM KCl, 6.25 mM $MgCl_2$, 10% (vol/vol) glycerol, 5 μM $ZnSO_4$, 0.05% (vol/vol) NP-40, and 0.5 mM EDTA containing 0.2 nM ^{32}P-end-labeled viral CRE probe and 250 ng/mL poly(dA)·poly(dT) in a 20 μl reaction volume. Binding reactions were incubated on ice for 30 min and resolved on 5% nondenaturing polyacrylamide gels [49:1 (wt/wt), acrylamide:N,N'-methylenebisacrylamide] in a buffer containing 0.04 M Tris·HCl, 0.306 M glycine (pH 8.5), and 0.1% (vol/vol) Nonidet P-40. Gels were dried and visualized by PhosphorImager (Molecular Dynamics).

2.5g CELL CULTURE AND TRANSIENT-TRANSFECTION ASSAY

Both HTLV-1 infected (SLB-1, MT-2, C8166) and uninfected (Jurkat, CEM, Molt-4) human T-cell lines were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin. For whole cell extract preparation, cells were serum-starved by cultivation in the presence of 0.5% serum 24 hr prior to harvest. Where indicated, cells were stimulated with 10 μM forskolin. Cells were lysed and resuspended in SDS sample dyes. Proteins were separated by 10% SDS-PAGE and analyzed by Western blot. For transient-transfection assays, cells were transfected with a constant amount of DNA using Fugene6 (Roche). After 24 hr, the cells were serum-starved (0.5% FBS) for an additional 24 hrs. The cells were harvested, lysed, and analyzed by Western blot. Cells were transfected with expression plasmids for Tax (pSG-Tax),[116] Gal4-CREB (Stratagene), and pUC19 as indicated in the experiment.

2.5h IMAGE PROCESSING

The ImageQuant program (Molecular Dynamics) was used to quantify results. Images were processed in Adobe Photoshop, with minor adjustments made to brightness/contrast as needed (gamma was kept at 1). No bands were obscured or altered. Images were annotated in PowerPoint. All experiments presented in this manuscript were shown to be reproducible in at least three independent trials.

2.6 ACKNOWLEDGMENTS

We thank Dinaida Lopez for help with protein purification. We also thank Mara Miller and other members of the laboratory for helpful discussions. This work was supported by a grant from the National Institutes of Health (CA55035, J.K.N.) and the W. M. Keck Foundation. J.A.R. was supported by a minority supplement (CA55035-S1).

SUPPLEMENTAL FIGURES

The supplemental figures contained in this section include experiments I performed that did not appear in the previous chapter "Molecular characterization of the Tax-containing HTLV-1 enhancer complex reveals a prominent role for CREB phosphorylation in Tax transactivation". However, these experiments present complementary data that expands on the results shown in chapter 2.

Figure 2.5 A-C *Forskolin modestly activates transcription from the HTLV-1 promoter.* CHO-K1 cells containing a chromosomally-integrated HTLV-1 LTR-luciferase construct were used in these experiments.[117] The cAMP agonist forskolin and/or the nonspecific transcriptional activator TPA (12-*O*-tetradecanoylphorbol-13-acetate) were used to stimulate cells in figure 2.5 A and B. Although TPA apparently acts synergistically with forskolin to activate HTLV-1 transcription in the absence of Tax, no further conclusions as to the mechanism(s) of this effect can be drawn due to the nonspecific effects of TPA. Figure 2.5B shows stimulation of CHO-K1 cells transfected with a Tax expression vector. In figure 2.5C a PKA expression vector was co-transfected into the CHO-K1 cells with or without Tax. Forskolin stimulation/PKA activity should not have a significant effect on Tax-mediated transcription if CREB is maximally phosphorylated in the presence of Tax. Indeed, these experiments demonstrate only a modest effect of forskolin on HTLV-1 transcription. On average, in the absence of Tax, forskolin activates HTLV-1 transcription three-fold over unstimulated levels (nine independent experiments) demonstrating our forskolin is active. However in the presence of Tax, forskolin stimulation only increased

HTLV-1 transcription an average of 1.3-fold compared to unstimulated Tax transcription in six independent experiments. Similarly, PKA transfection increased HTLV-1 transcription in the absence of Tax an average of three-fold (eight independent experiments), while in the presence of Tax PKA increased Tax transactivation an average of two-fold (six independent experiments).

A.

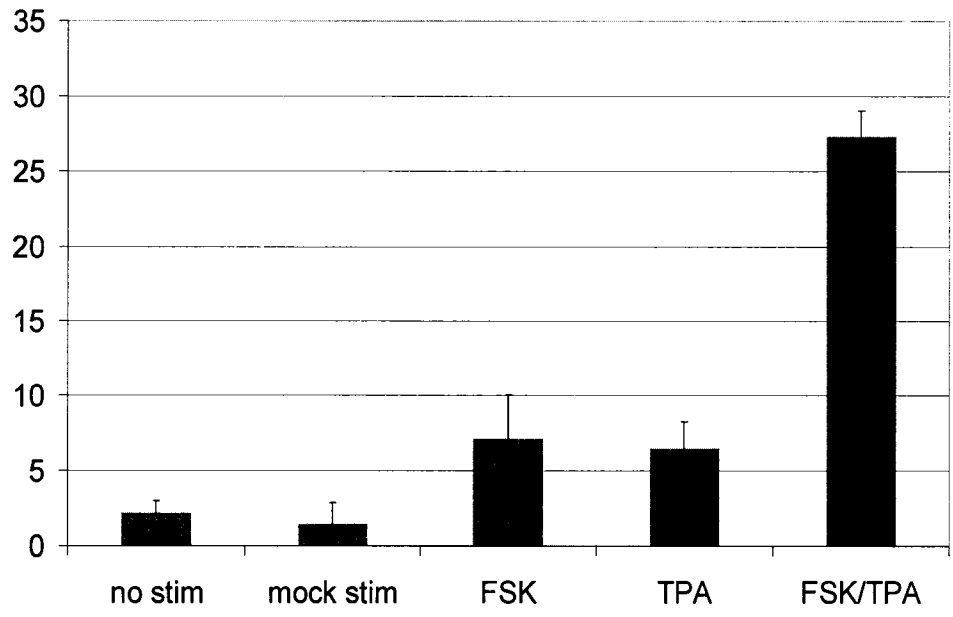


Figure 2.5A. Forskolin modestly activates transcription from the HTLV-1 promoter. CHO-K1 cells with a chromosomally integrated HTLV-1 LTR-luc were stimulated as shown. Cells were harvested and luciferase activity was measured after 12 hours of stimulation with 20 μM forskolin and/or 50 nM TPA, as indicated. Each condition was repeated in triplicate.

B.

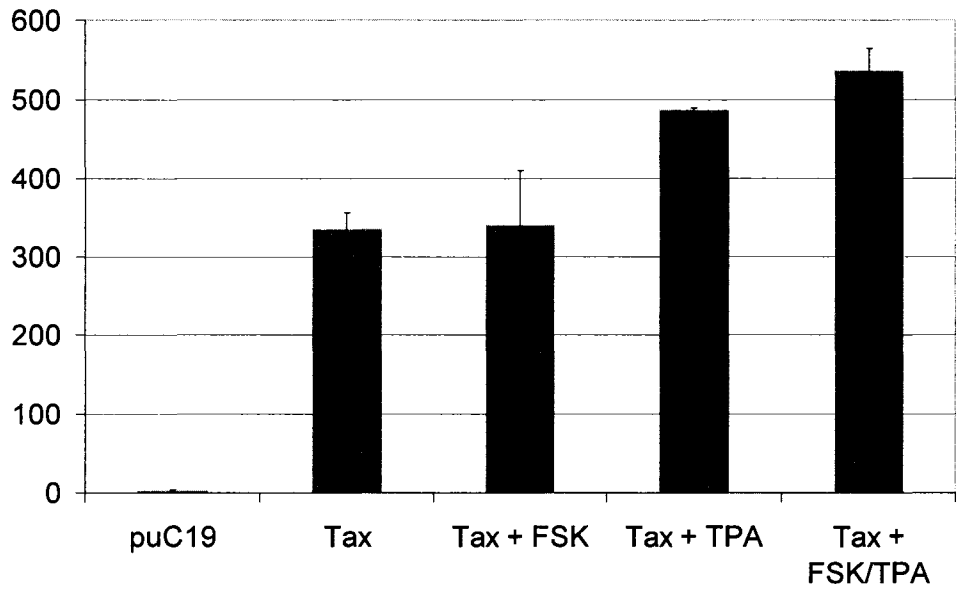


Figure 2.5B. *Forskolin has no effect on Tax transactivation.* CHO-K1 cells with an integrated HTLV-1 LTR-luc were transfected with a Tax expression vector (pSG-Tax, 10 ng) or pUC19 (10 ng) as a control. Cells were harvested and luciferase activity was measured after 24 hours of transfection and 12 hours of stimulation with 20 μM forskolin and/or 50 nM TPA, as indicated. Each transfection was repeated in triplicate.

C.

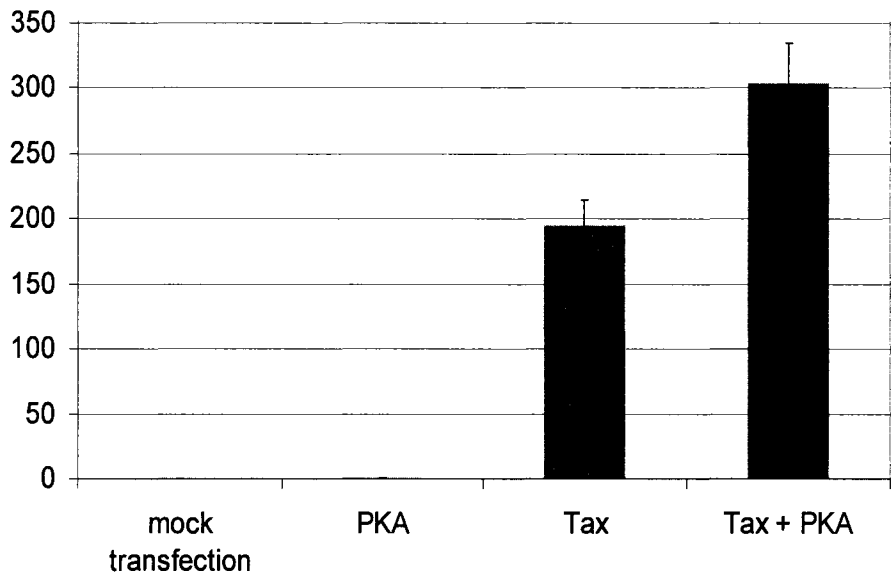


Figure 2.5C. PKA transfection modestly enhances Tax transactivation. CHO-K1 cells with an integrated HTLV-1 LTR-luc were co-transfected with expression vectors for Tax (pSG-Tax, 10 ng), PKA (RSV-PKA, 50 ng), or pUC19 (60 ng). Cells were harvested and luciferase activity was measured 24 hours after transfection. Each transfection was repeated in triplicate.

Figure 2.6 *Tax enhances pCREB levels in vitro*. This experiment was originally conducted to determine if Tax had an effect on CREB phosphorylation under the conditions of an *in vitro* transcription. Although I was able to obtain these results three times, I was unable to see any effect in the vast majority of experiments and so this result was never published. In light of our recently submitted paper (Chapter 2) and Young-mi's recent results, the effect shown in this experiment is likely real. I suspect this result is difficult to consistently obtain as it is highly sensitive to nuclear extract composition, concentration, and factor activity.

Figure 2.7 *Tax enhancement of pCREB levels does not involve PP1 or PKA*. These experiments were conducted to determine the mechanism of the observed Tax-mediated enhancement of pCREB levels. *In vitro* kinase and phosphatase assays were performed with recombinant PKA catalytic subunit and PP1, respectively. Each experiment was repeated at least three times. While Tax had no effect on PP1-mediated CREB dephosphorylation, in a seeming paradox it actually *decreased* the rate of PKA phosphorylation, possibly through steric hindrance or competition. This set of experiments demonstrates that the effect of Tax on pCREB levels seen *in vitro* is not mediated by PKA or PP1. Other kinase(s) and/or phosphatase(s) are likely involved.

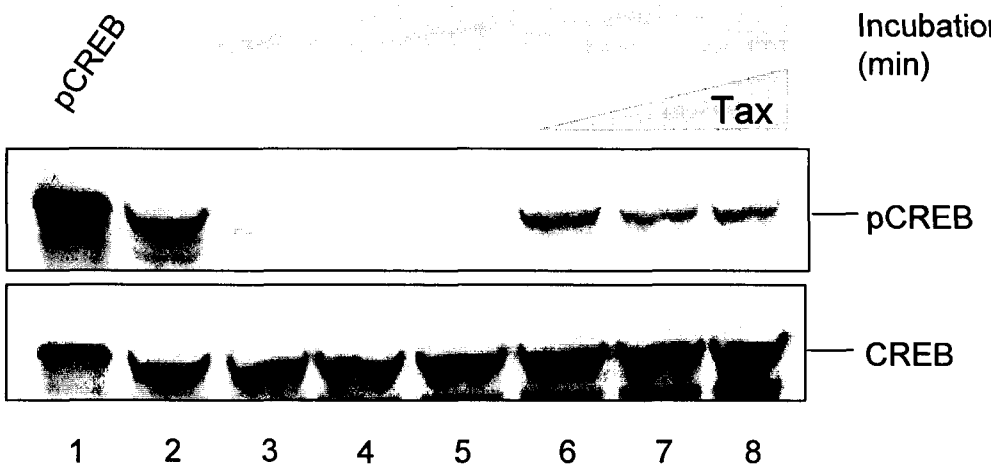


Figure 2.6 Tax enhances pCREB levels *in vitro*. A fixed amount (1 pmol, 33nM) of pCREB was incubated at 30°C in the presence of CEM T-cell nuclear extract for 1, 30, 60, and 90 min as indicated. Tax was added in increasing amounts (33, 66, and 99 nM, lanes 6-8) to identical reactions and preincubated 20 min at 30°C before the addition of CEM nuclear extract. All three reactions containing Tax were incubated 90 min at 30°C. A Western blot was performed, and the membrane was incubated with anti-pCREB (upper panel) and imaged, then incubated with anti-CREB and imaged again (lower panel). 25% pCREB input is shown in lane 1.

