DISSERTATION

TAX DEREGULATION OF HOST-CELL PROTEINS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY YOUNG-MI KIM ENTITLED *TAX DEREGULATION OF HOST-CELL PROTEINS* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

TAX DEREGULATION OF HOST-CELL PROTEINS

Human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus etiologically linked to an aggressive and generally fatal malignancy called adult T- cell leukemia (ATL) and to a chronic inflammatory neurological disease. Only a small percentage of infected individuals develop ATL following a prolonged latency period of up to 30 years post infection. The dominant mechanism of virus transmission in an infected individual is through clonal expansion of HTLV-1 infected cells. The HTLV-1-encoded protein Tax is the prominent player in promoting mitotic replication. Tax is also directly linked to malignant transformation and the etiology of ATL.

Tax is a potent transcriptional activator that stimulates HTLV-1 viral gene expression. Three 21 base pair repeat enhancer elements called viral cyclic AMP response elements (vCREs), located in the HTLV-1 transcriptional control region, are critical to Tax-activated transcription. Tax associates with the vCREs through protein-DNA interactions and through protein-protein interaction with the cellular transcription factor cAMP response element binding (CREB) protein. Together this complex recruits the cellular coactivators CBP/p300. The role of Ser133 phosphorylated CREB in mediating Tax function in HTLV-1 transcription has long been controversial. Our data reveal that CREB phosphorylation is absolutely required for viral Tax transactivation. Consistent with this, Tax induces

constitutively elevated levels of phosphorylated CREB *in vivo* and *in vitro*. We further investigated the mechanism of Tax-mediated CREB phosphorylation and uncovered a novel function of Tax: stimulation of CREB phosphorylation via the Ca2+/Calmodulin (CaM)-dependent protein kinase (CaM kinase) pathway to promote viral transcription. In addition to Tax-dependent CREB phosphorylation, we found that Tax upregulates B-cell lymphatic leukemia protein 3 (Bcl-3) and cyclin D1 expression, two key determinants of cell fate. Furthermore, Tax interacts with Bcl-3 *in vivo* and *in vitro*. Deregulation of these key host-cell proteins by Tax may contribute to the transformation of T-cells.

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INTRODUCTION TO HTLV-1: ETIOLOGY, DISEASE, & VIRAL FUNCTION

1.1 HUMAN T-CELL LEUKEMIA VIRUS TYPE 1

Adult T-cell leukemia (ATL) was first identified as a distinct clinical entity in Japan in 1977 (1). In 1980 and 1981, human T-cell leukemia virus type 1 (HTLV-1) was discovered as the causative agent of ATL in independent studies from the United States and Japan (2). Consequently, HTLV-1 became the first retrovirus shown to be directly associated with a human cancer. Moreover, HTLV-1 was also linked as the causative agent of a neurological progressive inflammatory syndrome called tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) (3). HTLV-1 is a member of the deltaretrovirus genus, which also includes human T-cell leukemia virus type 2 (HTLV-2), bovine leukemia virus (BLV), and simian T-cell leukemia virus (STLV). Both BLV and STLV induce neoplastic diseases whereas HTLV-2 is not associated with any human malignancies. Currently, approximately, 10-20 million individuals worldwide are infected with HTLV-1 (4), which is endemic in southwestern Japan, parts of Central Africa, the Caribbean islands, and South America.

HTLV-1 is transmitted between individuals via three major routes: 1) mother- to-infant transmission through breast milk, 2) sexual transmission, 3) parenteral transmission through blood transfusions and intravenous drug use (5). The infectivity of cell-free viral particles is very inefficient and virions are not detected in the serum of HTLV-1 infected individuals. Therefore, HTLV-1 is

mainly spread by cell-to-cell contact. Contact between infected and target cells induces polarization of microtubule organizing center (MTOC) in infected cells to the cell-cell junction and formation of a "virological synapse". Then, HTLV-1 gag protein and the viral genome are transferred to target cells through the virological synapse (6).

In vitro studies have shown that HTLV-1 can infect a variety of cell types such as T-lymphocytes, B-lymphocytes, monocytes and fibroblasts (7). Accordingly, glucose transporter 1 (GLUT1) and surface heparin proteoglycan, which are ubiquitously expressed on cell surfaces, have been identified as receptors for HTLV-1(8, 9). However, the HTLV-1 provirus is almost exclusively detected in $CD4^+$ T-lymphocytes *in vivo* (10). This suggests that although HTLV-1 may potentially infect various cell types, $CD4^+$ T-cells may be selectively targeted for infection, leading to more efficient proliferation *in vivo*.

After infection, the virus enters the clinically latent stage, rendering most HTLV-1 carriers asymptomatic throughout their lives. However, in a low percentage of HTLV-1 carriers (2-5%), the infected $CD4^+$ T-cells proliferate, resulting in the onset of ATL following typically 20-50 years after infection (11). The onset of disease following such a long latency suggests that many cellular alterations or mutations are required for T-cell transformation.

1.1a ADULT T-CELL LEUKEMIA

ATL is a highly aggressive malignancy of $CD4^+$ T-lymphocytes. ATL is characterized clinically by skin lesions (due to infiltrating leukemic cells), lytic

bone lesions, and greater than 5% abnormal T-cells (with large, multi–segmented nuclei). ATL is molecularly characterized by the presence of a chromosomally-integrated HTLV-I provirus in a highly aneuploid T-cell (5). There are four clinically distinct subtypes of ATL: chronic, smoldering, acute and lymphoma (12). Chronic and smoldering types have mild clinical courses. However, the latter two subtypes, acute and lymphoma have a poor prognosis due to aggressive clinical courses and the patients with these types are resistant to extensive chemotherapy. Therefore, the median survival time of the latter types is reported to be approximately 13 months (13).

Like other retroviruses, the HTLV-1 genome is reverse transcribed and then randomly integrated into the host chromosome (14), resulting in a unique integration site in each infected cell. Analysis of these integration sites revealed that the proliferation of infected cells is clonal (15) and, furthermore, that some clones persist *in vivo* (16). Importantly, clonal expansion is directly associated with the onset of ATL in some carriers.

The observation that extracellular virions are rare, coupled with a high proportion of CD4+ T-cells carrying integrated provirus, implicates mitotic replication as the primary mode of viral transmission within an infected individual. In support of this observation, variation in proviral sequence in the infected population is relatively small, despite the fact that HTLV-1 encodes an error-prone reverse transcriptase (17). Furthermore, a recent study found that proviral loads were refractory to the administration of reverse transcriptase inhibitors (18). Taken together, these studies support the conclusion that the dominant

mechanism of proviral transmission in an infected individual is through clonal expansion of the provirus. The HTLV-1-encoded protein Tax appears to be the prominent player in achieving clonal proliferation. Tax is also directly linked to malignant transformation (19).

1.1b HTLV-1 GENOME

HTLV-1 is a single-stranded diploid RNA virus, which carries a genome of approximately 9 kb. Similar to other retroviruses the HTLV-1 proviral genome encodes structural proteins and enzymes including group-specific antigen (gag), envelope (env), reverse transcriptase, protease and integrase, which are flanked by long terminal repeat (LTR) sequences at both the 5′ and 3′ends (20). The HTLV-1 LTR contains the viral promoter and other regulatory elements and is divided into U3, R, and U5 regions. A schematic of the HTLV-1genome and provirus is shown in figure 1.1. The U3 region contains three highly conserved 21 bp-repeats called viral cyclic AMP response elements (vCREs) that control proviral transcription (21). Additionally, the genome contains several open reading frames within the pX region which is located between the env open reading frame (ORF) and the 3′LTR. This region encodes nonstructural proteins including Tax, Rex, p12, p30, p21 and HBZ. Among these, the Tax protein is critical for viral replication, cellular proliferation, and cellular transformation leading to ATL.

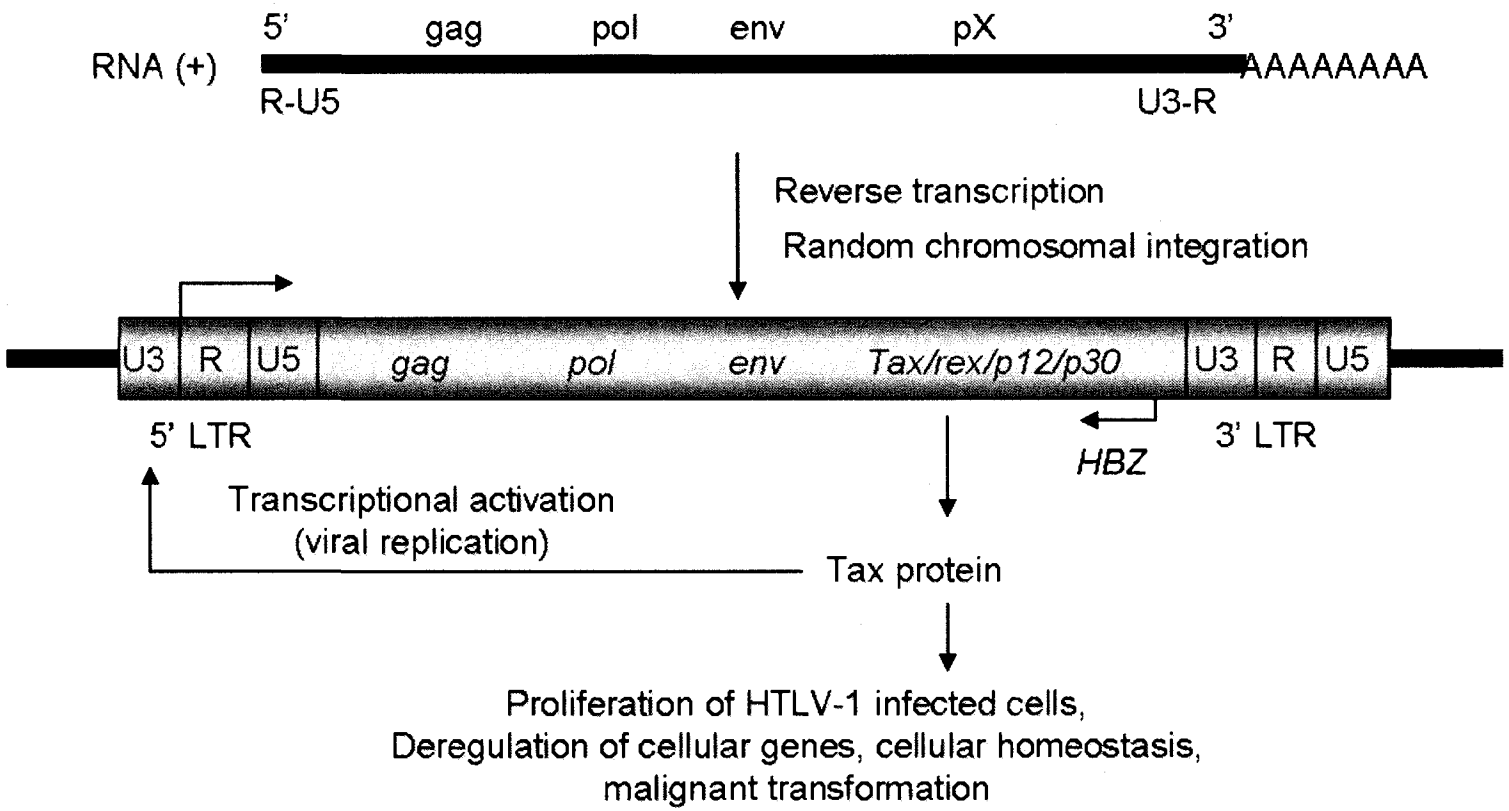


Figure 1.1. Schematic representation of the HTLV-1 viral genome and provirus. The gag and pol proteins are expressed from the full-genomic viral transcript, whereas the env protein is translated from a singly spliced viral RNA. The Tax and Rex proteins are expressed from a doubly spliced viral RNA.

1.2 PLEIOTROPIC FUNCTION OF TAX

Tax is a 40 kDa protein predominantly localized to the nucleus and is widely regarded as the primary oncogenic mediator of HTLV-1 due to its multiple actions on viral and cellular genes (22, 23). Tax is a potent transcriptional activator of HTLV-1 gene expression, contributing to viral replication (24). Tax also stimulates transcription of a large variety of cellular genes through deregulating cellular signal transduction pathways including the transcription factors NF-κB, CREB, and serum response factor (SRF) (23). For example, Tax stimulates these pathways and enhances the expression of several cytokines and receptors involved in T-cell growth and proliferation. Tax also stimulates the expression of several other transcription factors and proto-oncogenes, as well as inhibitors of nucleotide excision repair and apoptosis. Furthermore, Tax represses transcription of certain cellular genes involved in DNA repair and apoptosis. In addition to regulation of transcription, Tax is also known to interact with numerous cellular proteins to activate or inactivate their functions. For instance, Tax binds to and inactivates negative regulators of cyclin-dependent kinases such as $p16^{INK4a}$ and $p15^{INK4b}$(25).

Many seemingly divergent functions of Tax likely merge to promote cellular proliferation and may cooperate to facilitate malignant T-cell transformation. Taken together, Tax induces abnormal cell proliferation by activating growth promoting genes and repressing growth suppressing genes as well as by inhibiting the function of tumor suppressors. Tax also reduces DNA repair activity and bypasses cell cycle checkpoints, thus enhancing the

accumulation of mutations. These cooperative pleiotropic functions of Tax may correspond to the multiple steps required for the development of cancer. The overview of the pleiotropic functions is shown in figure 1.2.

HTLV-1 infected T cell lines constitutively express viral proteins at high levels. However, freshly isolated ATL cells express near undetectable levels of viral proteins (26). Even when viral transcripts are detected in leukemic cells by RT-PCR, Tax expression is so low and in only a small percent of in ATL cells (27). However, once the cells were cultured *ex vivo*, viral expression is quickly activated. We still do not understand the mechanism about how viral expression is maintained as a mostly latent form *in vivo* and is reactivated *in vitro*. Since Tax is a main target of cytotoxic T-cell mediated immune response (28, 29), ATL cells that have lost Tax expression may escape from the host immune surveillance and be preferentially selected *in vivo* during disease progression. Thus, it is speculated that Tax expression is required to initiate transformation, but not required to maintain transformation.

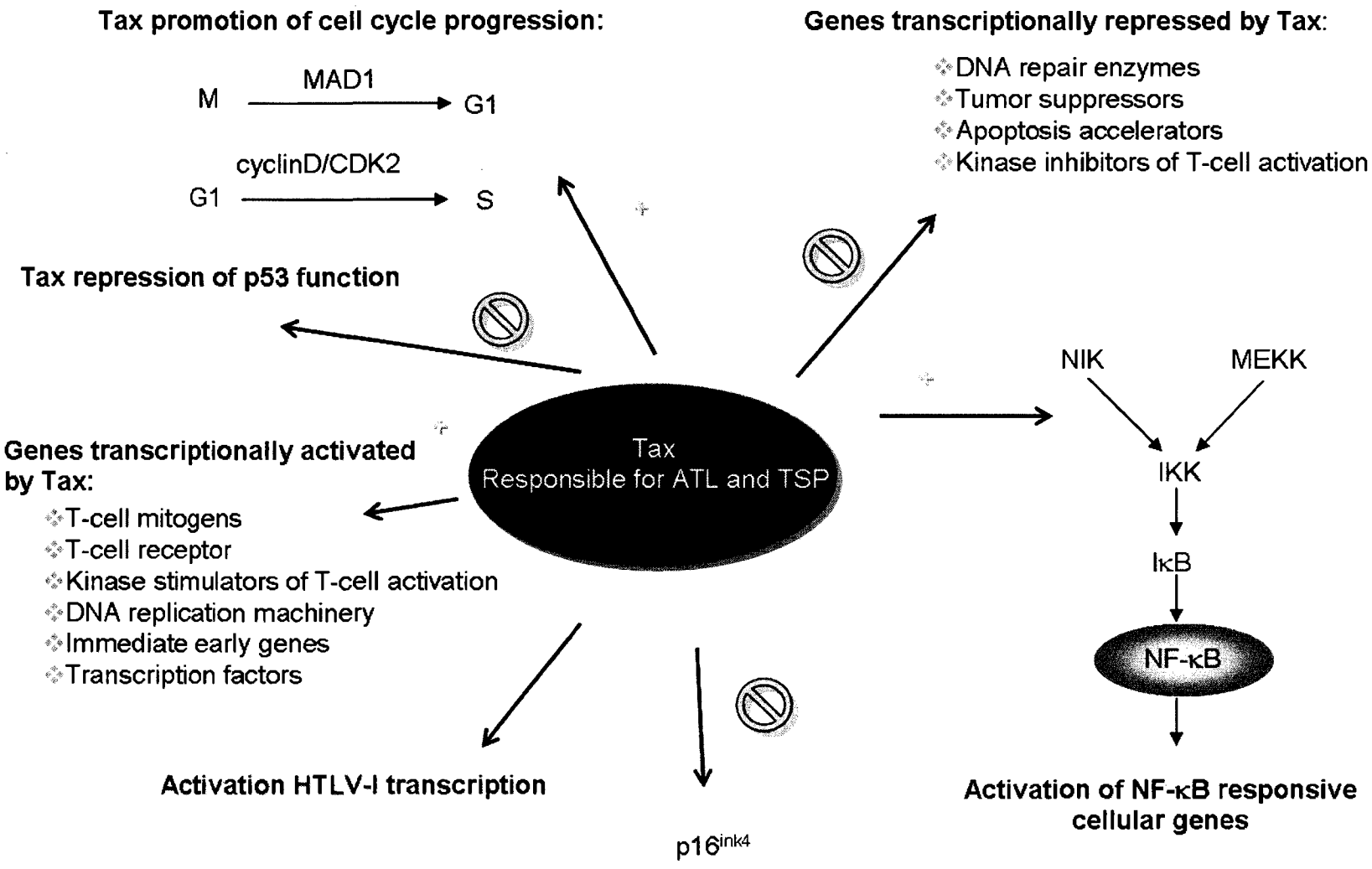


Figure 1.2. Overview of the pleiotropic functions of HTLV-1 Tax protein. Molecular mechanisms of viral transcription and cellular deregulation associated with the HTLV-I Tax protein.

1.2a TAX MEDIATED HTLV-1 TRANSCRIPTION

Following infection, the virus integrates into the host cell chromosome in a largely random fashion and the provirus is generally maintained in a latent state. Expression of Tax is essential whether the integrated virus progresses to replicate via clonal expansion or virion production. Both host-cell proliferation and HTLV-1-associated disease positively correlate with Tax expression, and individuals with the highest proviral load carry cells that express the highest levels of Tax (30, 31). An ill-defined cellular stimulus initially promotes modest transcription of the chromosomally–integrated provirus, leading to Tax expression. As Tax levels increase, there is a concomitant increase in viral transcription.

Tax is a very robust activator of HTLV-1 transcription. Three highly conserved 21 bp enhancer elements, located upstream of the RNA initiation site, are crucial for Tax-activated transcription. The 21 bp repeat elements carry a core off–consensus octanucleotide CRE that is immediately flanked by GC–rich DNA sequences. These GC-rich flanking sequences are conserved in all HTLV family members (HTLV-II, BLV, etc.). Together these sequences comprise the enhancer element, referred to as a viral CRE (vCRE). The CRE core sequence serves as the binding site for the cellular transcription factor CREB and/or other ATF/CREB family members. CREB is the prototypic member of the basic leucine-zipper family of cellular transcription factors. The transcriptional activity of CREB is regulated, in part, by a large number of protein kinases that phosphorylate the protein at Ser133, leading ro activation of target genes. Tax physically associates

with the vCREs through interacting directly with CREB (32, 33) as well as through interacting with GC-rich sequences that flank the core CREs (34, 35). The complex composed of Tax, CREB, and the viral CRE recruits the multifunctional coactivator, CREB binding protein (CBP) or its homolog p300 to the HTLV-1 promoter (36, 37). A schematic of the HTLV-1 promoter with viral CREs shown binding pCREB and Tax is shown in figure 1.3. CBP/p300 are very large (~300kD) structurally and functionally homologous coactivator proteins that are central mediators of gene expression in metazoans. Tax also directly interacts with CBP/p300, stabilizing the formation of a quaternary complex composed of Tax, CREB, CBP/p300 on the viral CRE, and thereby activating transcription (37-39). Recruitment of CBP/p300 to chromatin by Tax likely results in modification of histones via acetylation and may open chromatin structure to initiate transcription (40-43).

Many studies have shown a role for the isolated KIX region of CBP/p300 in Tax-mediated coactivator recruitment (36, 37, 44, 45). KIX is composed of three α-helices that form a compact hydrophobic core with two discrete transcription factor binding surfaces (46, 47). A recent study from our labratory found that full-length phosphorylated CREB (pCREB) and Tax interact simultaneously at each of the two distinct binding sites on KIX, forming a stable quaternary complex with the vCRE DNA (39). The simultaneous contacts made by Tax and pCREB with KIX likely occur due to their close proximity when bound to the vCRE, as well as intimate protein-protein interactions between the two activators.