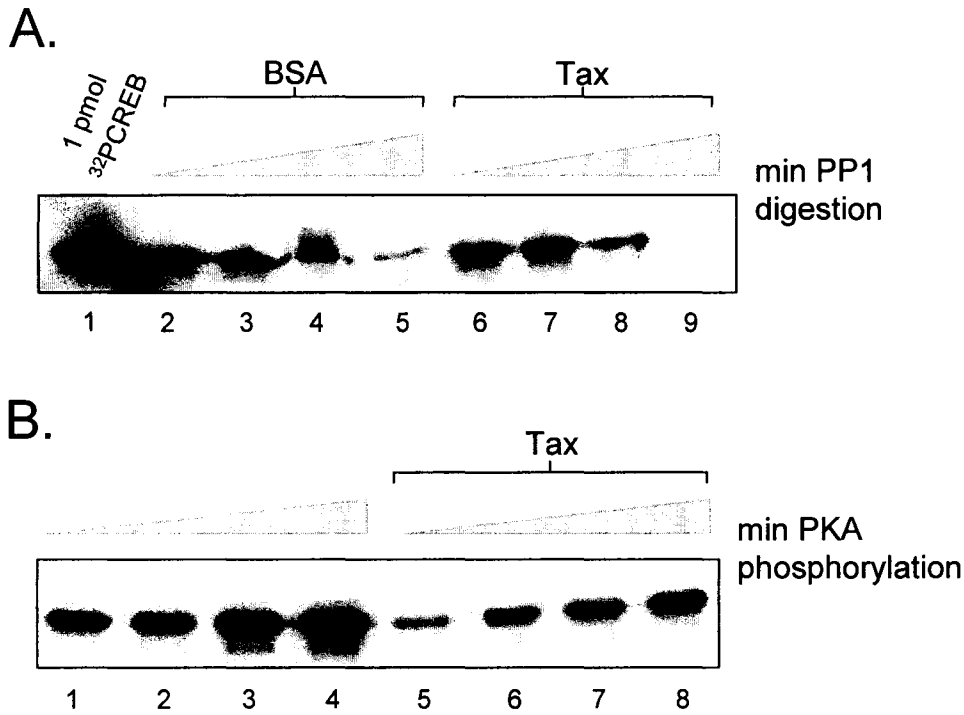


Figure 2.7 Tax enhancement of pCREB levels does not involve PP1 or PKA. A. *Tax does not affect the rate of CREB dephosphorylation by PP1.* ^{32}PCREB (1 pmol) was preincubated with an excess of Tax (8.8 pmol, lanes 6-9) or BSA (8.8 pmol, lanes 2-5) 20 min at 30°C. Recombinant PP1 was added to reactions for 2, 3, 5, and 10 min as indicated. Reactions were stopped by addition of SDS dyes. Proteins were resolved on a 12% SDS-polyacrylamide gel and analyzed by Phosphorimager. **B.** *Tax decreases the rate of CREB phosphorylation by PKA.* CREB (10 pmol) was phosphorylated with recombinant PKA catalytic subunit and γ-^{32}P ATP for 2, 3, 5, and 10 min as indicated. Tax (8.8 pmol) was added in lanes 5-8. Reactions were stopped by addition of SDS dyes. Proteins were resolved on a 12% SDS-polyacrylamide gel and analyzed by Phosphorimager.

CHAPTER 3
MOLECULAR CHARACTERIZATION OF HTLV-1 TAX INTERACTION WITH THE KIX DOMAIN OF CBP/p300

Chapter three describes a study of the interaction between the KIX domain of CBP with full-length Tax when bound in complex with full-length pCREB and the viral CRE. This study utilizes several KIX mutants to show that pCREB and Tax simultaneously interact with separate surfaces of the KIX molecule. Additionally, the possible role of Tax inhibition in KIX binding the mixed-lineage leukemia protein (MLL) activation domain is discussed. I performed all of the experiments shown in this chapter. Dinaida Lopez provided purified Tax protein. This work has been submitted to the *Journal of Molecular Biology* for publication. The citation for the manuscript is:

Ramírez, J.A., & Nyborg, J.K. (2007). Molecular characterization of HTLV-1 Tax interaction with the KIX domain of CBP/p300. *J. Mol. Biol.* in press, June 2007.

3.1 ABSTRACT

The viral oncoprotein Tax mediates transcriptional activation of human T-cell leukemia virus type 1 (HTLV-1). Both Tax and the cellular transcription factor CREB bind to viral cyclic AMP response elements (vCREs) located in the viral promoter. Tax and serine 133 phosphorylated CREB (pCREB) bound to the HTLV-1 promoter facilitate viral transcription via the recruitment of the large cellular coactivators CBP/p300. While the interaction between the phosphorylated kinase inducible domain (pKID) of pCREB and the KIX domain of CBP/p300 has been well-characterized, the molecular interactions between KIX, full-length Tax, and pCREB have not been examined. In this study we biochemically characterized the interaction between Tax and KIX in a physiologically relevant complex containing pCREB and vCRE DNA. Our data show that Tax and pCREB simultaneously and independently bind two distinct surfaces on the KIX domain: Tax binds KIX at the previously-characterized mixed-lineage leukemia (MLL) protein interaction surface while pCREB binds KIX at the pKID-KIX interface. These results provide evidence for a model in which Tax and pCREB bind distinct surfaces of KIX for effective CBP/p300 recruitment to the HTLV-1 promoter. We also show that MLL competes with Tax for KIX binding, suggesting a novel mechanism of Tax oncogenesis in which normal MLL function is disrupted by Tax.

3.2 INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus responsible for development of a highly aggressive and fatal malignancy known as adult T-cell leukemia (ATL).[99] The HTLV-1-encoded Tax protein causes cellular transformation and is required for strong transcriptional activation of the provirus. Tax stimulates HTLV-1 transcription through interaction with three conserved 21-base pair enhancer elements known as viral cyclic AMP response elements (vCREs) located within the HTLV-1 promoter. The vCRE sequences bind Tax in complex with the cellular transcription factor CREB. Tax binds to minor groove GC-rich sequences flanking the core octanucleotide CRE bound by CREB and interacts with CREB via protein-protein interactions.[29; 30; 56; 57] Both CREB and Tax associate with the vCRE element as dimers.[59; 60] Stimulation of many different kinase pathways results in CREB phosphorylation at serine 133.[47] Together, Tax and phosphorylated CREB (pCREB) bound to the vCRE elements serve as a high affinity binding site which recruits the large cellular coactivators CBP/p300 to the HTLV-1 promoter.[65; 91] The quaternary complex consisting of CBP/p300, DNA-bound Tax, and pCREB is believed to strongly activate viral transcription. The KIX domain of CBP/p300 is crucial for Tax-mediated coactivator recruitment. Tax has been shown to interact with three of four major transcription factor interaction domains located within CBP/p300, though the interaction between Tax and KIX is the only one that has been demonstrated to occur when Tax is assembled into the pCREB/Tax/vCRE DNA ternary complex.[65; 89; 91; 92; 94; 118] The fact that DNA-bound Tax and pCREB

simultaneously form a complex with KIX suggests that each transcription factor makes independent contacts with KIX.

The KIX domain of CBP/p300 is composed of three α-helices that form a compact hydrophobic core.[93] It has two well-characterized transcription factor binding surfaces. One surface has been shown to bind pCREB and c-Myb[93; 119] and the other has been shown to bind MLL and c-Jun binding interfaces.[96; 98] The phosphorylated kinase-inducible domain (pKID) of CREB, a region that includes the serine 133 phosphorylation site, binds KIX in a hydrophobic groove defined by KIX helices α1 and α3. The transactivation domain of the c-Myb transcription factor binds this hydrophobic groove in a similar fashion as pKID, though the two sequences share no clear similarity.[119] The structure of a ternary complex containing KIX, a domain of the c-Myb transcription factor, and the minimal activation domain of mixed-lineage leukemia (MLL) protein has been determined supporting a two-site model for transcription factor binding to KIX.[96] De Guzman *et al.* found that MLL binds a distinctly separate surface of KIX than the c-Myb/pKID binding surface.[96] These observations suggest the pCREB/Tax/KIX ternary complex may be analogous to the c-Myb/MLL/KIX ternary complex. Another transcription factor, c-Jun, has been shown to occupy the MLL binding site on KIX.[97] Tax was found to compete with c-Jun for KIX binding,[98] providing further support for a two-site binding model in which Tax and pCREB simultaneously bind separate surfaces of KIX. c-Myb and MLL have also been shown to assemble cooperatively with KIX,[120] while similarly, KIX binds Tax most effectively when it is assembled into a complex with full-length pCREB and viral

CRE DNA.[121] While both the pKID domain of CREB and a small N-terminal Tax peptide[122] have been shown to interact with separate surfaces on KIX, the full-length proteins have not previously been used to characterize these binding sites, nor have they been examined together.

In this report we sought to better characterize the interaction of Tax and KIX in the context of the physiologically relevant quaternary complex that also contains pCREB and vCRE DNA. We show that full-length pCREB and Tax bind KIX at two separate sites. MLL competition with Tax for KIX binding demonstrates Tax and MLL share the same interaction surface of KIX. In addition, KIX constructs carrying mutations in one of the two transcription factor binding sites have enabled us to distinguish independent Tax and pCREB binding to KIX. These findings support a model in which KIX binds the ternary complex composed of full-length Tax, pCREB, and vCRE DNA through simultaneous interaction with both Tax and pCREB on separate interfaces. Our observations also suggest a novel mechanism for Tax oncogenesis through disruption of MLL function.

3.3 RESULTS

3.3a TAX AND PCREB SIMULTANEOUSLY BIND THE KIX DOMAIN OF CBP

Our first objective was to determine whether full-length pCREB and Tax bind KIX both simultaneously and independently. We reasoned that if Tax and pCREB simultaneously bind to two sites on KIX, saturated binding of one protein

to KIX would have no effect on the binding of the second protein. Purified GST-KIX (CBP aa 588-683) was bound to glutathione-agarose beads and used in a GST pull-down assay. Increasing amounts of purified Tax were added to binding reactions until saturation of GST-KIX was achieved (Figure 1A). We performed several experiments to establish that this concentration of Tax saturated GST-KIX (data not shown). pCREB was then titrated into reactions containing the highest concentration of Tax (Figure 1A). pCREB bound to GST-KIX without displacing Tax. The converse experiment was then performed in which immobilized GST-KIX was incubated with increasing amounts of pCREB until saturation was achieved (Figure 1B). As before, several experiments were performed to determine the concentration of pCREB that saturated GST-KIX (data not shown). vCRE DNA was also included in these binding reactions as we were interested in examining the binding of Tax to GST-KIX in the context of the more physiologically-relevant ternary complex (pCREB/Tax/vCRE DNA) (Figure 1C). For simplicity, figure 1C depicts one KIX molecule binding to the ternary complex though it is unknown whether one or two KIX molecules bind the complex. Tax was then titrated into reactions containing the highest concentration of pCREB (Figure 1B). Tax bound to GST-KIX with no effect on pCREB binding. It should be noted that approximately 40-fold lower concentrations of Tax were used to achieve GST-KIX binding in Figure 1B than in Figure 1A, reflecting the relatively low affinity of free Tax for KIX. The presence of vCRE DNA and pCREB dramatically increases Tax affinity for GST-KIX.[121] These experiments show that full-length Tax and pCREB each bind KIX

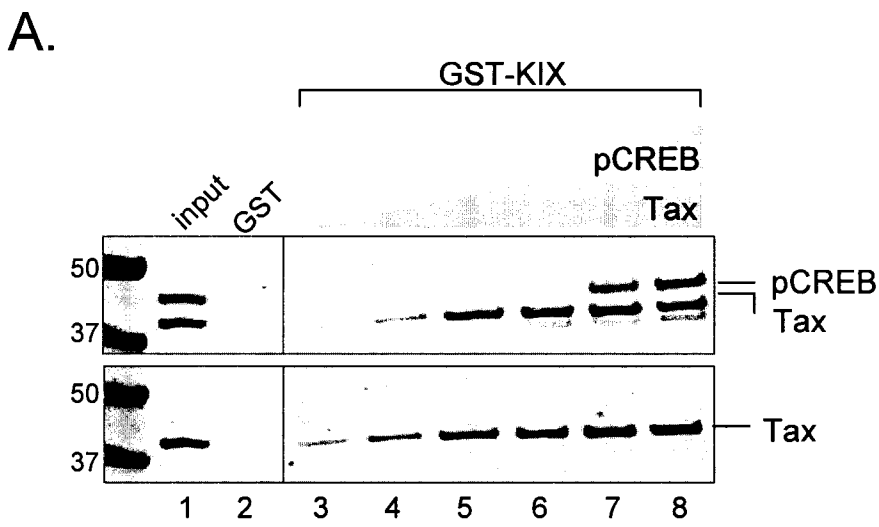


Figure 3.1. Tax and pCREB simultaneously bind the KIX domain of CBP. A. *pCREB binds to KIX without displacing bound Tax.* GST-$KIX_{588-683}$ (100 nM) was bound to glutathione agarose beads and incubated with increasing amounts of full-length purified Tax (0.7, 1.4, 2.1, 2.8 μM, lanes 3-6) until GST-KIX was saturated with Tax. pCREB was added in increasing amounts (10 and 20 nM, lanes 7-8) to reactions containing the highest amount of Tax. Tax and pCREB input amounts (25%) are shown in lane 1. As a negative control, pCREB and Tax binding to GST was tested (lane 2). Samples were washed and bound proteins were resolved by 10% SDS PAGE and analyzed by Western blot. An anti-His_6 antibody was used first to detect Tax (lower panel). The blot was then incubated with an anti-CREB antibody and both antibodies were imaged (upper panel). The line between lanes 2 and 3 denotes cropping between lanes of the same experiment.