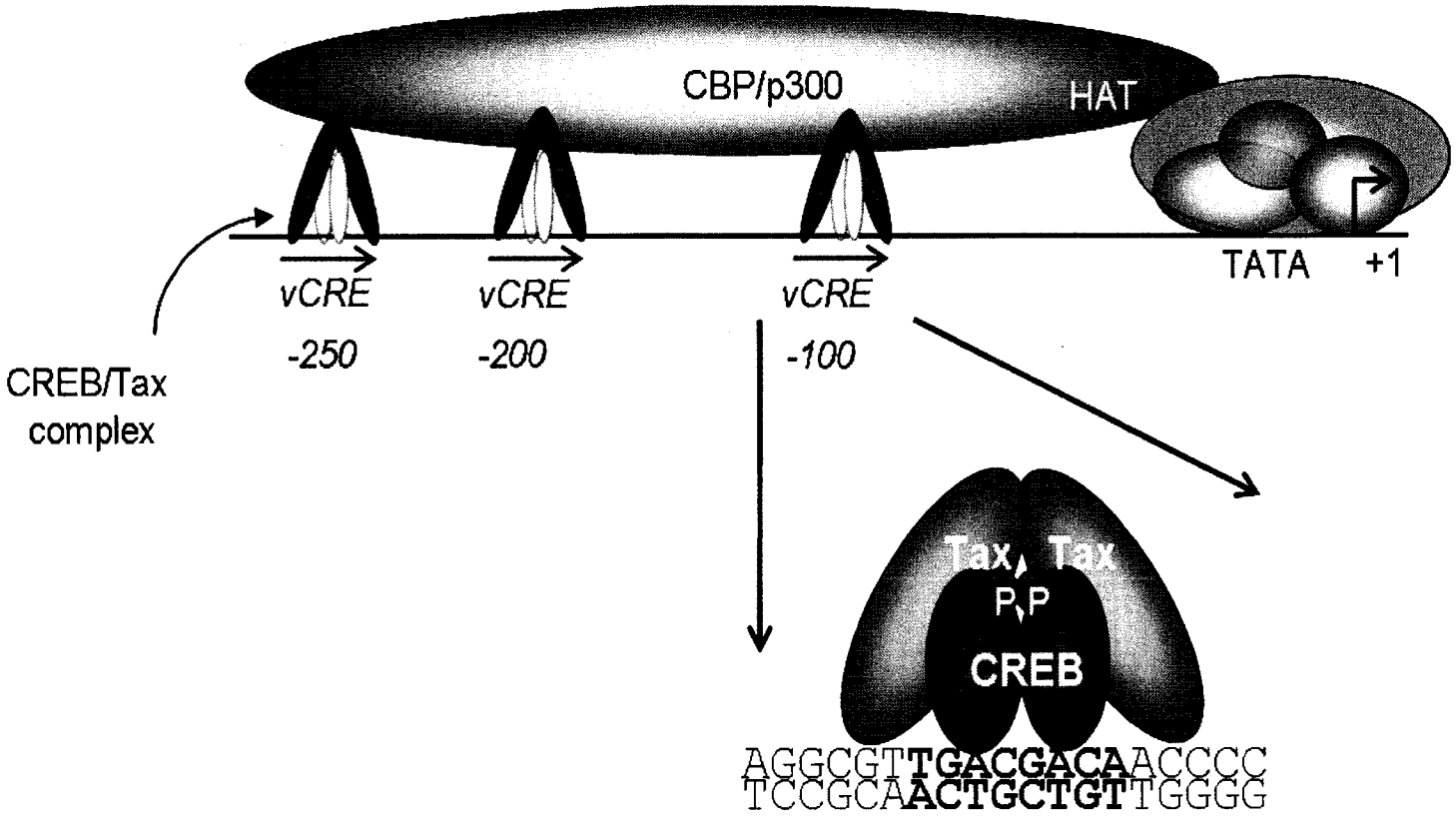
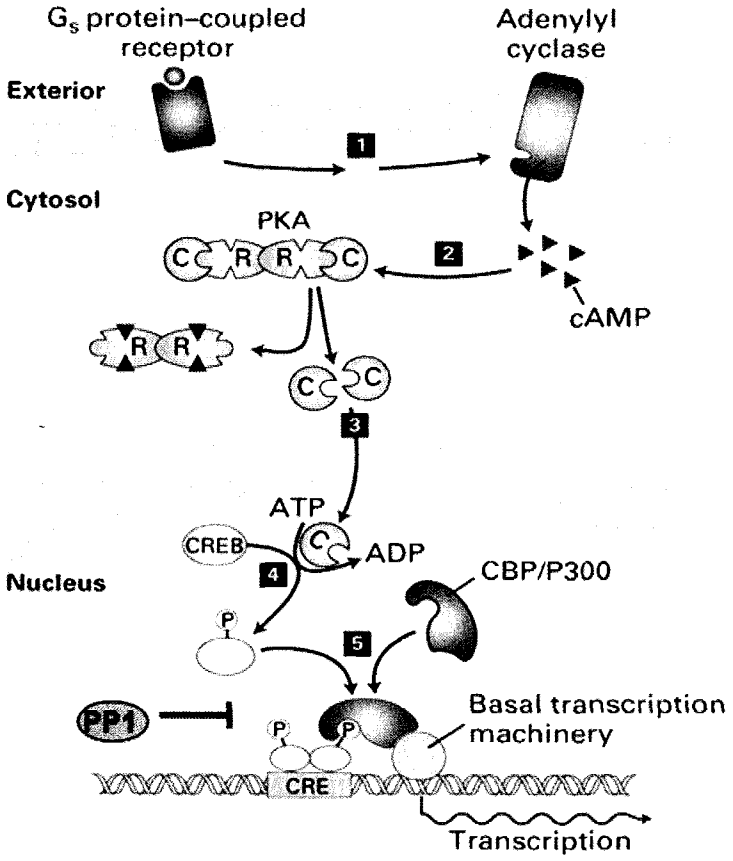


Figure 1.3. Schematic of the HTLV-1 promoter with viral CREs shown binding pCREB and Tax.

Many cellular genes containing CRE at their promoters are activated by signals that increase the cellular cAMP level. The elevated cAMP activates protein kinase A (PKA) to phosphorylate CREB, binding to CRE and to CBP/p300, thus leading to activation of transcription (48). Therefore, CRE-dependent transcription becomes signal dependent. In contrast, the viral gene containing vCRE at the promoter is constitutively expressed in HTLV-1 infected cells. A diagram of this signaling pathway is shown in figure 1.4.

The role of CREB phosphorylation in Tax-mediated HTLV-1 transcription has been controversial. Previous studies have suggested that Tax bypasses the need for CREB phosphorylation in the recruitment of p300/CBP to the vCRE, while other studies have found that PKA stimulation increases Tax transactivation (49-51). 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. Previous studies suggested that Tax significantly enhanced the recruitment of KIX to unphosphorylated CREB (36, 37). This led to the hypothesis that Tax bypasses the requirement for CREB phosphorylation in CBP/p300 recruitment to the viral promoter. In contrast to this model, however, we recently found that Tax expression directly enhances Ser133 CREB phosphorylation (pCREB) and the pCREB is an essential partner in Tax-dependent CBP/p300 recruitment and transcriptional activation of HTLV-1. These studies are described in further detail in chapter 2 and chaper 3.

Cellular promoter



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

Viral Promoter

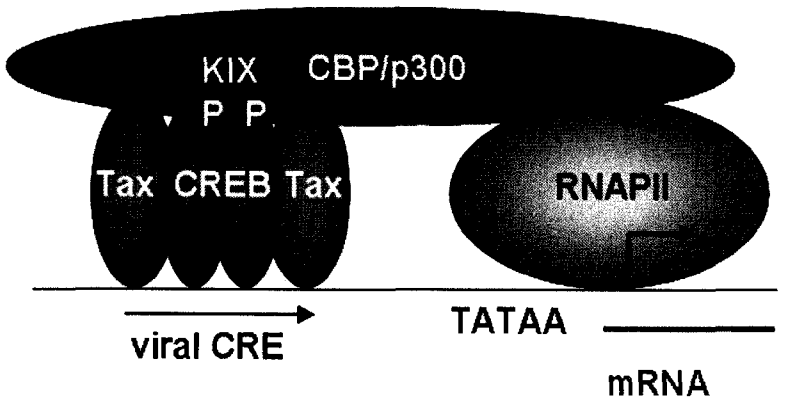


Figure 1.4. Transcriptional activation through CREB. Ser133 CREB phosphorylation by a large number of kinases is required to activate transcription of many cellular genes containing CRE at their promotes. However, the role of CREB phosphorylation in Tax transactivation of HTLV-1 has been controversial.

1.2b TAX MEDIATED CELLULAR GENE TRANSCRIPTION

Tax induces or represses various cellular genes through modulating transcription factors such as NF-κB, CREB, and SRF. Among them, NF-κB has been shown to be a key mediator of Tax mediated deregulation of cellular genes (52). Tax constitutively activates NF-κB, which induces transcription of various cytokines and their receptors as well as cell cycle and apoptosis related genes (53). For example, Tax can induce expression of interleukin (IL)-2Rα, IL-6, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), and B-cell lymphoma (Bcl)-X_L via the NF-κB pathway (54). Dysregulation of these genes can contribute to cancer development (55). NF-κB family members are composed of p50/NF-κB1 (its precursor: p105), p52/NF-κB2 (its precursor: p100), p65 (Rel A), Rel B, and c-Rel. Family members share a Rel homology domain and can homo or heterodimerize to bind the consensus DNA motif GGGRNNYYCC (N=any base, R=purine, and Y=pyrimidine), known as the κB site. In normally regulated T-cells and most other cell types, NF-κB factors are sequestered in the cytoplasm by association with inhibitors such as IκBα and IκBβ (56). Cytokines and mitogens stimulate two alternative pathways, the canonical and noncanonical NF-κB activation pathways (57, 58). The canonical pathway activates an IκB kinase (IKK) complex that induces IκB phosphorylation, ubiquitination and degradation. This leads to release of NF-κB factors from IκBs and translocation of these factors into the nucleus where they activate the transcription of NF-κB dependent genes (59). The noncanonical pathway activates NF-κB through NF-κB inducing kinase (NIK) mediated and IKKα

mediated site-specific phosphorylation and proteasome mediated processing of the p52 (NF-κB2) precursor protein p100 (60, 61).

Although NF-κB is transiently activated by external signals in normal T-cells, NF-κB is constitutively activated in HTLV-1 infected T-cells, Tax expressing cells, and freshly isolated ATL cells (62, 63). Many studies have suggested that Tax can regulate NF-κB activity in the cytoplasm as well as the nucleus. Tax induces the canonical pathway of NF-κB activation in several ways in the cytoplasm. For example, Tax can physically interact with the IκB kinase (IKK) complex and its upstream kinases, mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1) and NIK to enhance their kinase activities, which results in phosphorylation of IκBs (64). Tax also can bind directly to the IκBs through the ankyrin repeat domains, which leads to their degradation (65). Thus, Tax enhances the subsequent translocation of NF-κB factors to the nucleus by inducing degradation of IκBs in the cytoplasm without cellular stimuli. In addition to Tax-mediated canonical NF-κB activation, Tax stimulates noncanonical NF-κB signaling pathway (61, 66). Activation of the noncanocical pathway member p52 by processing of its p100 precursor occurs primarily in B-cells but not in T-cells under physiological conditions. In contrast, constitutive p52 production is detected in HTLV-1 infected T cell lines and furthermore, Tax expression induces p100 processing through physical interaction with IKK (67).

Some studies have shown that Tax can directly associate with NF-κB factors p50 (68), p65 (68), p52 (69), and c-rel (68). Additionally a study showed Tax colocalization with NF-κB p50, p65, and RNA polymerase II in the nucleus

(70). However, the significance and function of the physical interactions between Tax and NF-κB factors in the nucleus is still unclear.

Extensive studies have shown that persistent NF-κB activation is associated with many human cancers and is also known to mediate cancer cell growth and survival (71). Thus, constitutive NF-κB activation by Tax may be responsible for the abnormal growth and survival of HTLV-1 infected T-cells.

In addition to dysregulation of NF-κB family, expression of immediate early genes such as c-fos and c-egr is elevated in HTLV-1 infected T-cells and Tax expressing cells (72). SRF transactivates these genes through serum response element (SRE) promoter elements containing the CC(AT)6GG motif (CArG box). Tax directly interacts with the residues 422-435 of SRF on SRE and enhances transcription of these immediate early genes (73).

1.2c TAX MEDIATED DEREGULATION OF THE CELL CYCLE

Cell cycle deregulation is a hallmark of transformed cells. The cell cycle is composed of four major phases; G1, S, M and G2. Transition from one stage of the cell cycle to the next is controlled by different cyclin/cyclin dependent kinase (CDK) complexes, which are regulated by cdk inhibitory proteins (CDKI) such as $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$, $p21^{CIP/WAF1}$, and $p27^{KIP1}$. The cell cycle is regulated by sequential activation and inactivation of cyclin/CDK complexes. The levels of CDK are stable; however cyclin levels vary with the phases of the cell cycle. D-type cyclins (D1, D2, and D3) and cyclin E regulate the G1 to S phase progression by binding to CDK2, 4, and 6. The active cyclin D/CDK or cyclin

E/CDK complexes hyperphosphorylate the retinoblastoma (Rb) tumor suppressor protein. This in turn causes the release of the E2F transcription factor. E2F then activates transcription of S phase critical genes such as dihydrofolate reductase, DNA polymerase α, and cyclins (74). The cyclin B/CDK complex regulates mitosis by activating anaphase promoting complex (APC). APC degrades cyclin B and securin (an anaphase inhibitor) by ubiqutination mediated proteasome degradation (75). The levels of cyclin B and securin increase in the S phase, reach a maximum at the G2/M and are degraded rapidly at the end of M phase (76).

In HTLV-1 infected T-cells, normal cell cycle regulation of DNA replication and cell division is disrupted via Tax-mediated dysregulation of cell cycle control (77). Tax has been shown to deregulate the G1 phase and accelerates the G1/S transition (78) through a variety of mechanisms. For example, Tax enhances the activity and expression levels of cyclin D/CDK partners. It achieves this by transactivation of the cyclin D1 and cyclin D2 genes (79, 80) and physical interaction with CDK4 to promote its association with cyclin D (81), which leads to phosphorylation of RB and subsequent release of E2F transcription factor (82, 83), leading to E2F-dependent transcription (84, 85). Tax transcriptionally represses CDK inhibitors such as p18 ^{INK4c}, p19^{INK4d}, and p27^{Kip1} through the E-box element of their promoters (86-88). In fact, Tax directly binds p16^{INK4a} and disrupts its CDK inhibitory function (25). Intriguingly, Tax also induces the expression of p21^{WAF1}, which was originally thought to be a cell cycle inhibitor. However, some studies have demonstrated that p21^{WAF1} can promote G1/S

transition by promoting and stabilizing an active $p21^{WAF1}$-cyclinD2-CDK complex (89, 90). Finally, Tax reduces the expression of cyclin A, which normally accumulates at the beginning of S phase to ensure only one round of DNA replication during S phase via its associated kinase activity (91). Thus reduced cyclin A levels allow redundant DNA replication, contributing to aneuploidy (92).

Expression of Tax in rodent, in human cells, and in budding yeast has been shown to delay the G2/M transition and result in development of aneuploidy (93-95). In addition, Tax physically interacts with the checkpoint kinase 2 (Chk2) tumor suppressor and inactivates its function, leading to arrest of cells at G2/M (94). Several studies have demonstrated that Tax has multiple roles in the development of aneuploidy. Tax can suppress the mitotic spindle assembly checkpoint (MSC) by directly binding to a component of MSC, mitotic arrest deficiency protein 1(MAD1) and inactivating it (96). Tax also reduces the expression level of cyclin B1 (a mitotic cyclin) and securin (an anaphase inhibitor) before entering mitosis. Moreover, Tax can directly interact with, and activate the cdc20-associated anaphase promoting complex (APC CDC20), promoting degradation of cyclin B and securin (97). This, in turn, may cause improper sister chromatid segregation and contribute to aneuploidy.

1.3 TAX MEDIATED CELLULAR TRANSFORMATION

Numerous studies have demonstrated that Tax has oncogenic potential in several experimental systems including multiple cell culture and transgenic-mouse models. Transformation was determined by formation of foci of

morphologically transformed cells, by colony formation in soft agar and by tumor formation in nude mice. For example, Tax is both necessary and sufficient to induce immortalization of primary human T-lymphocytes derived from peripheral blood or cord blood (98). Tax also induces transformation of Rat-1 fibroblasts (99, 100) and cooperates with the Ras oncogene in transformation of primary rat embryo fibroblasts, which were shown to be highly tumorigenic in nude mice (101, 102). Moreover, Transgenic mice in which Tax was expressed under the control of the HTLV-1 promoter developed soft agar tissue tumors (103). Additionally, transgenic mice expressing Tax under granzyme B promoter, targeting to the mature T-cells, developed leukemia (104). Notably, studies using infectious clones of the entire HTLV-1 genome revealed that Tax is involved in cellular transformation of primary human T-cells (105). Although the mechanism of Tax- meditated transformation remains unclear, these studies clearly show that Tax is a viral oncogene without a cellular homologue.

Tax contributes to cell proliferation and transformation by modulating different regulatory pathways including transcription factors NF-κB, CREB and SRF and deregulating many cellular genes. To understand the contribution of Tax-mediated CREB or NF-κB dependent transcription to cellular transformation, Tax mutants specific for activation of only the CREB or NF-κB pathway have been utilized. Some of these studies supported that Tax-mediated NF-κB activation is required for cellular transformation (99, 106, 107). On the other hand, other studies have suggested that CREB/ATF is critical for Tax-induced transformation (100, 108). Moreover, a recent study demonstrated CREB can

function as a proto-oncogene by promoting abnormal proliferation, cell cycle progression, and survival of hematopoetic cells (109). It has also been reported that Tax-mediated NF-κB activation is sufficient for growth response to IL-2, but that Tax-mediated CREB/ATF and SRF activation is also required for clonal expansion of CD4+ T-cells (110).

1.3a TAX MEDIATED INHIBITON OF TUMOR SUPPRESSORS

Oncoproteins must overcome the actions of cellular tumor suppressors before cellular transformation can occur. Likewise, the viral oncoprotein Tax has been shown to abrogate the function of tumor suppressors, p53 and hDLG.

p53 is a DNA- binding transcription factor which guards cells against DNA damage and transformation. In response to DNA damage, p53 mediates cell cycle G1/S arrest or apoptosis (111). The gene for p53 is one of the most frequently mutated in human cancers, occurring in roughly 50% of tumors. However, p53 mutation is reported in only a small fraction (30%) of ATL cells (112-114). Interestingly, HTLV-1 infected T-cells and Tax expressing cells contain high levels of wild type of p53 (115). Many studies have demonstrated that Tax can impair the transactivational and apoptotic functions of p53 (116-118). However, Tax does not interact with p53, alter its nuclear location, or disrupt its DNA-binding activity when assayed by electorphoretic mobility shift assays (EMSA) (116, 117, 119). Several mechanisms of Tax-mediated loss of p53 function have been proposed. Tax competes with p53 for binding CBP/p300 and inhibits p53-dependent transcription (120-122). Tax inactivates p53 through

the NF-κB pathway leading to the phosphorylation of p53 at Ser15 and Ser392 (123) which blocks interaction between p53 and general transcription factors such as TFIID (124). While Tax inactivation of p53 is not fully understood, the loss of p53 function may contribute to Tax-mediated cellular transformation by preventing proper G1/S arrest, p53-mediated apoptosis and DNA repair.

hDLG, a human homologue of the *Drosphila* discs large PDZ (PSD-95/DISCS LARGE/Z0-1) containing tumor suppressor (125), functions downstream of the Wnt/Frizzle pathway (126). hDLG interacts with the C-terminus of the tumor suppressor protein, adenomatous polyposis complex (APC) and regulates cellular proliferation and cell cycle progression (127). Tax was also found to bind to another tumor suppressor, hDLG, through a PDZ binding motif (PBM) at its C-terminus (125, 128). Tax competes with APC for interaction with hDLG and inhibits cell cycle arrest induced by hDLG, possibly promoting abnormal cell growth (125). Moreover, the interaction between Tax and hDLG correlates with Tax-mediated colony formation in rat fibroblasts(129). These data suggested that the complex of Tax and hDLG might also play a relevant role in cellular transformation

1.3b TAX MEDIATED DEREGULATION OF CELL SIGNALING PATHWAYS

A comparison of HTLV-1 infected versus uninfected T- cells reveals many differences in the expression of signaling molecules including cytokine receptors and cytokines (130, 131). Additionally, infected cells have increased expression of multiple genes involved in growth promoting and antiapoptotic functions due to

induction by Tax (132). IL-15 and IL-15 Rα expression are elevated by Tax in HTLV-1 infected cells (133, 134), therefore, an IL-15 autocrine loop may also contribute to HTLV-1 pathogenesis (135). Upon IL-15 binding, the IL-15 receptor associated Janus tyrosine kinase (JAK3) phosphorylates the two signal transducers of activated T-cells (STAT5a, b), which, in turn, translocate to the nucleus and stimulate target gene transcription. Both HTLV-1 infected T-cells and lymphocytes from HTLV-1 patients contain hyper-activated STAT5a and STAT5b, which are critical for normal T-cell proliferation (136). IL-13 is also upregulated in HTLV-1 transformed cells as well as in cultured ATL cells (137, 138) and is linked to leukemogenesis probably through an autocrine mechanism. Both a member of the TNF-receptor family, OX40 and the OX40 ligand are overexpressed in HTLV-1 infected cells, suggesting that costimulatory signals from OX40 contribute to transformation (139, 140).

Cytokine receptors and T-cell receptor activation stimulate the phosphoinositide 3-kinase (PI3K) pathway (141), which provides cell growth and survival signals. PI3K activates Akt, a serine/threonine kinase, through site-specific phosphorylation, primarily on Ser473. Akt is constitutively activated in T-cells isolated from ATL patients (142) and HTLV-1 infected T-cells (143). Tax promotes Akt phosphorylation by directly binding PI3K, thereby increasing cell proliferation and survival (143). Therefore, Tax-mediated Akt activation is important for HTLV-1 transformation.

1.4 THESIS RESEARCH OBJECTIVES AND SIGNIFICANCE

The objective of this thesis research was to better understand the multiple functions of Tax in both HTLV-1 transcription and cellular gene deregulation. My approach was three-fold: *i)* examine the role of CREB phosphorylation in Tax-dependent viral transcription, *ii) examine* the interaction *of* Tax and Bcl-3, a protein intimately associated with T-cell survival and growth, and, *iii*) examine the direct effect of Tax on cyclin D1 transcription, an important cell-cycle control gene. To achieve these objectives, I performed *in vivo* and *in vitro* assays, including transient transfection assays, co-immunoprecipitation assays (Co-IP), chromatin immunoprecipitation assays (ChIP), *in vitro* kinase assays, GST pull-down assays and DNA pull-down assays. The data presented herein revealed that Tax induces CREB phosphorylation at Ser-133, that the CaM kinase pathway is implicated in Tax stimulated CREB phosphorylation. In addition to Tax-mediated deregulation of the CaM kinase pathway, we examined the effects of Tax on cellular regulator, Bcl-3 and cyclin D1. We demonstrated that Tax and Bcl-3 each deregulate the expression of the other, that Tax and Bcl-3 interact in a stable complex, and that Tax activates cyclin D1 via the NF-κB and CREB pathway. Since elevation of Ser-133 CREB phosphorylation and expression of both Bcl-3 and cyclin D1 are all known to be associated with tumorigenic potential, Tax may utilize these mechanisms to transform T-cells.

CHAPTER 2

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

Chapter 2 describes a study of the role of Ser133 phosphorylated CREB in Tax-mediated transactivation using a variety of *in vitro* and *in vivo* assays. I performed the experiments shown in figures 2.4A-C. 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.* 2007. 282:18750-7.

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 (5). 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 (34, 35, 144, 145). 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 (36, 37, 42, 45, 146). CBP/p300 is 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)(147). 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 (47). 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 (36, 37, 44, 45).