B.

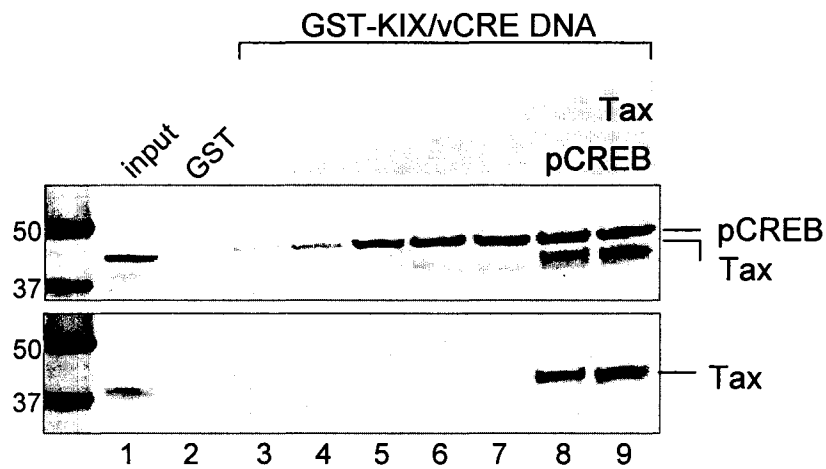
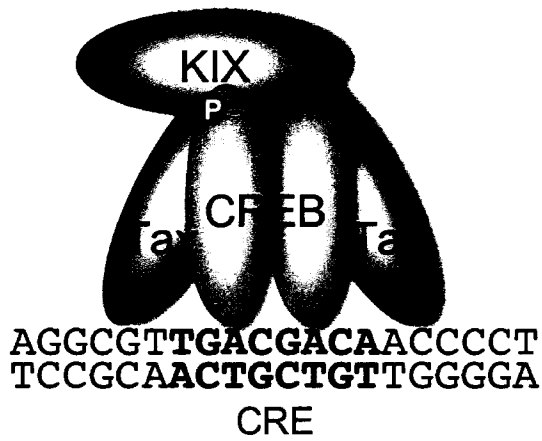


Figure 3.1B. *Tax binds to KIX without displacing bound pCREB.* GST-$KIX_{588-683}$ (25 nM) was used in a GST pull-down assay along with a constant amount of vCRE DNA (500 nM). Full-length purified pCREB was added in increasing amounts (2.5, 5, 25, and 50 nM, lanes 3-6) until GST-KIX was saturated with pCREB. Full-length purified Tax was added in increasing amounts (5, 50, and 75 nM, lanes 7-9) to reactions containing vCRE DNA and the highest amount of pCREB. As a negative control, pCREB and Tax binding to GST in the presence of vCRE was tested (lane 2). Tax and pCREB input amounts (10%) are shown in lane 1. A Western blot was first performed using an anti-His_6 antibody to detect Tax (lower panel). The blot was then incubated with an anti-CREB antibody and both antibodies were imaged (upper panel).

C.



D.

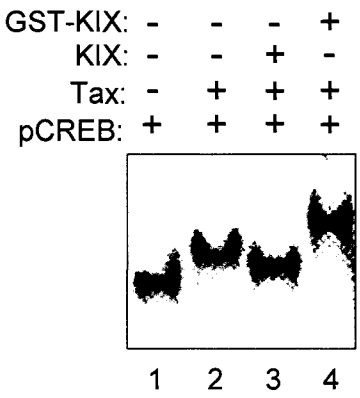


Figure 3.1C. *Schematic representation of the quaternary complex*. A diagram of the quaternary complex containing Tax, pCREB, KIX, and the viral CRE depicts our model of Tax and pCREB binding to separate surfaces of KIX when in complex with DNA. Although only one KIX molecule is shown, it is unknown whether one or two KIX associate with the ternary complex.

D. *Both untagged KIX and GST-KIX bind the ternary complex*. Electrophoretic mobility shift assays were performed with γ-^{32}P-end labeled vCRE probe (0.15 nM), pCREB (3 nM), Tax (250 nM) and $KIX_{586-680}$ (250 nM) or GST-$KIX_{588-683}$ (250 nM).

independently and can occupy their respective sites simultaneously, supporting a two-site model for pCREB and Tax binding to KIX.

Since we use GST-KIX throughout this study, we performed an electrophoretic mobility shift assay (EMSA) as a control to compare untagged KIX vs. GST-KIX binding to the pCREB/Tax/vCRE DNA ternary complex. Radiolabeled vCRE DNA was incubated with purified pCREB, Tax, and GST-$KIX_{588-683}$ or untagged $KIX_{586-680}$ to form quaternary complexes (Figure 1D, lanes 3-4). The addition of both untagged KIX and GST-KIX to ternary complexes produced an easily observable alteration in mobility, indicating their binding to the complexes. GST-KIX binding resulted in a more slowly migrating quaternary complex than untagged KIX, reflecting the size difference between the proteins and the shape of the resulting complexes. Notably, untagged KIX migrates faster than the ternary complex, likely due to its more compact structure. Comparable amounts of both proteins produced a complete shift in the mobility of the ternary complex (~50 nM, data not shown). As further support for this result, we performed gel filtration chromatography on complexes assembled with either GST-KIX or untagged KIX using a Superdex 200 HR 10/30 column, and found the peak elution volume of each quaternary complex was exactly the same (V_E = 10.24 mL, data not shown). The components of each peak were verified using SDS-PAGE.

3.3b A SMALL ACTIVATION DOMAIN OF MIXED-LINEAGE LEUKEMIA PROTEIN (MLL) COMPETES WITH TAX FOR KIX BINDING

We were next interested in determining whether Tax and the MLL activation domain bind the same site on KIX, as previously hypothesized.[96] A small synthetic peptide corresponding to human MLL (aa 2842-2858) was used for these experiments. This sequence was selected based on previous studies.[96; 120] The peptide was synthesized with a C-terminal FLAG tag to facilitate concentration determination by absorbance spectroscopy and to provide a convenient negative control for experiments. We first determined whether our MLL peptide actively bound KIX using a fluorescence polarization assay. An N-terminal fluorescein-tagged MLL peptide was incubated with various concentrations of purified untagged $KIX_{586-680}$ peptide and the fluorescence polarization signal was measured (Figure 2A). The K_d for MLL binding to KIX was 2.5 ± 0.3 μM, almost identical to the previously published value determined by isothermal titration calorimetry.[120] We next tested whether MLL peptide bound to KIX could prevent Tax binding, and thus formation of the quaternary complex. Purified GST-$KIX_{588-683}$ was bound to glutathione-agarose beads as in previous GST pull-down experiments and MLL peptide was titrated into the binding reactions. FLAG peptide was added as a negative control. Following GST-KIX/MLL binding, full-length purified pCREB, Tax, and vCRE DNA were added to the binding reactions. The presence of MLL reduced the amount of Tax bound to GST-KIX while pCREB binding remained unaffected (Figure 2B). The addition of FLAG peptide corresponding to the highest concentration of MLL had no effect

A.

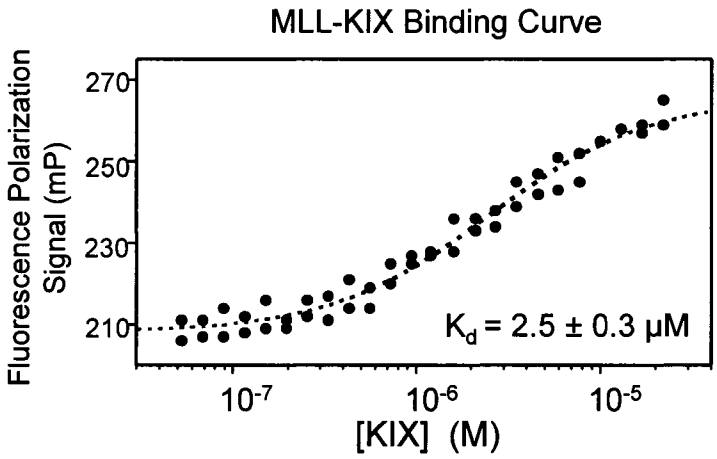


Figure 3.2. A small activation domain of Mixed-Lineage Leukemia protein (MLL) competes with Tax for KIX binding. **A.** *Fluorescence polarization shows MLL peptide binds KIX.* The binding affinity of the MLL activation domain for purified untagged $KIX_{586-680}$ peptide was determined by fluorescence polarization using an N-terminal fluorescein-tagged MLL peptide (aa 2842-2858). The equilibrium dissociation constant was determined by directly fitting the polarization data to a single-site binding curve.

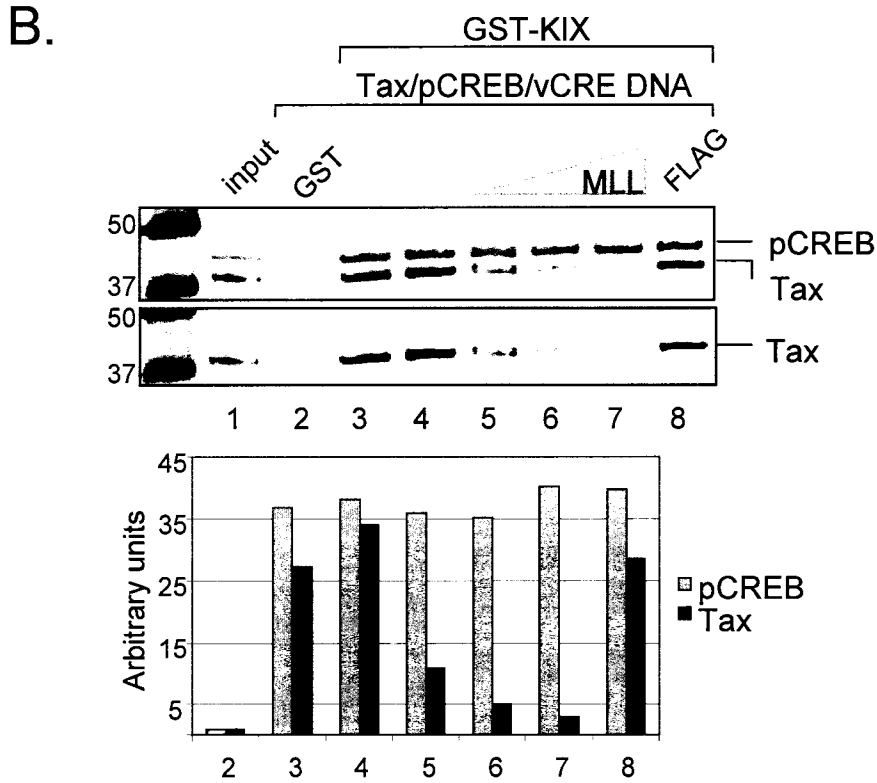


Figure 3.2B. *MLL peptide competes with Tax but not pCREB for KIX binding.* GST-$KIX_{588-683}$ (25 nM) was bound to glutathione-agarose beads as in previous GST pull-down experiments and FLAG-tagged MLL peptide (aa 2842-2858) was added in increasing amounts (9.5, 19, and 38 μM, lanes 5-7) to binding reactions. Following GST-KIX/MLL binding, full-length purified pCREB (25 nM), Tax (35 nM), and vCRE DNA (500 nM) were added to the binding reactions. FLAG peptide (38 μM, lane 8) was tested as a negative control for Tax binding inhibition. pCREB and Tax binding to GST in the presence of vCRE DNA was also tested as a negative control (lane 2).Tax and pCREB input amounts (20%) are shown in lane 1. A Western blot was first performed using an anti-His_6 antibody to detect Tax (lower panel). The blot was then incubated with an anti-CREB antibody and both antibodies were imaged (upper panel). A densiometric analysis of pCREB and Tax binding in this blot is shown below. Numbers on the x-axis correspond to lanes shown in the Western blot. The GST control was set to 1. This data is representative of multiple experiments.

on either Tax or pCREB binding, indicating MLL binding to KIX specifically inhibited the binding of Tax, but not pCREB, to GST-KIX.

As an alternative method to examine whether MLL and Tax share the same binding site on GST-KIX, an EMSA was performed. Tax does not stably bind vCRE DNA by itself, therefore, the CREB basic leucine zipper (bZIP) domain was used together with Tax in these assays. We chose to use the bZIP domain to avoid any possibility of an indirect Tax association with KIX via protein-protein interaction with pCREB. Use of the bZIP domain also allowed us to examine the Tax-KIX interaction in the absence of the pKID/KIX interaction. Radiolabeled vCRE DNA was incubated with purified CREB bZIP, GST-$KIX_{588-683}$, and Tax forming a quaternary complex (Figure 2C, lane 3). Formation of this more slowly migrating complex is dependent upon the binding of KIX to the ternary complex formed with bZIP, Tax, and vCRE DNA. The MLL peptide was titrated into the binding reactions. Figure 2C shows that the MLL activation domain disrupted the interaction between Tax and KIX. To rule out the possibility that the MLL peptide might be interfering with formation of the ternary complex composed of Tax, pCREB, and vCRE DNA, another EMSA was performed in which the MLL peptide was titrated into reactions containing full-length pCREB, Tax, and vCRE DNA. We used full-length pCREB in this experiment as Tax does not bind the bZIP/vCRE DNA complex strongly enough in the absence of KIX to form a detectable ternary complex. The MLL peptide did not interfere with formation of the Tax/pCREB/vCRE ternary complex (Figure 2D). These results strongly suggest that Tax and MLL bind to the same site on the KIX domain.