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 (49-51). 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-

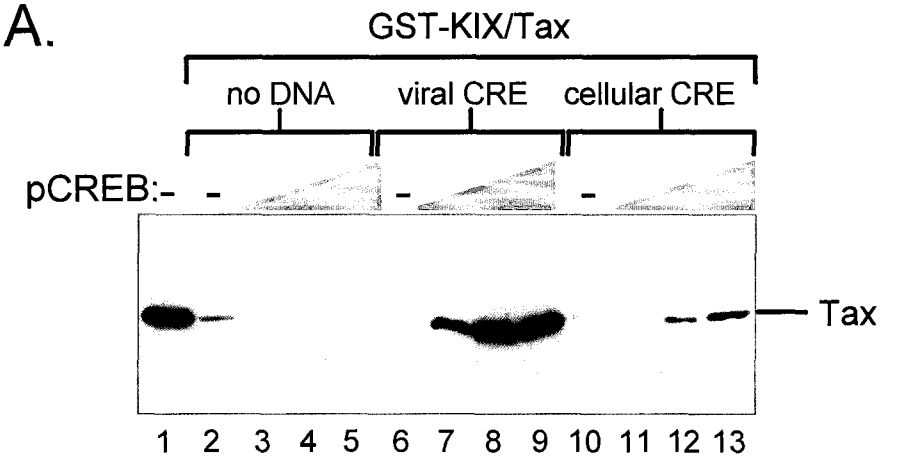


Figure 2.1. The viral CRE and pCREB enhance Tax binding to the KIX domain of CBP. GST pull-down assay. **A.** *Tax binding to KIX is strongly enhanced by vCRE DNA and pCREB.* Tax (25 nM) was incubated with GST-KIX $aa_{588-683}$ (25 nM) in the absence (lanes 2-5) or presence of vCRE (lanes 6-9) or consensus 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.

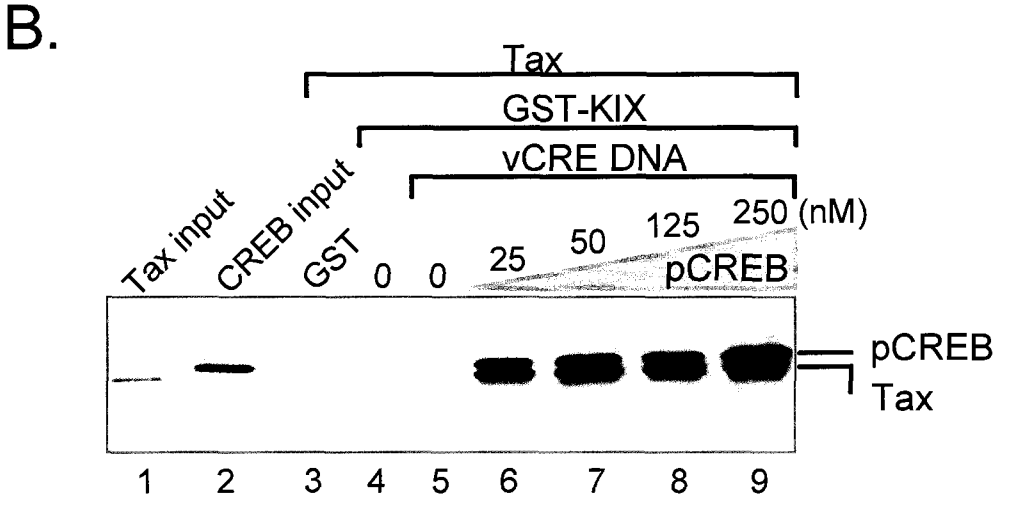


Figure 2.1B. *Tax and pCREB binding to KIX precisely correlates in the presence of DNA.* GST pull-down assay. Tax (45 nM), vCRE DNA (250 nM), and the indicated amount of pCREB were bound to GST- KIX (250 nM), and assayed as described in Figure 2.1A. As a negative control, Tax binding to GST (250 nM) 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.

rich flanking sequences in the Tax-DNA interaction (34, 35, 144, 145). We extend these 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 (44, 45, 122, 148, 149). 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

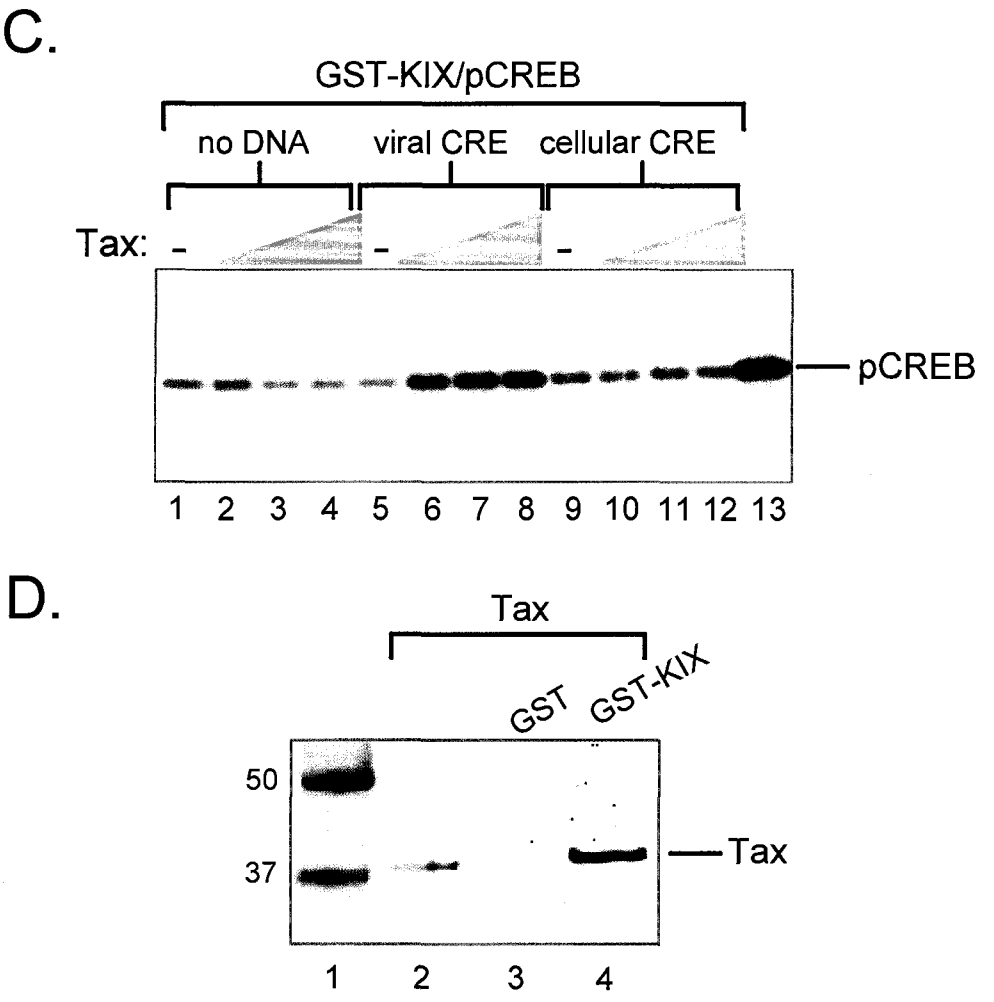


Figure 2.1C. *pCREB binding to KIX is modestly enhanced by Tax and vCRE DNA.* The experiment shown here is the reciprocal of the experiment shown in Figure 2.1A. pCREB (25 nM) was bound to GST-KIX $aa_{588-683}$ (25 nM) in the absence (lanes 1-4) or presence of vCRE (lanes 5-8) or consensus CRE DNA (lanes 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. Experiment performed by J. A. Ramirez

vCRE. We first used the DNA pull-down assay to evaluate the ability of KIX to 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

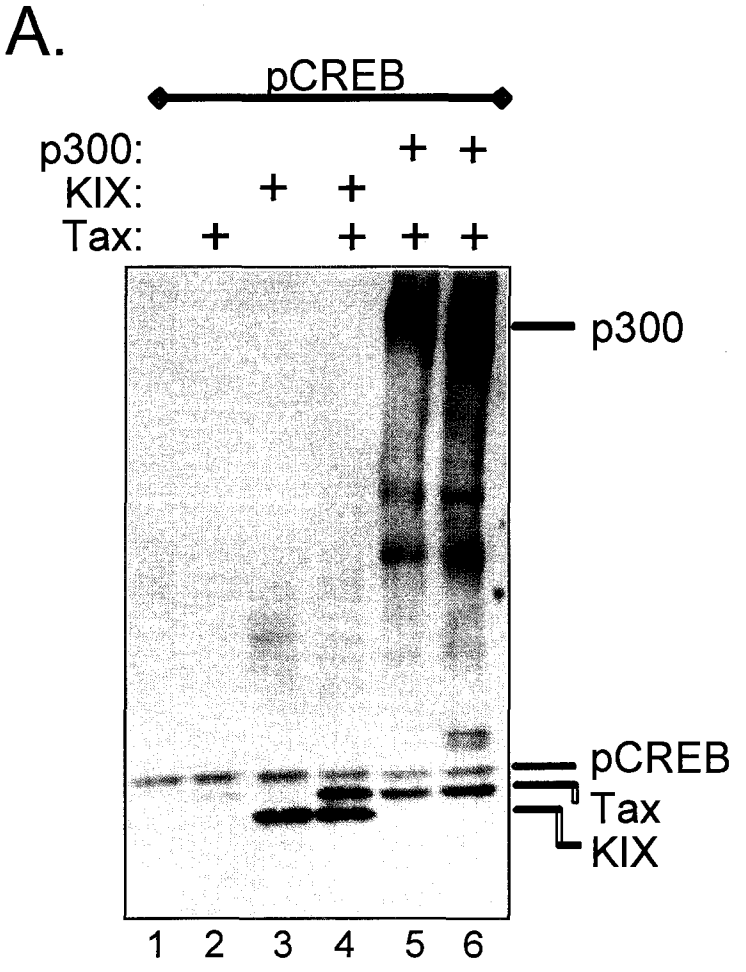


Figure 2.2. Strong Tax binding to the viral CRE requires the KIX domain of CBP. A. *The KIX domain and full-length p300 stabilize Tax binding.* Immobilized vCRE' DNA (5 nM) was incubated with pCREB (25 nM), Tax (25 nM) and either GST-KIX (25 nM) or full-length p300 (25 nM), as indicated. Lanes 5 and 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.

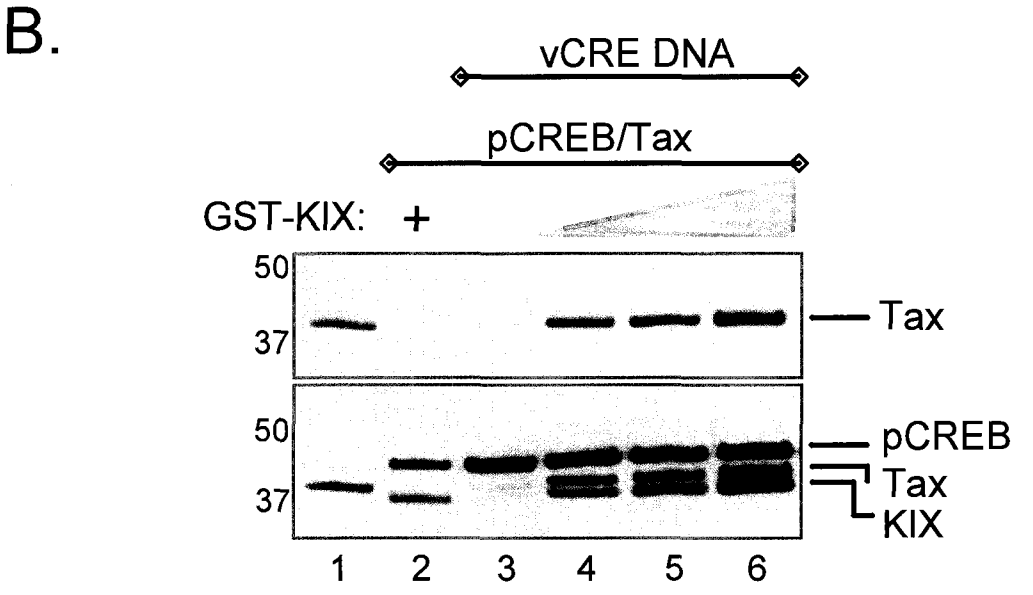


Figure 2.2B. *Tax binding precisely correlates with KIX binding to the vCRE.* Increasing amounts of GST-$KIX_{588-683}$ (2.5, 5, 12.5 nM) were added into binding reactions containing biotinylated vCRE' DNA (5 nM), pCREB (12.5 nM), and Tax (12.5 nM). A biotinylated modified half-CRE with no GC-rich flanks (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. Experiment performed by J. A. Ramirez.

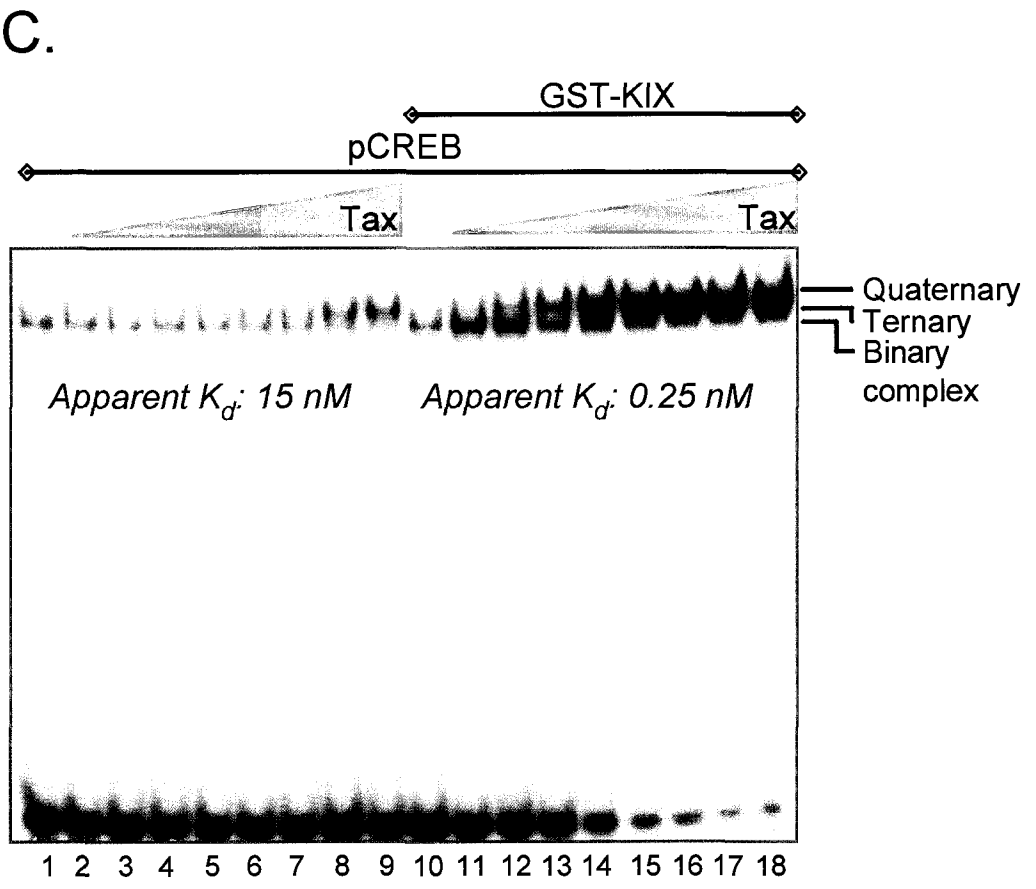


Figure 2.2C. *KIX increases the affinity of Tax for the vCRE/pCREB complex.* EMSAs were performed with γ-^{32}P-end labeled vCRE probe (0.15 nM), pCREB (1.5 nM), and increasing amounts of Tax (25 pM to 75 nM). Binding reactions were carried out in the absence (lanes 1-9) or presence (lanes 10-18) of GST-KIX$_{588\text{-}683}$ (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.

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 higher position on the gel than pCREB/Tax/DNA (36). 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-$KIX_{588-683}$

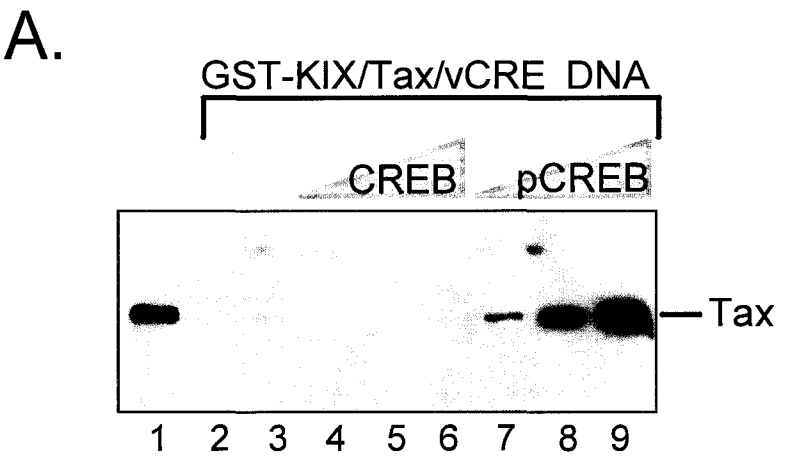


Figure 2.3. CREB phosphorylation is necessary for stable Tax binding to viral CRE DNA. A. *pCREB strongly enhances Tax binding to KIX.* Increasing amounts of CREB or pCREB (2.5, 25, 250 nM) was added to GST pull-down reactions containing Tax (25 nM), vCRE DNA (500 nM), and GST-$KIX_{588-683}$ (25 nM)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

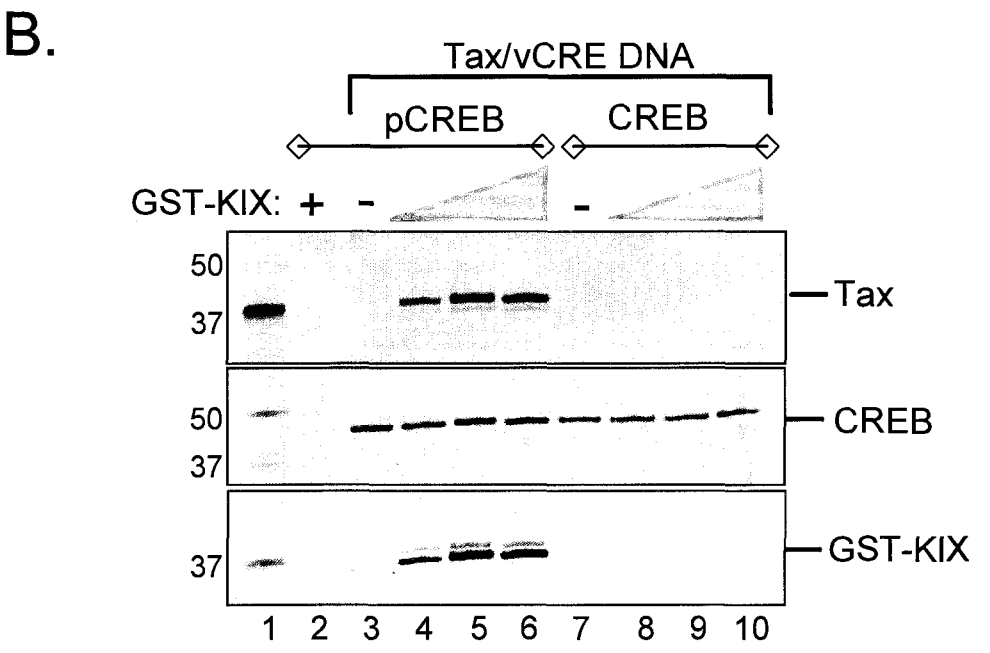


Figure 2.3B. *pCREB is necessary for Tax binding enhancement by KIX.* Immobilized vCRE' DNA (5 nM) was added to DNA pull-down reactions containing Tax (12.5 nM), pCREB or CREB (12.5 nM) and increasing amounts of GST-$KIX_{588-683}$ (2.5, 5, and 12.5 nM). As before (Fig. 2B), a reaction containing a modified 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. Experiment performed by J. A. Ramirez.

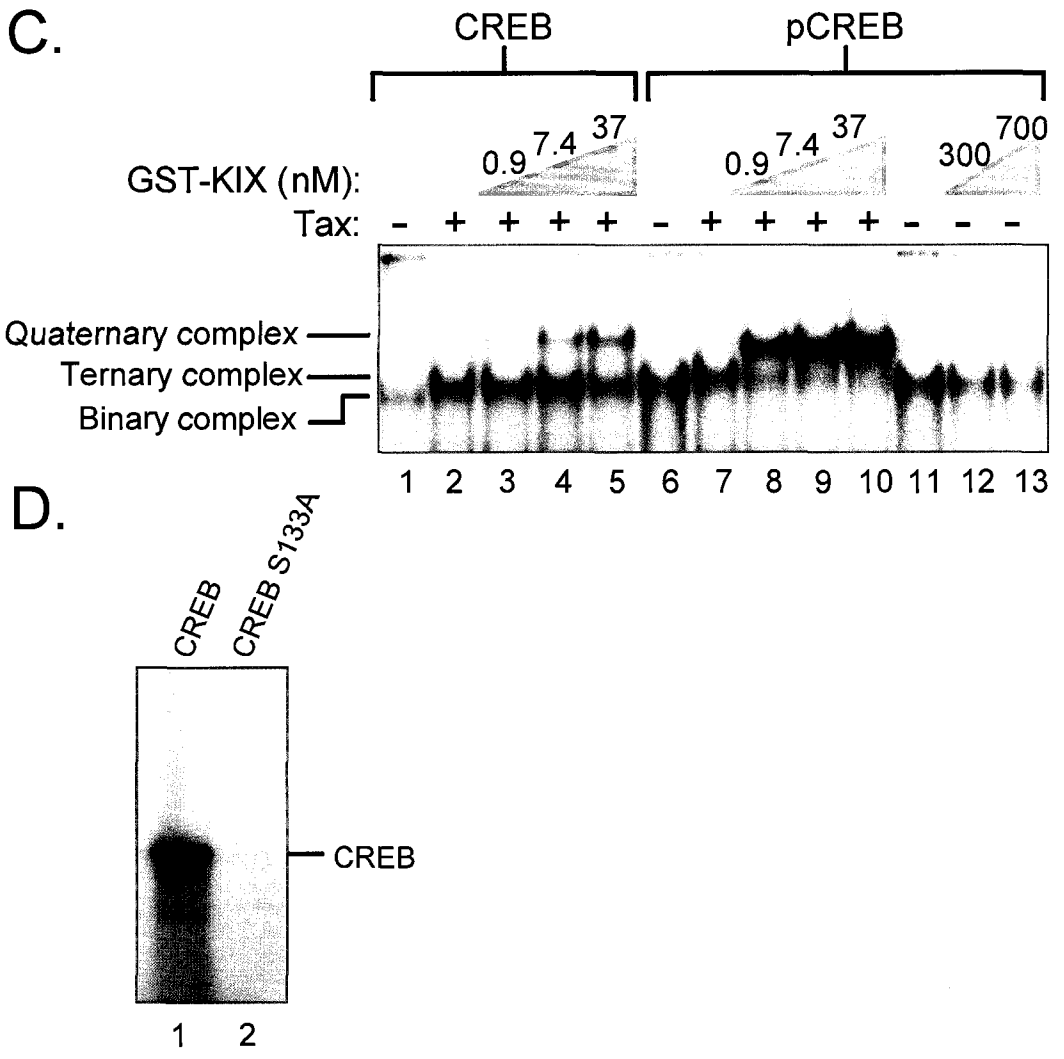


Figure 2.3C. *pCREB, Tax, and KIX are all needed for strong quaternary complex formation.* Binding reactions for EMSAs contained γ-^{32}P-end labeled viral CRE DNA probe (0.3 nM/reaction) and constant amounts of CREB (3.6 nM) or pCREB (1.8 nM), in the presence or absence of Tax (95 nM). GST-$KIX_{588-683}$ 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. Experiment performed by J. A. Ramirez.