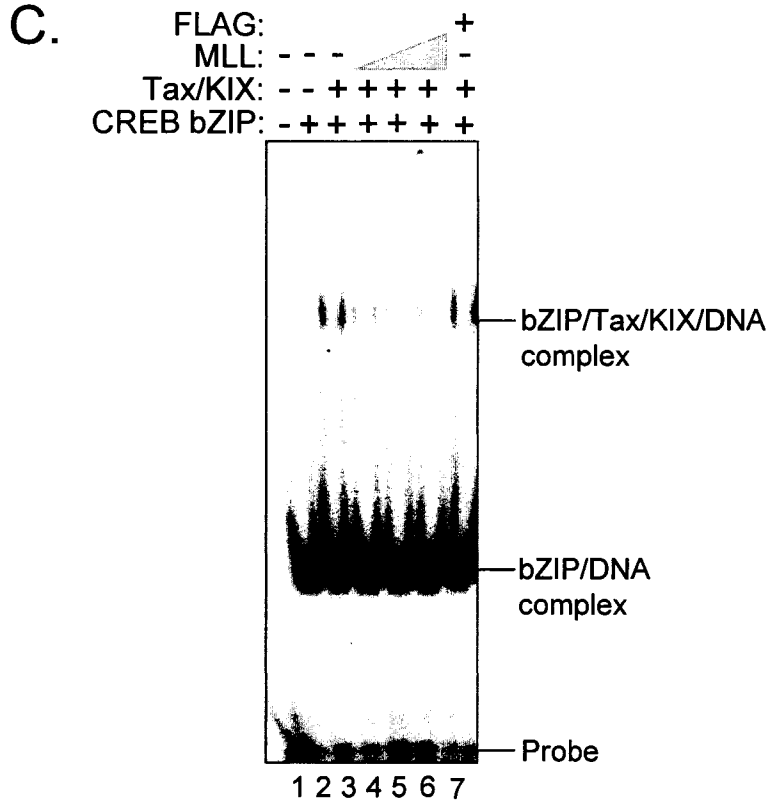


Figure 3.2C. *MLL peptide inhibits quaternary complex formation.* Electrophoretic mobility shift assays were performed with γ-^{32}P-end labeled vCRE probe (0.15 nM), bZIP (7.8 nM), Tax (350 nM) and GST-KIX$_{588\text{-}683}$ (312 nM). FLAG-tagged MLL peptide was added to reactions in increasing amounts (12, 24, and 47 μM, lanes 4-6). FLAG peptide (47 μM) was added to lane 7 as a negative control. Each nucleoprotein complex is indicated.

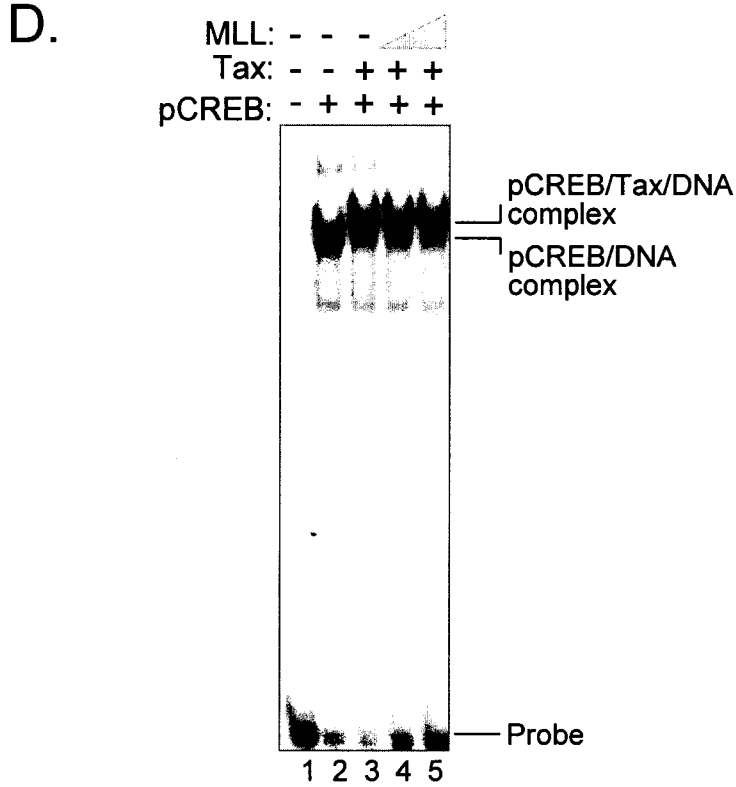


Figure 3.2D. *MLL peptide does not interfere with ternary complex formation.* An EMSA was performed as before with vCRE probe (0.15 nM), pCREB (2.5 nM), and Tax (250 nM). FLAG-tagged MLL peptide was added to reactions in increasing amounts (20 and 40 μM, lanes 4 and 5). Nucleoprotein complexes are indicated.

3.3c A CARBOXY-TERMINAL TRUNCATION OF KIX DEMONSTRATES SIGNIFICANTLY REDUCED TAX BINDING

The C-terminal half of the KIX α_3 helix is extended from E665 to R669 upon MLL binding, creating additional contacts between MLL and KIX (residues 612, 664, 667, and 668).[96] An earlier study found this region of the KIX domain was important for Tax binding, as a truncated KIX mutant (GST-KIX short, CBP aa 588-665) was defective for Tax but not pCREB binding.[94] We were interested in investigating whether this mutant is also defective for Tax binding in the context of the quaternary complex. Purified wild-type GST-$KIX_{588-683}$ (GST-KIX *wt*) and the truncated GST-$KIX_{588-665}$ (GST-KIX short) proteins are shown in Figure 3A. A GST pull-down assay was first performed to simultaneously test Tax and pCREB binding to GST-KIX short in the presence of vCRE DNA. Tax was titrated into reactions containing GST-KIX *wt* or GST-KIX short and a constant amount of pCREB and vCRE DNA. GST-KIX short showed dramatically reduced Tax binding while pCREB binding remained intact (Figure 3B). We next performed a DNA pull-down assay to test the ability of GST-KIX short to stabilize Tax binding in the pCREB/vCRE complex. For this assay we used a biotinylated vCRE double-stranded oligonucleotide bound to streptavidin-agarose beads. A biotinylated DNA fragment carrying a weak CREB binding site was used as a negative control for Tax binding. The presence of GST-KIX short in the binding reaction did not stabilize Tax on the immobilized template as effectively as wild-type GST-KIX (Figure 3C), demonstrating the importance of the C-terminal half of

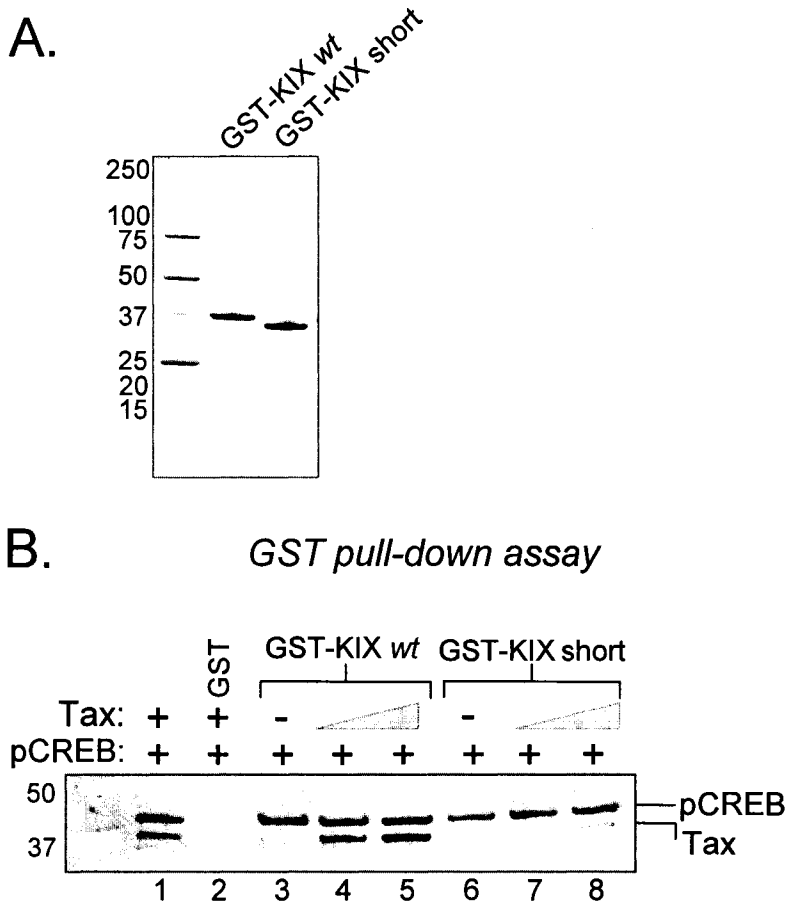


Figure 3.3. A carboxy-terminal truncation of KIX demonstrates significantly reduced Tax binding. A. Purification of GST-KIX *wt* and GST-KIX short. Purified wild-type GST-$KIX_{588-683}$ (GST-KIX *wt*), and GST-$KIX_{588-665}$ (GST-KIX short) are shown Coomassie-stained in a 10% SDS polyacrylamide gel as indicated.

B. *Tax binding to GST-KIX short is significantly reduced compared to wild-type GST-KIX, while pCREB binding remains unaffected.* GST-KIX *wt* (25 nM, lanes 3-5) or GST-KIX short (25 nM, lanes 6-8) were used in a GST pull-down assay in the presence of pCREB (5 nM) and vCRE DNA (500 nM). Full-length purified Tax was added in increasing amounts (5 and 10 nM) as indicated. pCREB and Tax input amounts (20% each) are shown in lane 1. A Western blot was performed using a mixture of antibodies against CREB and His_6.

C. *vCRE DNA pull-down assay*

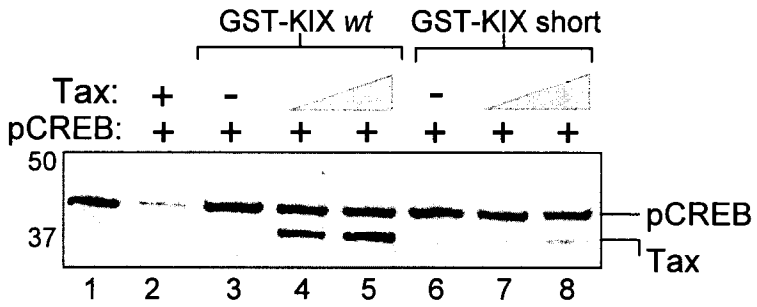


Figure 3.3C. *Tax binding at the vCRE is significantly reduced in the presence of GST-KIX short relative to wild-type GST-KIX.* Increasing amounts of Tax (20 and 40 nM) were added as indicated to binding reactions containing either GST-KIX *wt* (5 nM, lanes 3-5) or GST-KIX short (5 nM, lanes 6-8), biotinylated vCRE DNA (5 nM), and pCREB (7.5 nM). A biotinylated DNA fragment with a weak CREB binding site (lane 2) was included as a negative control for Tax. The pCREB input amount (13%) is shown in lane 1. A Western blot was performed using a cocktail of antibodies against CREB and His_6.

the KIX α_3 helix for Tax binding in the context of the vCRE-containing quaternary complex.

3.3d MUTATION OF KIX AMINO ACIDS IMPORTANT FOR MLL INTERACTION REDUCES TAX BINDING

In light of our findings that the MLL peptide competes with Tax for KIX binding and KIX α_3 helix residues are important for Tax as well as MLL binding, we designed a KIX triple point mutant based on conserved, solvent-exposed KIX residues found to interact strongly with MLL (aa F612, D622, and R624).[120] We hypothesized that this mutant would be defective for Tax binding. Using site-directed mutagenesis, we constructed GST-$KIX_{588-683}$ ΔT (Tax binding site mutant) with these three residues mutated to alanine. The KIX residues targeted for mutation are shown in blue in Figure 4A. Purified GST-KIX ΔT is shown in Figure 4B. A GST pull-down assay was performed using GST-KIX *wt* or GST-KIX ΔT bound to glutathione-agarose beads. Tax was titrated into reactions containing a constant amount of pCREB and vCRE DNA (Figure 4C). Tax binding to GST-KIX ΔT was reduced by approximately two-thirds relative to GST-KIX *wt*, while pCREB binding remained unaffected. These data provide further support for the hypothesis that full-length Tax and MLL share the same binding site on KIX. We also performed a DNA pull-down assay using biotinylated vCRE DNA bound to streptavidin-agarose beads. A biotinylated DNA fragment carrying a weak CREB binding site was used as a negative control for Tax binding.