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 with previously published data (36). 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

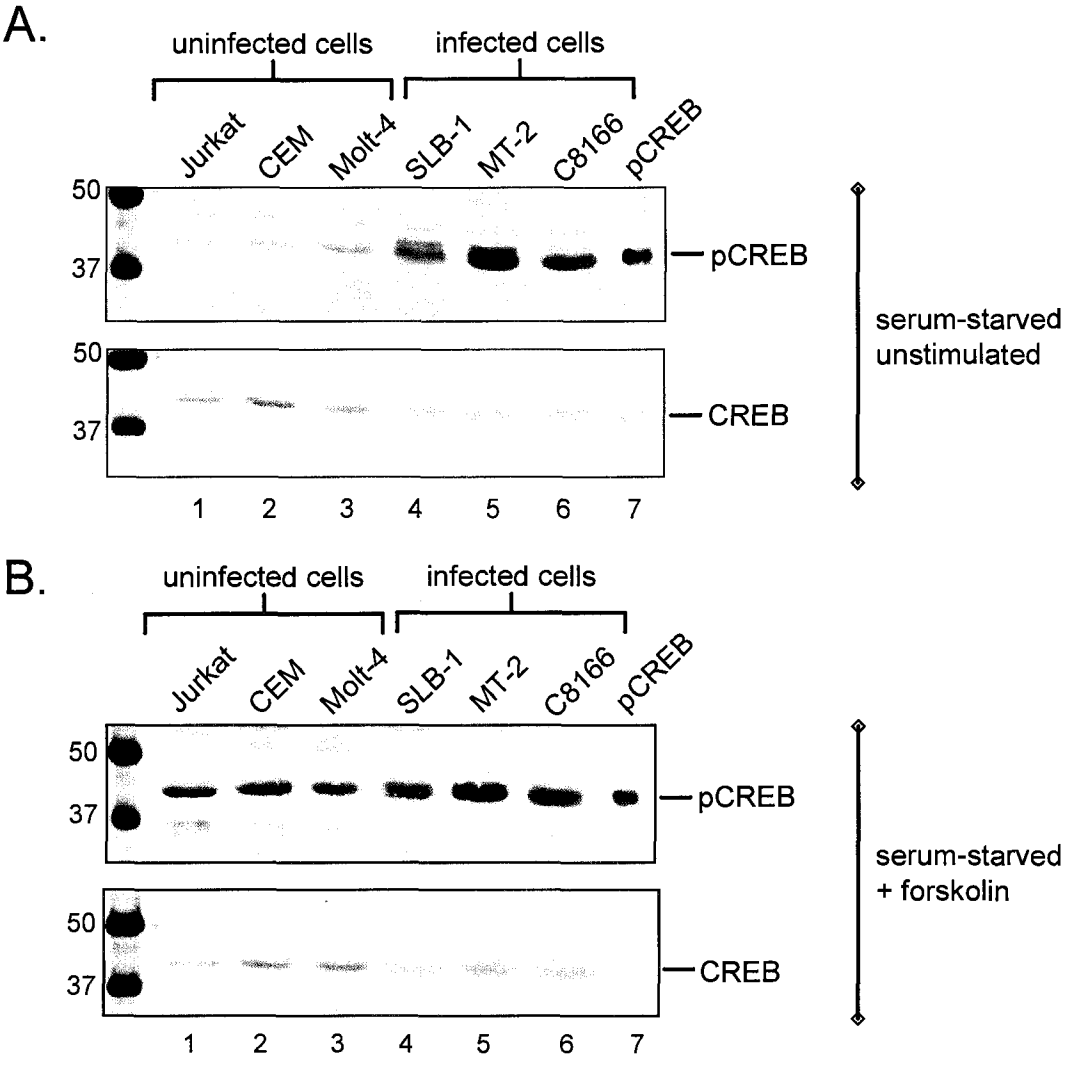


Figure 2.4. Tax enhances CREB phosphorylation *in vivo*. A. *HTLV-1 infected T-cells have increased levels of pCREB compared to uninfected cells.* Western blot analysis of whole cell extracts from three HTLV-1 infected (SLB-1, MT-2, and C8166) and uninfected (Jurkat, CEM, and Molt-4) T-cell lines. All 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. **B.** *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.

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, 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.

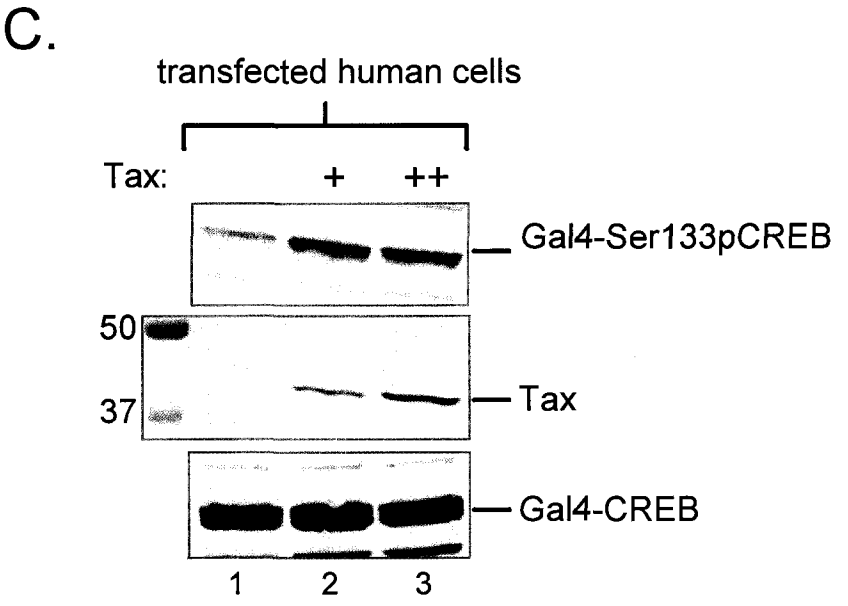


Figure 2.4C. *Tax expression increases CREB phosphorylation in vivo.* Human 293 cells were transfected with a Tax expression vector (pSG-Tax, 0.5 µg and 1 µg) or pUC19 (1 µg) as a control. After a 24 hour transfection, cells were serum-starved (0.5% FBS) for an additional 24 hours. Cells were then harvested, 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.

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 (44, 45, 122, 148, 149), 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 (36, 37). 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 (109, 150). 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 (107, 108, 110, 151-153). 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 (154).

Consonant with our hypotheses that Tax promotes CREB phosphorylation *and* utilizes pCREB for transactivation, kinase inhibitors have been shown to block Tax function (51, 155). 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 (156). Trevisan *et al.* (156, 157) proposed that Tax may prolong the phosphorylation state of CREB via the inactivation of specific phosphatases or activation of specific kinases.

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 (32), CREB S133A, Tax-His_6 (158) and GST-KIX (36) proteins were purified to >95% homogeneity as previously described (36). Full-length His_6-tagged p300 was expressed from recombinant baculovirus in Sf9 cells and purified as previously described (159). 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) (160). 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 (161). 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) (162), 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).

CHAPTER 3
THE HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 TAX PROTEIN CONFERS CBP/P300 RECRUITMENT AND TRANSCRIPTIONAL ACTIVATION PROPERTIES TO PHOSPHORYLATED CREB

Chapter 3 describes a study of the role played by pCREB and Tax in recruitment of full-length p300 to the viral promoter and viral transacriptional activation. I performed the experiment shown in figure 3.3E. This work has been published in the *Molecular Cell Biology*, and is presented here exactly as it appeared in the journal. The citation for the publication is:

Geiger, T.R., Sharma, N., Kim, Y.M., & Nyborg, J.K. The HTLV-1 Tax protein confers CBP/p300 recruitment and transcriptional activation properties to phosphorylated CREB. *Mol. Cell. Biol.* 2008. 28:1383-92.

3.1 ABSTRACT

The human T-cell leukemia virus-encoded oncoprotein Tax is a potent activator of viral transcription. Tax function is strictly dependent upon the cellular transcription factor CREB, and together they bind cAMP-response elements within the viral promoter and mediate high-level viral transcription. Signal-dependent CREB phosphorylation at Ser^{133} (pCREB) correlates with the activation of transcription. This activation has been attributed to recruitment of the coactivators CBP/p300 via physical interaction with the KIX domain. Here we show that the promoter-bound Tax/pCREB complex strongly recruits the recombinant, purified full-length coactivators CBP and p300. Additionally, the promoter-bound Tax/pCREB (but not Tax/CREB) complex recruits native p300 and potently activates transcription from chromatin templates. Unexpectedly, pCREB alone failed to detectably recruit the full-length coactivators, despite strong binding to KIX. These observations are in marked contrast to published studies that have characterized the physical interaction between KIX and pCREB, and extrapolated these results to the full-length proteins. Consistent with our observation that pCREB is deficient for binding CBP/p300, pCREB alone failed to support transcriptional activation. These data reveal that phosphorylation of CREB is not sufficient for CBP/p300 recruitment and transcriptional activation. The regulation of transcription by pCREB is therefore more complex than generally recognized, and co-regulators, such as Tax, likely play a critical role in the modulation of pCREB function.

3.2 INTRODUCTION

cAMP-response element binding protein (CREB) is one of the most widely studied transcription factors in metazoans. The basic leucine zipper region of CREB binds to cAMP response elements (CREs) and potentially regulates the expression of a significant percentage of genes in the human genome (163-165). The large number of cellular genes regulated by CREB exemplifies the critical role this archetypal transcription factor plays in vital cellular processes such as development, differentiation and cellular homeostasis. Over 300 distinct extracellular stimuli converge on several kinases, including protein kinase A and C, that phosphorylate CREB at Serine-133 (Ser^{133}) to activate the transcription function of CREB (154). Phosphorylated CREB (pCREB) then recruits the cellular coactivator paralogues CREB-binding protein (CBP) and p300 to activate transcription (147, 166-168).