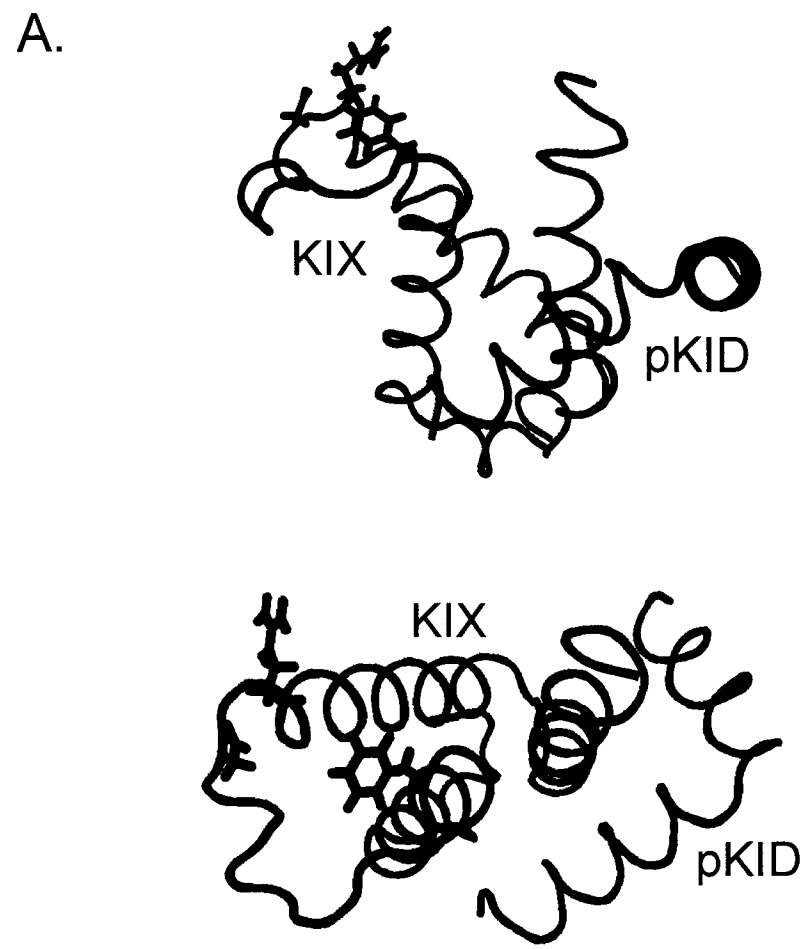
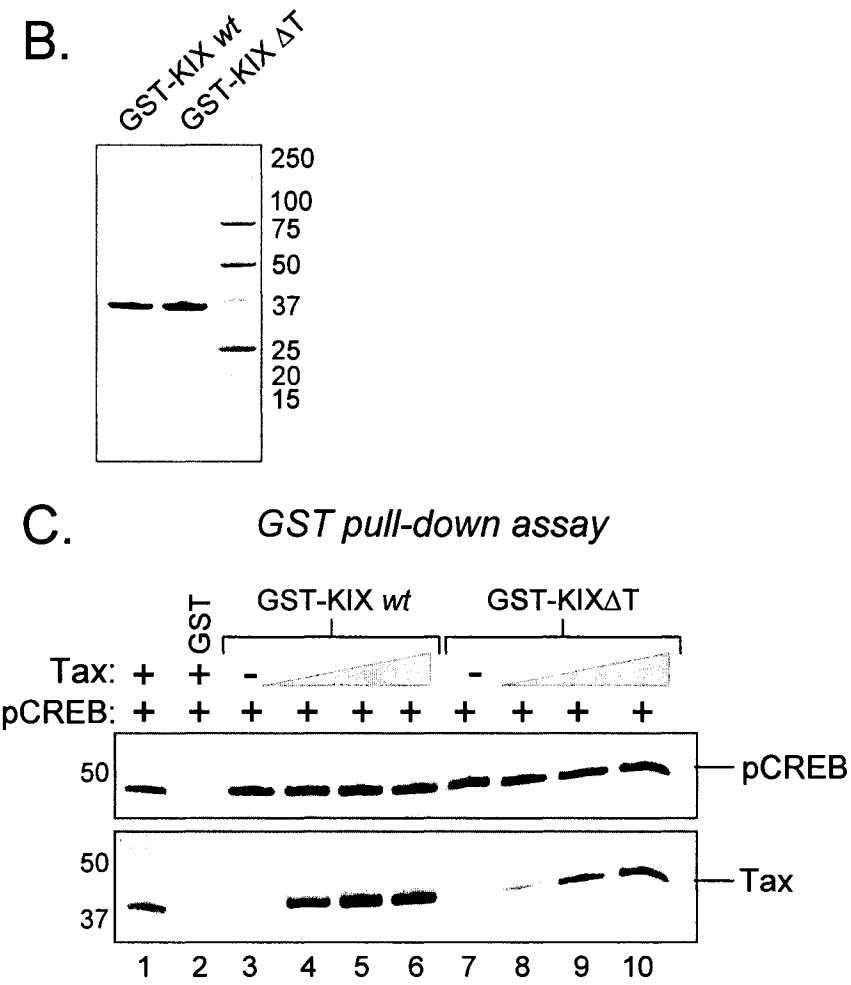


Figure 3.4. Mutation of KIX amino acids important for MLL interaction reduces Tax binding. A. *Schematic of wild-type KIX residues mutated in GST-KIX ΔT.* Two orthogonal views of KIX (in green) and pKID (in fuschia) are shown in complex with each other as determined by solution NMR (Protein Data bank accession code 1kdx). The three KIX residues shown in blue (F612, D622, and R624) strongly interact with MLL (De Guzman *et al.* 2006, Goto *et al.* 2002). These amino acids were mutated to alanine for construction of GST-KIX ΔT.



3.4B. *Purification of GST-KIX ΔT.* Purified wild-type GST-KIX$_{588-683}$ (GST-KIX *wt*) and GST-KIX ΔT (F612A, D622A, and R624A) are shown Coomassie-stained in a 10% SDS polyacrylamide gel as indicated.

C. *GST-KIX ΔT demonstrates significantly reduced Tax binding.* GST-KIX *wt* (25 nM, lanes 3-6) or GST-KIX ΔT (25 nM, lanes 7-10) was used in a GST pull-down assay along with pCREB (5 nM) and vCRE DNA (500 nM). Full-length purified Tax was added in increasing amounts (5, 10, and 20 nM, lanes 4-6 and 8-10). Tax and pCREB input amounts (50%) are shown in lane 1. As a negative control, pCREB and Tax binding to GST was tested (lane 2). A Western blot was first performed using an anti-His$_6$ antibody to detect Tax (lower panel). The blot was then incubated with an antibody to detect CREB (upper panel).

As in Figure 4C, Tax was titrated into reactions containing either GST-KIX *wt* or GST-KIX ΔT in the presence of a constant amount of pCREB (Figure 4D). Tax binding at the vCRE was significantly reduced in the presence of GST-KIX ΔT relative to GST-KIX *wt* (lanes 6-8 vs. 3-5). This experiment demonstrates that mutation of the putative Tax binding site on KIX significantly reduced Tax association with the DNA-bound complex.

3.3e A KIX MUTANT DEFECTIVE FOR pCREB BINDING DEMONSTRATES WILD-TYPE TAX BINDING

We constructed another KIX mutant, GST-KIX ΔpC, which carries a single point mutation (Y658A) that has previously been shown to be defective for pCREB binding (Figure 5A, purified protein shown in Figure 5B).[93] We reasoned that if a KIX mutant defective for Tax binding can bind pCREB, then similarly, a KIX mutant defective for pCREB binding should be able to bind Tax. To test this theory, a GST pull-down experiment was first performed to confirm that our KIX mutant was indeed defective for pCREB binding. pCREB was titrated into reactions containing either glutathione-bound GST-KIX *wt* or GST-KIX ΔpC (Figure 5C). As expected, we did not detect pCREB binding to GST-KIX ΔpC. The lower panel shows comparable amounts of GST-KIX *wt* and ΔpC were used in the experiment. We next performed another GST pull-down assay to test the ability of GST-KIX ΔpC to bind full-length Tax. Tax was titrated into reactions containing either GST-KIX *wt* or GST-KIX ΔpC (Figure 5D). Tax binding to GST-

KIX ΔpC and GST-KIX *wt* was comparable, indicating mutation of the pCREB binding site on KIX does not affect Tax binding.

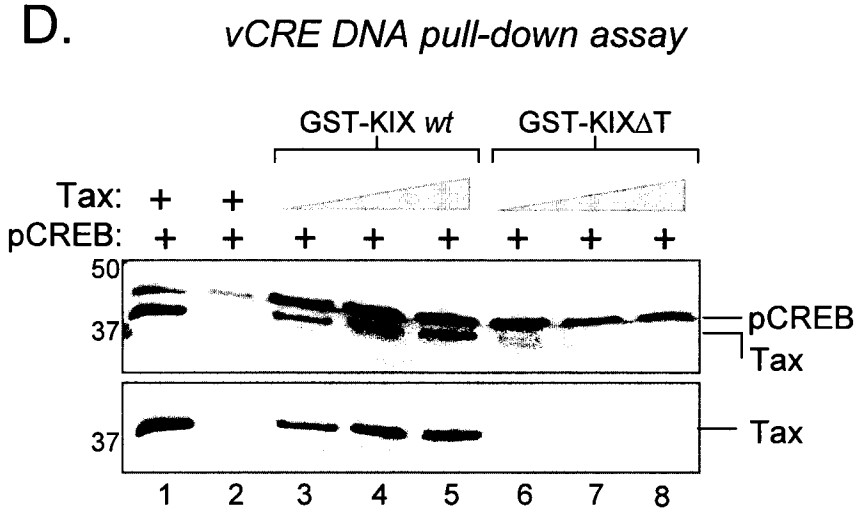


Figure 3.4D. *Tax binding at the vCRE is significantly reduced in the presence of GST-KIX ΔT relative to wild-type GST-KIX.* Increasing amounts of Tax (5, 10, and 20 nM) were added to binding reactions containing either GST-KIX *wt* (12.5 nM, lanes 3-5) or GST-KIX ΔT (12.5 nM, lanes 6-8), biotinylated vCRE DNA (5 nM), and pCREB (5 nM). A biotinylated DNA fragment carrying a weak CREB binding site (lane 2) was included as a negative control for Tax. pCREB and Tax input amounts (25%) are shown in lane 1. A Western blot was first performed using an anti-His_6 antibody to detect Tax (lower panel). The blot was then incubated with an anti-CREB antibody and both antibodies were imaged (upper panel).

A.

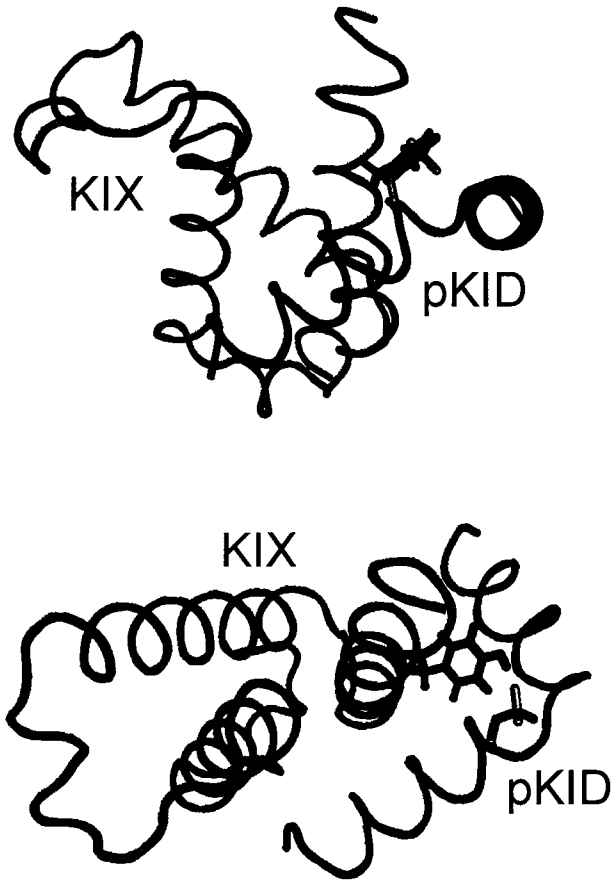


Figure 3.5. A KIX mutant defective for pCREB binding demonstrates wild-type Tax binding. A. *Schematic of wild-type KIX residues mutated in GST-KIX Δp.* Two orthogonal views of the KIX-pKID complex are shown with the KIX Y658 residue (in blue) interacting with pKID phosphorylated S133 (in orange). This KIX residue was mutated to alanine for construction of GST-KIX Δp.

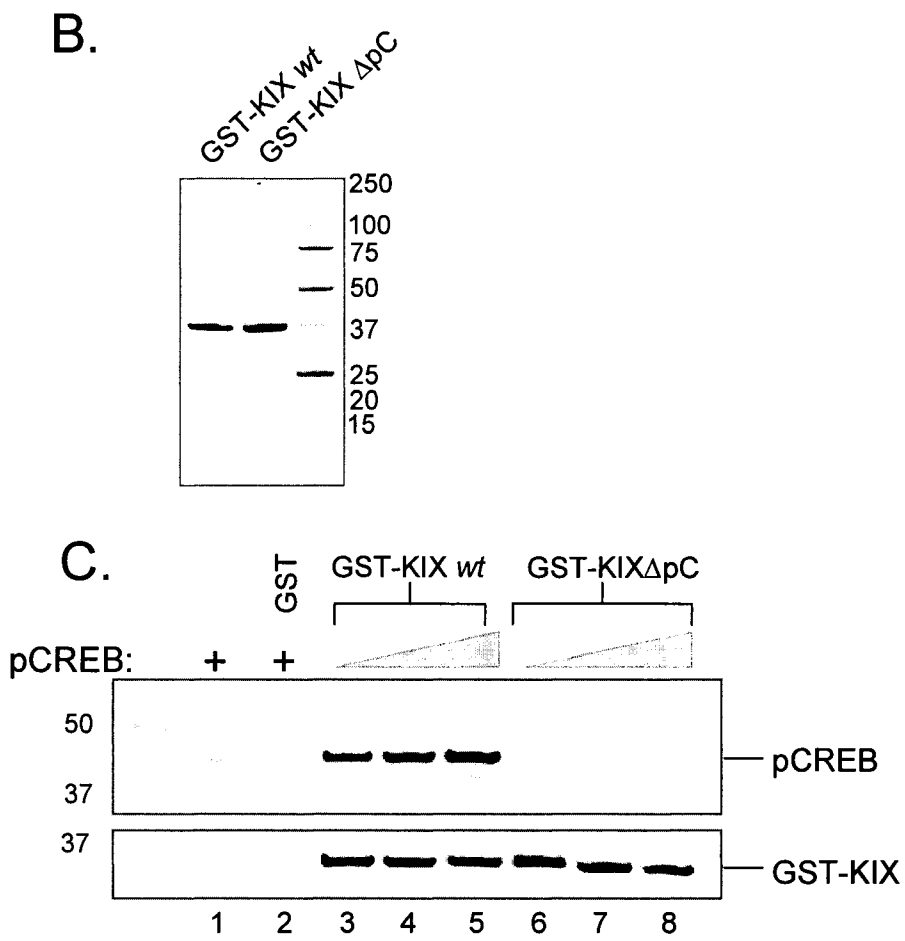


Figure 3.5B. *Purification of GST-KIX ΔpC.* Purified wild-type GST-KIX$_{588-683}$ (GST-KIX *wt*) and GST-KIX ΔpC (Y658A) are shown Coomassie-stained in a 10% SDS polyacrylamide gel as indicated.

C. *GST-KIX ΔpC does not detectably bind pCREB.* GST-KIX *wt* (100 nM, lanes 3-5) or GST-KIX ΔpC (100 nM, lanes 6-8) was used in a GST pull-down assay. Full-length purified pCREB was added in increasing amounts (10, 20, and 40 nM) as indicated. pCREB input (25% lowest concentration) is shown in lane 1. As a negative control, pCREB binding to GST was tested (lane 2). A Western blot was first performed using an antibody recognizing CREB phospho-Ser133 to detect pCREB (upper panel). The blot was then incubated with an anti-GST antibody to detect GST-KIX (lower panel).

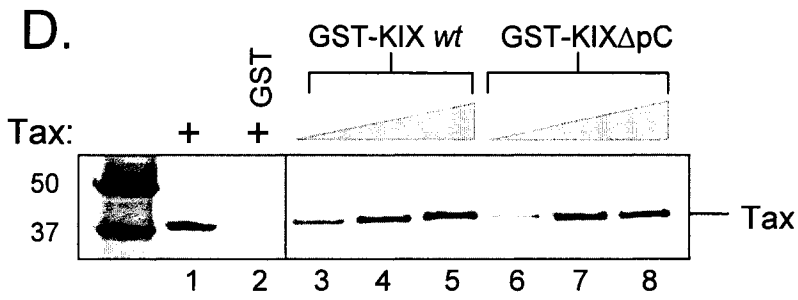


Figure 3.5D. *Tax binds GST-KIX ΔpC as well as wild-type GST-KIX.* GST-KIX *wt* (100 nM, lanes 3-5) or GST-KIX ΔpC (100 nM, lanes 6-8) was used in a GST pull-down assay. Full-length purified Tax was added in increasing amounts (0.7, 1.4, and 2.1 μM) as indicated. The pCREB input amount (3% lowest concentration) is shown in lane 1. As a negative control, Tax binding to GST was tested (lane 2). A Western blot was performed using an anti-His_6 antibody to detect Tax. The line between lanes 2 and 3 denotes cropping between lanes.

3.3f EMSA CONFIRMS TAX BINDS KIX AT AN INDEPENDENT SITE FROM PCREB

Although similar results were obtained from two different types of immobilization experiments, we confirmed our Tax binding results for the GST-KIX ΔT and ΔpC mutants using an additional method. EMSAs were performed with purified CREB bZIP, Tax, and the various GST-KIX proteins. As in Figure 2C, the bZIP domain of CREB was used in lieu of full-length pCREB to separate the Tax-KIX interaction from the pCREB-KIX and pCREB-Tax interactions. GST-KIX ΔpC and GST-KIX *wt* formed nearly equivalent amounts of the quaternary complex (KIX/Tax/bZIP/vCRE DNA) (Figure 6). However, the GST-KIX ΔT triple point mutant reduced the amount of quaternary complex to approximately one-third that of GST-KIX *wt*. As previously shown,[94] the truncated GST-KIX short construct did not support detectable quaternary complex formation.

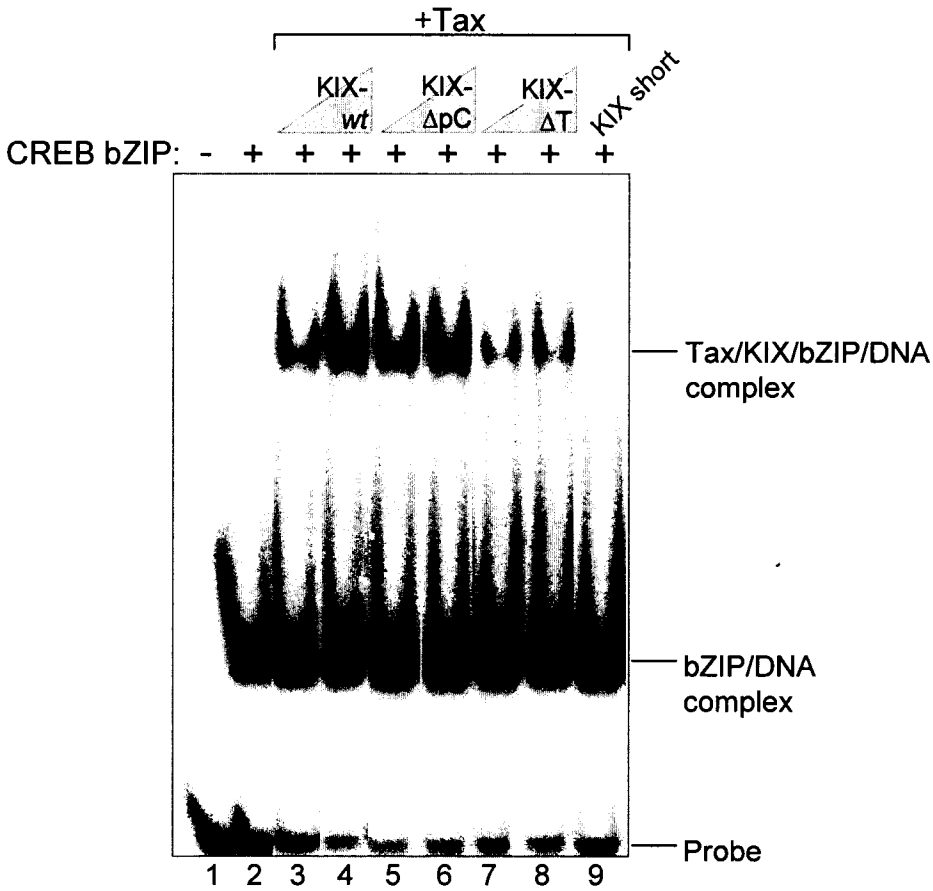


Figure 3.6. EMSA confirms Tax binds KIX at an independent site from pCREB. Electrophoretic mobility shift assays were performed with γ-^{32}P-end labeled vCRE probe (0.15 nM), CREB bZIP (5 nM), and Tax (246 nM). GST-KIX *wt* (0.2 and 0.3 μM), GST-KIX ΔpC (0.2 and 0.3 μM), GST-KIX ΔT (0.2 and 0.6 μM), or GST-KIX short (1 μM) were added to reactions as indicated. Each nucleoprotein complex is indicated.

3.4 DISCUSSION

The KIX domain of CBP has two separate hydrophobic grooves on opposite sides of the molecule that serve as transcription factor binding sites. Previous studies have demonstrated that one site binds the pKID region of CREB in addition to the activation domain of c-Myb, and the second site binds the activation domain of MLL and a domain of c-Jun.[96; 97; 105; 119; 120] The HTLV-1 Tax protein, in complex with pCREB and vCRE DNA, serves as a high-affinity binding site for the KIX domain.[65; 91] The observation that this complex binds KIX with considerably higher affinity than either protein alone suggests that pCREB and Tax may simultaneously occupy separate sites on KIX. However, full-length Tax and pCREB are much larger than the small domains examined in structural studies of cooperative transcription factor binding to KIX, and steric hindrance could prevent both from simultaneously binding the small KIX domain. In this study, we investigated the molecular interactions between KIX and full-length Tax and pCREB. We also characterized the interaction of the Tax/pCREB/vCRE ternary complex with KIX. As predicted, we found that both Tax and pCREB independently and simultaneously bind KIX. In addition, the MLL activation domain competed with Tax for KIX binding. The C-terminal half of KIX is elongated upon MLL binding, creating additional contacts between MLL and KIX. Our findings suggest Tax interacts with the MLL binding site on KIX in a similar fashion, since a truncated KIX mutant (GST-KIX short) was found defective for Tax binding. In addition, a KIX construct (GST-KIX ΔT) with mutations in the MLL binding site bound Tax with reduced affinity while retaining wild-type pCREB

binding. In both cases, mutations in the MLL binding site on KIX disrupted Tax binding, both with Tax free in solution and as part of the transcriptionally-relevant ternary complex. While pCREB and Tax bind KIX in very close proximity, these findings support a model for CBP/p300 recruitment to the DNA-bound Tax/pCREB complex in which Tax and pCREB simultaneously interact with separate binding sites on KIX.

A recent study found that the MLL activation domain transitions from an unstructured to a more highly structured state upon KIX binding.[96] A similar mechanism may occur during Tax binding to KIX in the context of the Tax-containing ternary complex. We have recently found the affinity of Tax for KIX is dramatically increased in the presence of pCREB and vCRE DNA.[121] Independent and simultaneous transcription factor binding to KIX provides a mechanism by which promoter-bound Tax and pCREB synergistically recruit CBP/p300 to the HTLV-1 promoter.

MLL and Tax competition for the same binding site on KIX raises the possibility that these two transcription factors compete *in vivo* for KIX binding and subsequent promoter recruitment of CBP/p300. This carries implications for an additional oncogenic function of Tax. MLL is a large *Drosophila* trithorax homologue that was originally identified as a gene frequently rearranged in therapy-induced acute myeloid leukemias and childhood leukemias. It positively regulates *Hox* genes and opposes the repressive effect of proteins such as *Bmi-1.* [123; 124; 125] Tax may disrupt normal MLL function by competition for CBP/p300 binding in HTLV-1 infected cells. Although MLL is usually not

rearranged in adult T-cell leukemia patients unless they develop secondary therapy-related myelodysplasia or leukemias,[126] Tax expression may mimic the effects of MLL rearrangement in HTLV-1 infected cells by preventing it from activating its target genes. Evidence for Tax-MLL competition for KIX binding thus provides a novel theoretical model for Tax oncogenesis involving disruption of MLL function early in the course of leukemogenesis, during high levels of Tax expression in HTLV-1 infected T-cells.

3.5 MATERIALS AND METHODS

3.5a EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

Bacterially-expressed CREB A[112], Tax-His_6[113] and all GST-KIX[91] proteins were purified to >95% homogeneity as previously described.[91] GST-KIX mutant expression vectors were prepared by site-directed mutagenesis (QuikChange II®, Stratagene), and the incorporation of mutations was verified by sequence analysis. CREB A is a naturally occurring splice variant (aa 1-327) where Ser^{119} corresponds to Ser^{133} in human CREB B (aa 1-341).[115] To avoid confusion, we will use the Ser^{133} nomenclature throughout this work. CREB and GST-KIX proteins were dialyzed against TM buffer (50 mM Tris pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 20% (vol/vol) glycerol, 0.025% (vol/vol) Tween-20, and 4 mM DTT), aliquoted and stored at –70° C. Tax-His_6 was dialyzed against buffer containing 50 mM Tris pH 8.0, 100 mM KCl, 0.5 M imidazole, 5 μM $ZnSO_4$ 20% (vol/vol) glycerol, and 4 mM DTT. It was also aliquoted and stored at –70° C. CREB was phosphorylated using the catalytic subunit of protein kinase A by incubating 1.6

μM CREB in a reaction containing 3.3 μM ATP, 5 mM $MgCl_2$, and 55 units of PKA (New England Biolabs) in a 25 mM potassium phosphate buffer, pH 6.6. Successful CREB phosphorylation was confirmed by the absence of γ-^{32}P-ATP incorporation following cold phosphorylation. The expression vector for untagged KIX (mouse $CBP_{586-680}$), a gift from Kevin J. Lumb,[127] was expressed in *E. coli* BL21(DE3)pLysS. Cultures were harvested by centrifugation, and resuspended in 0.015 volumes of 50 mM Tris-HCl pH7.5, 10 mM EDTA pH 7.5, 0.1 mM PMSF, 8 ng/μl aprotinin, 8 ng/μl leupeptin, 1 mini EDTA-free protease inhibitor tablet (Roche, cat #1836170), and 2 mM DTT. Cells were sonicated, the lysate cleared by centrifugation, and applied to Q-sepharose. The flow through containing KIX was applied to two consecutive SP-sepharose columns. KIX was purified to ≥98% homogeneity. Cultures were harvested by centrifugation and resuspended in 0.015 volumes of 50 mM Tris-HCl pH7.5, 10 mM EDTA pH 7.5, 0.1 mM PMSF, 8 ng/μl aprotinin, 8 ng/μl leupeptin, 1 mini EDTA-free protease inhibitor tablet (Roche, cat #1836170), and 2 mM DTT. Cells were sonicated, the lysate cleared by centrifugation, and applied to Q-sepharose. The flow through containing KIX was applied to two consecutive SP-sepharose columns. KIX was purified to ≥98% homogeneity.

3.5b MLL PEPTIDE

A peptide containing the minimal human MLL activation domain (aa 2842-2858) with a C-terminal FLAG tag was synthesized by Global Peptide. An N-terminal fluorescein-tagged version of this peptide was also obtained from Global

Peptide for fluorescence polarization experiments.

3.5c OLIGONUCLEOTIDES

The top strand sequence of the complementary oligonucleotides used in the experiments described herein are as follows. The CRE core sequence is underlined and bolded. vCRE: 5'-GAAGATCTCTCAGGCGT**TGACGTCA**ACCCCTCACAGATCTTC-3'. Our vCRE construct carries the full vCRE sequence with a single base pair change that converts the off-consensus CRE core to a consensus CRE. It binds Tax indistinguishably from the natural vCRE. A DNA fragment containing a weak CREB binding site (underlined and bolded) was used as a negative control for Tax binding:

5'-GGGGATCT**CTCA**AATATTCTTAGGACCTTTCACCAGATCGGC-3'. The oligonucleotides were purchased from Integrated DNA Technologies (IDT). For the DNA pull-down reactions, a biotin group was chemically added to the 5' end of the upper strand oligonucleotide (IDT).

3.5d ANTIBODIES

The antibodies used in the Western blots presented herein were as follows: anti-His (H-3 and H-15), anti-CREB (C-21), anti-phospho-Ser^{133} CREB, and anti-GST (B-14). All were purchased from Santa Cruz Biotechnologies.

3.5e GST PULL-DOWN ASSAYS

GST pull-down experiments were performed using 10 µl of glutathione-agarose beads equilibrated in pull-down buffer (20 mM HEPES [pH 7.9], 12.5 mM $MgCl_2$, 10 µM $ZnSO_4$, 100 mM NaCl, 50 mM KCl, 10% [vol/vol] glycerol, 0.05% [vol/vol] Nonidet P-40). The purified GST proteins were incubated with the equilibrated beads for 1 h at 4°C, washed, and incubated with the second protein(s) for 1 h at 4°C. The reactions were washed two times with pull-down buffer. Bovine serum albumin (0.3 µM) was used to block glutathione agarose beads between GST fusion and experimental protein binding. Bound proteins were resolved by electrophoresis on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to nitrocellulose for subsequent Western blot analysis. The final concentrations of protein and DNA in each reaction are given in the figure legend.

3.5f FLUORESCENCE POLARIZATION ASSAYS

Fluorescence polarization measurements were conducted using a black 384 well flat-bottom polystyrene plate in a Perkin Elmer Victor V model 1420 multilabel counter using 480 nm excitation and 535 nm emission band-pass filters. The 50 µl reactions contained 50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM EDTA (pH 7.5), 20% glycerol, 4 mM DTT, 0.1% NP40, 10 nM N-terminal fluorescein-labeled MLL peptide, and purified untagged $KIX_{586-680}$ at various concentrations from 53 nM to 22 µM. Reactions were assembled at 4° C and allowed to equilibrate for 30 min at 25° C prior to reading the FP signals. The equilibrium dissociation constant was determined by directly fitting the

polarization data to a single-site binding curve using Kaleidagraph (Synergy Software). The K_d value obtained was >100-fold higher than the 10 nM MLL concentration and consequently the free and total KIX concentrations were considered equal when fitting the data. This experiment was performed several times with different KIX protein preparations and similar results were obtained.

3.5g DNA PULL-DOWN ASSAYS

DNA pull-down experiments were performed using streptavidin-coated agarose beads (Novagen). Biotinylated double-stranded oligonucleotides containing a single CRE element were bound to streptavidin-agarose beads by incubating 90 min at 25°C according to the manufacturer's directions. The amount of DNA bound was quantified by measuring the A_{260} of the DNA-containing supernatant before and after streptavidin-agarose bead binding. DNA-bound beads were stored in a 100 mM Na_2HPO_4 pH 7.5, 0.02% sodium azide solution and washed with 0.5X TM buffer before use in assays. Purified proteins were added to aliquots of the streptavidin-agarose bead-bound DNA in 0.5X TM buffer with 0.6 ng/μL poly(dA)·poly(dT) and 39 nM BSA added as nonspecific competitors, incubated 45 min at 4°C, and washed three times to remove unbound proteins. DNA-bound proteins were separated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to nitrocellulose for detection by Western blot analysis.

3.5h ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

EMSAs were performed by incubation of the indicated amount of purified CREB, CREB bZIP, Tax, GST-KIX (aa588-683), GST-KIX ΔT (D622A, R624A, F612A), GST-KIX Δp (Y658A), or GST-KIX short (aa 588-665) in 12.5 mM HEPES pH 7.9, 75 mM KCl, 6.25 mM $MgCl_2$, 10% (vol/vol) glycerol, 5 μM $ZnSO_4$, and 0.05% (vol/vol) NP-40 containing 0.2 nM ^{32}P-end-labeled vCRE probe and 250 ng/mL poly(dA)·poly(dT) in a 20 μl reaction volume. Binding reactions were incubated on ice for 15 min. and resolved on 5% nondenaturing polyacrylamide gels [49:1 (wt/wt), acrylamide:N,N'-methylenebisacrylamide] in a buffer containing 0.04 M Tris·HCl, 0.306 M glycine (pH 8.5), and 0.1% (vol/vol) Nonidet P-40. Gels were dried and visualized by PhosphorImager (Molecular Dynamics).

3.5i PROTEIN STRUCTURE GRAPHICS

Cn3D 4.1 was used to generate graphics depicting mutated residues in KIX ΔT and KIX ΔpC. The NCBI protein databank code used was 1KDX (MMDB #9136).[93]

3.5j IMAGE PROCESSING

The ImageQuant program (Molecular Dynamics) was used to quantify results. Images were processed in Adobe Photoshop, with minor adjustments made to brightness/contrast as needed (gamma was kept at 1). No bands were obscured or altered. Images were annotated in PowerPoint.

3.6 ACKNOWLEDGMENTS

We thank Olve Peersen for invaluable suggestions and discussion. We also thank Jeanne Mick for contributing the EMSA shown in Figure 1D, Dinaida Lopez for critical discussion and provision of purified Tax protein, Mara Miller for critical reading of the manuscript, and Sarah Horstmann for help with site-directed mutagenesis. This work was supported by a grant from the National Institutes of Health (CA55035, J.K.N.). J.A.R. was supported by a minority supplement (CA55035-S1).

SUPPLEMENTAL FIGURES

The supplemental figures contained in this section include experiments I performed that did not appear in the previous chapter "Molecular characterization of HTLV-1 Tax interaction with the KIX domain of CBP/p300". These experiments present complementary data that expands on the results shown in chapter 3.

Figure 3.7 *Wild-type KIX represses Tax transactivation more than KIX mutants defective for Tax interaction.* This experiment is an *in vitro* transcription on an assembled chromatin 4TxRE G-less cassette containing four tandem copies of the promoter proximal vCRE. It was performed to demonstrate a dominant-negative effect of exogenous KIX addition on Tax transactivation by blocking CBP/p300 KIX domain binding to the CREB/Tax/viral CRE DNA ternary complexes. The experiment clearly shows a dominant-negative effect of GST-KIX *wt*, which was readily reproducible. However, the effect of the GST-KIX mutants with reduced Tax binding on transcription was inconsistent both within and between experiments. The experiment shown is the best representative showing decreased ability of the KIX mutants to bind the ternary complex and repress transcription.

Figure 3.8 *KIX suppresses Tax transactivation in vivo.* Expression vectors for Tax and various GST-KIX constructs were co-transfected into CHO-K1 cells containing a chromosomally-integrated HTLV-1 LTR-luciferase reporter construct.[117] The intent of these experiments was to demonstrate the dominant-negative effect of KIX on Tax transactivation of the HTLV-1 promoter *in vivo* and

to show a difference in the ability of KIX *wt* and reduced Tax binding KIX mutants to affect Tax transactivation. KIX expression reduced Tax transactivation by more than 50%, indicating the interaction of the Tax/CREB/viral CRE ternary complex with the KIX domain of CBP/p300 plays an important role in Tax-mediated transactivation *in vivo*. However, the KIX mutants with reduced Tax binding did not demonstrate a significantly different effect on transcription than did *wt* KIX.

Figure 3.9 A-C. *V89A Tax has a lower affinity for KIX than wild-type Tax.* These three experiments test whether V89A Tax is truly defective for KIX binding, as has been previously published.[92] Figure 3.9A is an electrophoretic mobility shift assay (EMSA) with GST-KIX *wt* titrated into reactions containing full-length CREB and either wild-type (*wt*) Tax or V89A Tax. The apparent K_d of GST-KIX for the V89A ternary complex is ~30 nM greater than for the *wt* ternary complex. Figure 3.9B is another EMSA with either *wt* Tax or V89A Tax titrated into reactions containing CREB bZIP and GST-KIX *wt*. This experiment was performed with the bZIP domain to isolate the Tax-KIX interaction from the Tax-CREB interaction. V89A Tax shifts ~2/3 less probe than *wt* Tax at any given concentration. Figure 3.9C is a GST pull-down assay with either *wt* Tax or V89A Tax titrated into reactions containing pCREB, GST-KIX *wt*, and viral CRE DNA. V89A Tax bound the quaternary complex only ~1/3 as well as *wt* Tax. These experiments clearly show V89A Tax binds to the KIX domain of CBP with a weaker affinity than *wt* Tax. However, they also show that V89A Tax is not defective for KIX binding as was previously thought.

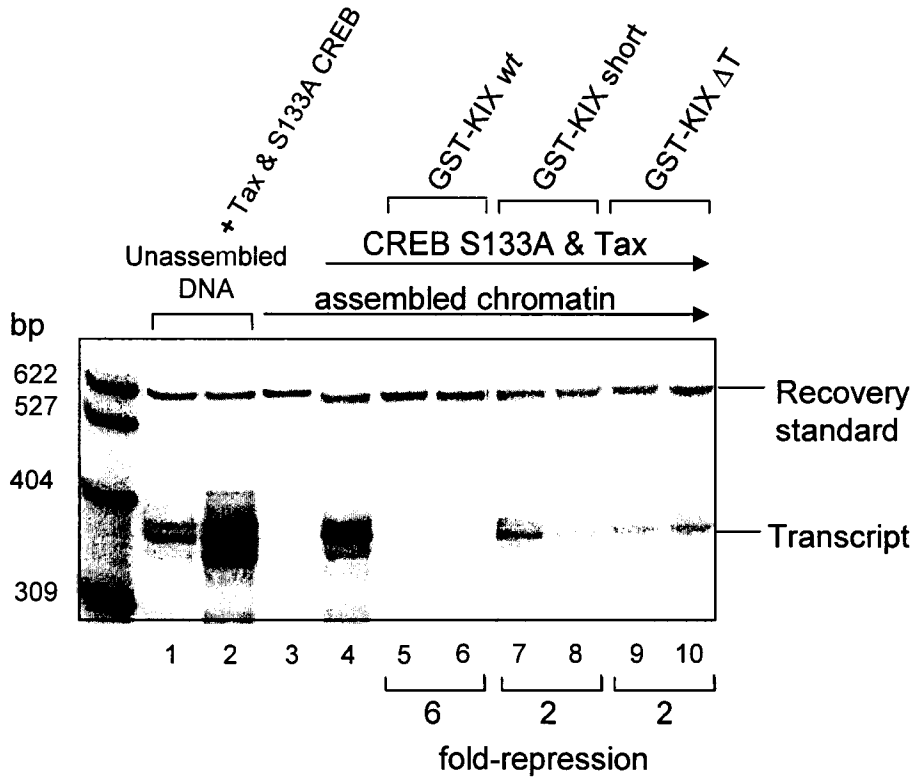


Figure 3.7 Wild-type KIX represses Tax transactivation more than KIX mutants defective for Tax interaction. *In vitro* transcription. Template DNA is 4TxRE G-less cassette assembled with *drosophila* core histones and dNAP. S133A CREB (67 nM) and Tax (100 nM) were added to reactions along with GST-KIX *wt,* short, or ΔT (167 nM ea.) and pre-incubated 20 min at 30° C with assembled DNA. CEM nuclear extract was then added. Lanes 5 and 6, 7 and 8, 9 and 10 are duplicates.

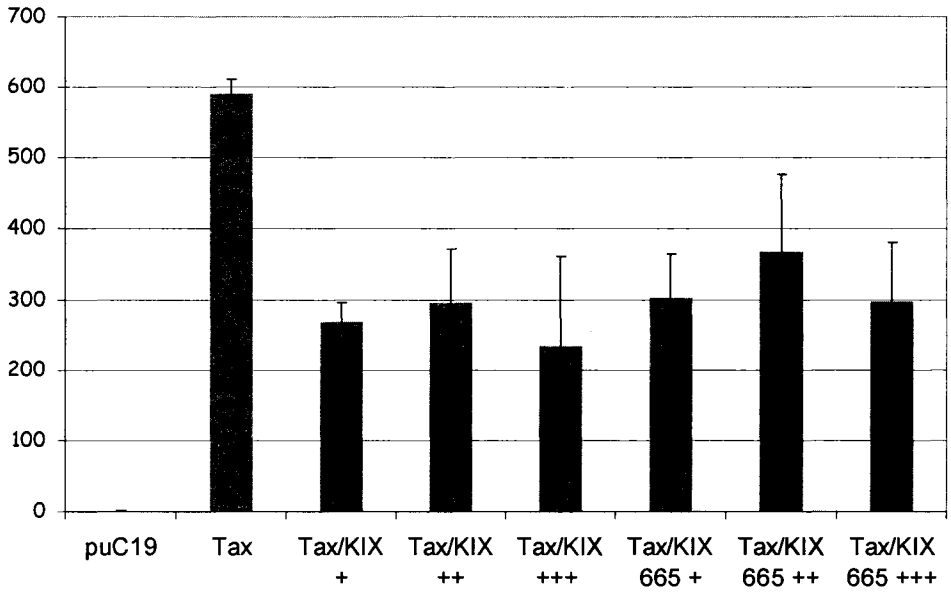


Figure 3.8 KIX suppresses Tax transactivation in vivo. CHO-K1 cells containing a chromosomally-integrated HTLV-1 LTR-luc were co-transfected with expression vectors for Tax (pSG-Tax, 100 ng) and KIX constructs (RSV-KIX *wt* or RSV-KIX short, 30, 150, and 300 ng) with pUC19 as a control. Cells were harvested and luciferase assays were performed 24 hours after transfection. Each transfection was repeated in triplicate.

A.

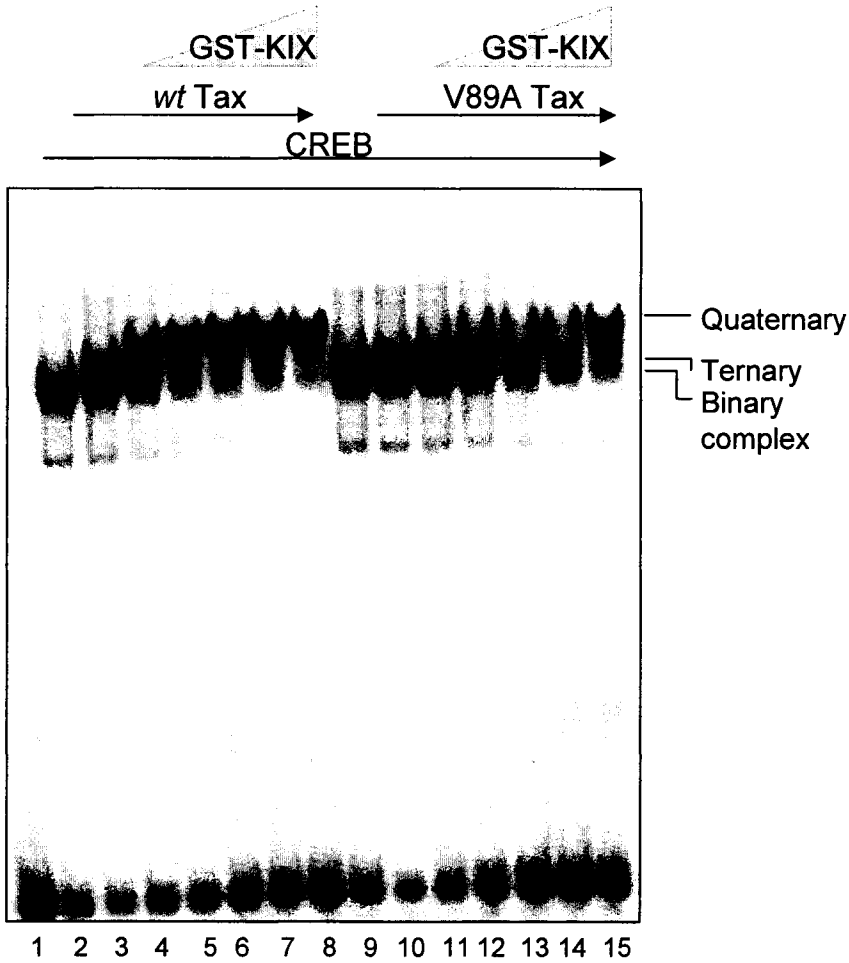


Figure 3.9A. V89A Tax has a lower affinity for KIX than wild-type Tax. An electrophoretic mobility shift assay was performed with γ-^{32}P-end labeled vCRE probe (0.15 nM), 3 nM CREB, 50 nM each *wt* and V89A Tax, and a titration of GST-KIX *wt* (10, 20, 30, 40, 50 nM).

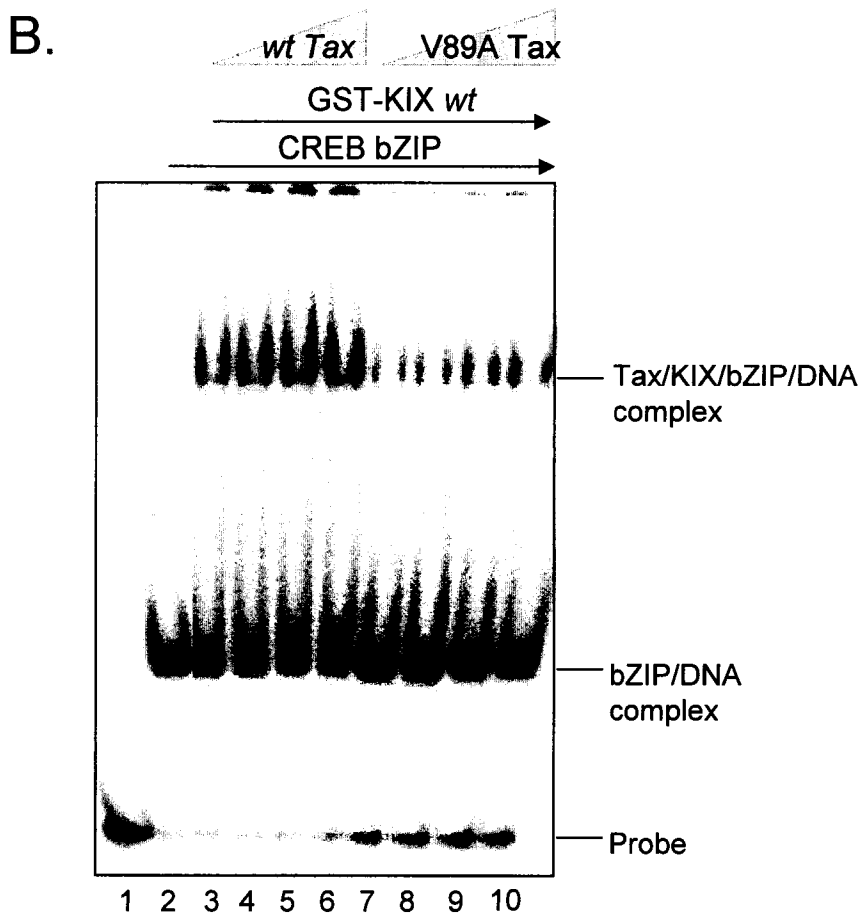


Figure 3.9B. *V89A Tax has a lower affinity for KIX than wild-type Tax.* An electrophoretic mobility shift assay was performed with γ-^{32}P-end labeled vCRE probe (0.15 nM), CREB bZIP (5 nM), GST-KIX *wt* (0.2 μM) and a titration of either *wt* or V89A Tax (0.1, 0.2, 0.4, 0.6 μM).

C.

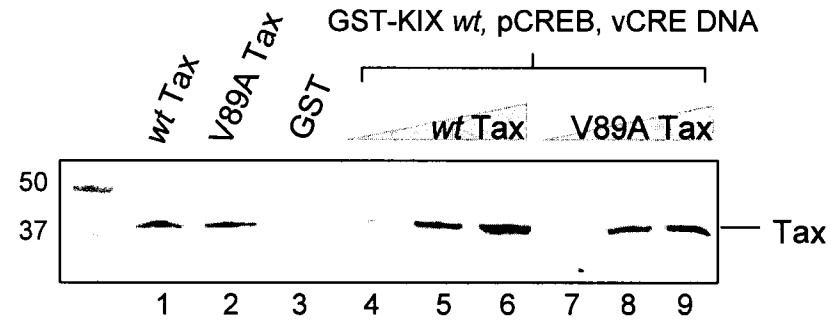


Figure 3.9C. *V89A Tax has a lower affinity for KIX than wild-type Tax.* GST pull-down assay. Full-length purified *wt* Tax (lanes 4-6) or V89A Tax (lanes 7-9) was added in increasing amounts (5, 20, and 40 nM) to reactions containing GST-KIX *wt* (25 nM), pCREB (25 nM), and vCRE DNA (0.5 μM). The Tax input amounts (6% each, highest concentration) are shown in lane 1. As a negative control, Tax binding to GST was tested (lane 3). A Western blot was performed using an anti-His_6 antibody to detect Tax.

CHAPTER FOUR
FUTURE DIRECTIONS

The *in vitro* data presented here reveal that *i)* vCRE DNA and the KIX domain of CBP, as well as phosphorylation of CREB at serine 133, are all necessary for strong Tax binding to the quaternary complex *in vitro*, *ii)* Tax and pCREB make separate and independent contacts with KIX *in vitro*, and *iii)* the MLL activation domain inhibits Tax binding to KIX. These important observations define the role of the individual components of the quaternary complex in maintaining complex stability. A better understanding the interactions between the quaternary complex components furthers our knowledge of HTLV-1 transcription. The results of this study also contribute to paradigms for interactions between the components of other protein complexes involved in transcription. In addition, this research provides strong evidence that Tax and MLL share the same binding surface on KIX. This finding immediately raises several critical questions: Do Tax and MLL compete for CBP binding *in vivo*? Does Tax expression interfere with normal MLL function *in vivo*? If so, does this interference contribute to leukemogenesis in HTLV-1 infected cells?

Tax may compete with MLL for CBP/p300 binding, affecting transcription of MLL-responsive promoters in Tax-expressing HTLV-1 infected cells. Although MLL likely has many downstream target genes, it is best known for maintaining *HOX* gene expression during development and hematopoiesis. It also exhibits histone methyltransferase activity, affecting gene expression by directly altering

chromatin structure. MLL has also been shown to interact with chromatin-associated proteins and transcriptional regulators including CBP, likely affecting chromatin structure indirectly as well. MLL is best-known as a common target of chromosomal translocation associated with several types of leukemia. The MLL activation domain is lost in the fusion proteins resulting from these translocations. Although MLL translocations are rarely observed in ATL patients, Tax competition with the MLL activation domain for CBP binding may imitate the loss of domain function evident in MLL fusion proteins. Tax binding to free and/or promoter-bound CBP may prevent the CBP/MLL interaction necessary for transcriptional activation of MLL-responsive promoters. Additionally, overwhelming CBP binding to the HTLV-1 promoter in the presence of Tax might sequester CBP, affecting its availability for incorporation in transcriptional pre-initiation complexes at other promoters. Tax inhibition of MLL-mediated transcriptional activation could represent a novel mechanism contributing to ATL leukemogenesis through down-regulation of MLL-responsive promoters.

Both fluorescence polarization and analytical ultracentrifuge fluorescence detection could be used to test the hypothesis that Tax inhibits fluorescein-MLL binding to KIX. I have investigated the binding of KIX to fluorescein-MLL with good results. Free MLL peptide and MLL bound to KIX produce two distinct sedimentation coefficients (0.55 S for free MLL, 1.2 S for MLL/KIX complex) that vary in amplitude depending on the concentration of KIX used. Different concentrations of Tax could be added to samples containing MLL and KIX to assess the effect of Tax on MLL-KIX binding.

Other assays that would test whether Tax inhibits MLL binding to KIX might involve the expression and purification of recombinant GST-MLL activation domain. This protein could then be used for pull-down assays and/or electrophoretic mobility shift assays. Co-immunoprecipitation or pull-out assays using T-cell nuclear extract and immobilized GST-KIX might be used to investigate whether endogenous MLL binding to KIX is reduced in the presence of recombinant purified Tax. To address whether Tax and MLL compete for CBP binding *in vivo*, an MLL activation domain expression vector such as RSV-$MLL_{2829-2883}$ could be constructed and used for transfection into cells with an integrated HTLV-1 LTR-luc reporter. Co-transfection of this plasmid along with a Tax expression vector should decrease Tax-mediated transactivation by preventing Tax binding to CBP. Transfection of this plasmid into Tax-expressing T-cells should also decrease Tax association with CBP as measured by chromatin immunoprecipitation (ChIP) of CBP at the HTLV-1 promoter when compared to control transfections.

The questions of whether Tax expression interferes with normal MLL function *in vivo* and whether this contributes to leukemogenesis in HTLV-1 infected cells will be difficult to answer. The expression of MLL-responsive genes could be measured via reverse-transcriptase PCR to evaluate mRNA levels of particular *HOX* genes. ChIP assays could also be used to evaluate MLL transcriptional activity through investigation of MLL, di-methylated histone H3 K4, and RNA pol II occupancy on *HOX* promoters. These assays could be used to

compare MLL transcriptional activity in Tax-expressing infected vs. uninfected T-cells as well as cells transfected with Tax vs. control transfections.

These data raise other intriguing possibilities. Our finding that the MLL activation domain interferes with Tax binding to CBP could raise new possibilities for drug development to combat the development and/or progression of ATL. A synthetic peptide that mimics the MLL activation domain might effectively inhibit the Tax-CBP interaction, leading to significantly reduced transcription of the viral promoter as well as cellular promoters regulated by Tax. Such a drug might prove an effective method to inhibit ATL development and/or progression in HTLV-1 infected patients that express Tax. This treatment might also lead to decreased CBP accessibility to endogenous MLL. If disruption of the CBP/MLL interaction is important in ATL leukemogenesis as previously proposed, such a drug might be ineffective or even accelerate ATL development. In addition, other cellular transcription factors such as cJun bind CBP at the same site as MLL and Tax. A drug such as this could easily interfere with the normal cellular functions of CBP. A more effective strategy for drug design might involve designing a molecule that directly binds Tax and prevents interaction with CBP.

This study merely begins to characterize the Tax/KIX interface. The region of interaction between Tax and KIX could be more clearly defined through further biochemical analysis of KIX mutations, crosslinking studies, protease digestion, multi-dimensional NMR spectroscopy using unlabeled full-length Tax and labeled KIX, or solving the crystal structure of the quaternary complex. In addition, the region of Tax that interacts with KIX remains poorly defined. Although current

evidence strongly suggests that a region of Tax involving K88 and V89 interacts with KIX, other contacts between Tax and KIX are likely as mutations of these Tax residues still retain the ability to bind KIX weakly. Further research is needed to advance our knowledge of Tax-mediated transcriptional activation, to direct small-molecule drug development targeting disruption of viral transcription, and to serve as a model for understanding the interactions of other protein complexes involved in transcription.

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