A significant number of studies support a model of CREB transcription function whereby the binding of pCREB to the KIX domain of CBP/p300 is concomitant with coactivator recruitment and transcriptional activation. This model is largely based on characterization of the physical interaction between pCREB and the isolated KIX domain (47, 168), and subsequent extrapolation of these data to the recruitment of the full-length proteins. Emerging evidence, however, indicates that CREB phosphorylation may not be sufficient for CBP/p300 recruitment and transcription function. First, stimuli that lead to Ser^{133} phosphorylation of CREB induce transcription of only a subset of the many CRE-containing genes *in vivo* (164, 169-173). Recent genome-wide analysis revealed

that exposure of cells to the cAMP agonist forskolin resulted in phosphorylation of promoter-bound CREB, but failed to induce transcription at the majority of CREB target genes identified (164, 165, 169). Consistent with these observations, a number of genes have been identified where phosphorylated CREB is bound at the promoter, yet CBP/p300 occupancy was not detected (164, 169, 170). Despite the evidence that CREB phosphorylation is insufficient for coactivator recruitment and gene activation at a large number of genes *in vivo*, pCREB recruitment of CBP/p300 remains the paradigm for signal-induced transcriptional activation in metazoans.

The expression of the human T-cell leukemia virus type 1 (HTLV-1) is inextricably linked with CREB. HTLV-1 transcription is regulated primarily through three conserved CRE-containing enhancer elements, called viral CREs (vCREs), located in the viral promoter. The vCREs are composed of an off-consensus CRE core flanked by a short run of GC-rich DNA. CREB binds the CRE and the virally-encoded oncoprotein Tax binds adjacent to CREB in the minor groove of the GC-rich DNA (34, 35, 144, 145). Tax and CREB also directly interact (174), and together the proteins recruit CBP/p300 (36, 37) and coordinate the assembly of the transcriptional apparatus at the HTLV-1 promoter, resulting in high-level viral transcription. *In vivo*, at a chromosomally-integrated HTLV-1 promoter, Tax recruits both CREB and p300, and activates transcription several hundred fold (175). These data are consistent with a long-standing model that Tax bypasses the requirement for CREB phosphorylation to recruit the coactivators and activate HTLV-1 transcription (36, 37). In contrast to this model, however, we recently

found that the phosphorylated form of CREB is critical to stable complex formation with Tax, the KIX domain of CBP, and vCRE DNA (38). Furthermore, Tax was shown to induce CREB phosphorylation *in vivo*, suggesting that Tax and Ser^{133}-phosphorylated CREB cooperate to promote the strong HTLV-1 transcriptional response (38, 156, 157).

In light of these observations, we set out to biochemically characterize the detailed molecular interactions between Tax, CREB, and full-length CBP/p300. Unexpectedly, we find that CRE-bound Ser^{133}-phosphorylated CREB is insufficient to recruit full-length p300 or CBP. Tax in complex with pCREB, however, serves as a high-affinity binding site for the coactivators. We also find that HTLV-1 promoter-bound pCREB is deficient for transcription from chromatin templates *in vitro*, consistent with its inability to bind the coactivators. Furthermore, forskolin fails to stimulate vCRE-dependent transcription *in vivo*. Tax promotes strong transcriptional activation that is dependent upon pCREB and concomitant with p300 recruitment. These data provide the first direct evidence that CRE-bound pCREB is unable to effectively bind full-length CBP/p300, and provide a molecular explanation for the *in vivo* observations described above that noted an absence of CBP/p300 occupancy at a large number of genes bound by phosphorylated CREB. The data presented herein support an emerging body of evidence that pCREB requires additional regulatory molecules, functionally analogous to Tax, to modulate precise patterns of cellular gene expression.

3.3 RESULTS

3.3a TAX REQUIRES PCREB FOR EFFICIENT RECRUITMENT OF FULL-LENGTH P300 AND CBP

Our initial goal was to study the coactivator recruitment characteristics of HTLV-1 promoter-bound Tax in complex with unphosphorylated and Ser^{133}-phosphorylated CREB. Nearly all previously published studies characterized the physical interaction of the Tax/CREB/vCRE complex to the KIX domain of CBP/p300. To investigate the recruitment of full-length p300 *in vitro*, we performed DNA pull-down assays using a promoter fragment carrying four tandem copies of the highly Tax-responsive vCREs (p4TxRE/G-less (176). A biotin group was incorporated at the upstream end of the DNA, and the fragment was immobilized on magnetic streptavidin-agarose beads. Highly purified, recombinant proteins (Fig. 3.1A) were incubated with the biotinylated DNA template, washed, and the bound proteins were analyzed by western blot. In these studies, we optimized the concentrations of purified Tax and CREB or PKA-phosphorylated CREB to saturate binding at the vCREs. We then added purified p300 at a physiologically-relevant concentration (50 nM) (177).

We observed dramatically enhanced recruitment of full-length p300 to the Tax/pCREB complex relative to the Tax/CREB complex (Fig. 3.1B, lanes 1 and 3), indicating that Tax does not bypass the need for CREB phosphorylation in the recruitment of p300. These data are consistent with recent studies from our laboratory showing that pCREB stabilizes the complex containing Tax, KIX and

A.

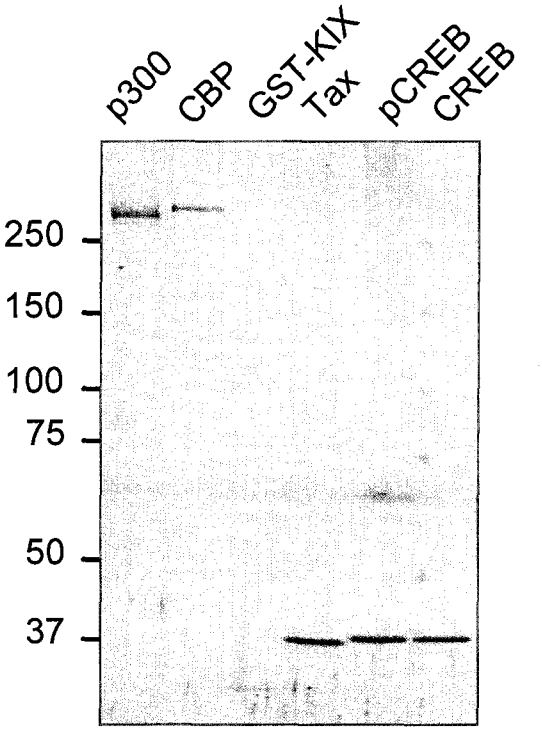


Figure 3.1. p300 and CBP recruitment to a viral CRE-containing promoter requires pCREB and Tax. A. Analysis of recombinant, purified proteins by SDS-PAGE and silver stain. Molecular weight markers are indicated. Experiment performed by T. R. Geiger.

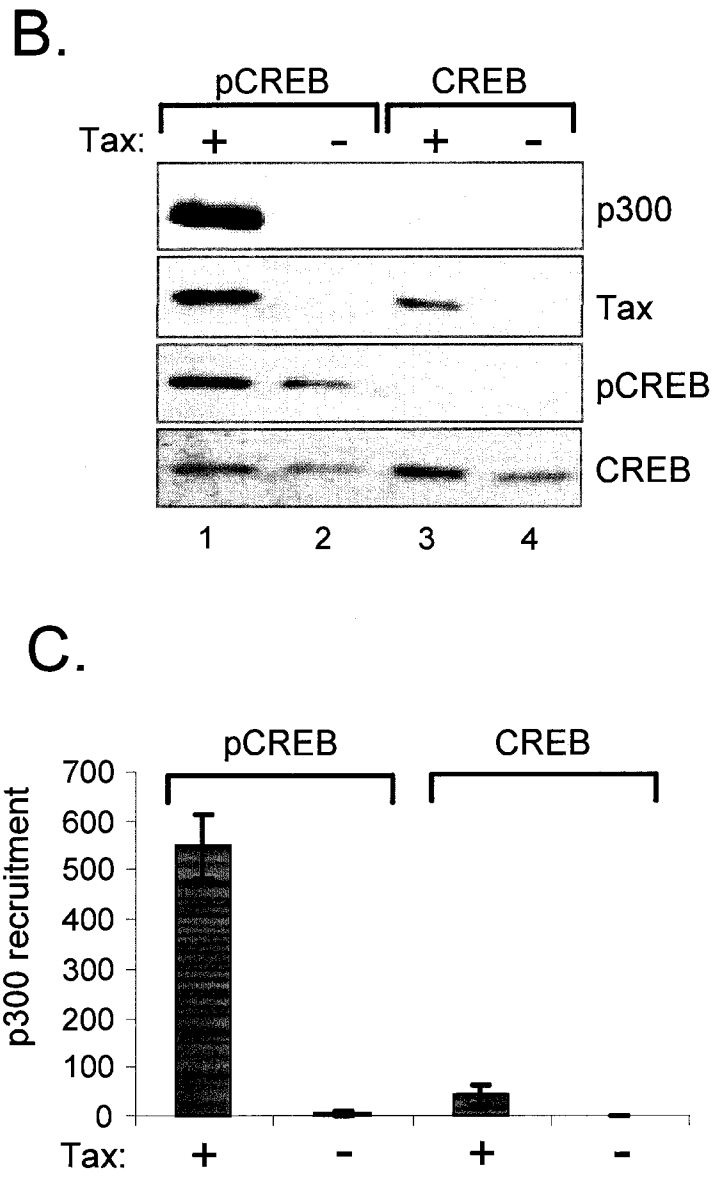


Figure 3.1B. Immobilized 4TxRE promoter DNA (1 pmol) was incubated with full-length p300 (2 pmol) in the presence of CREB, CREB+Tax, pCREB, and pCREB+Tax (8 pmol each) as indicated. Bound complexes were washed, and DNA-bound proteins were separated on a 6%-12% gradient SDS-polyacrylamide gel and analyzed by western blot. **C.** Densiometric analysis of p300 binding from three independent experiments is presented graphically. Experiment performed by T. R. Geiger.

the vCRE much more effectively than the unphosphorylated form of the protein (38). Furthermore, recent studies indicate that Tax is directly responsible for promoting elevated levels of CREB phosphorylation *in vivo* (38, 156, 157). Together, these data indicate that phosphorylated CREB may play a prominent role in Tax transactivation.

Unexpectedly, the binding of full-length p300 to the HTLV-1 promoter in the presence of pCREB was nearly undetectable (Fig. 3.1B, lane 2). The addition of Tax produced a significant increase in p300 binding (compare lanes 1 and 3). This is in contrast to studies that used the isolated KIX domain of CBP/p300 to conclude that pCREB strongly recruits the full-length coactivators. DNA pull-down data from three independent experiments was quantified, and a graph showing the relative recruitment of p300 by the various DNA-bound complexes is shown in figure 3.1C. Compared with unphosphorylated CREB, pCREB enhanced p300 binding six-fold. The Tax/CREB complex enhanced p300 binding 40-fold relative to CREB. The Tax/pCREB complex further enhanced p300 binding 90-fold relative to pCREB. These binding assays were performed under a variety of conditions, including the absence and presence of acetyl CoA and ATP. A recent study found that in the presence of acetyl CoA, p300 was released from promoter templates following autoacetylation (178). We found p300 recruitment was unaffected by acetyl CoA and ATP in our assays (data not shown).

The dramatic results obtained with full-length p300 prompted us to perform the parallel experiment with the full-length paralogue CBP. Both

coactivators have been implicated in Tax transactivation and signal-dependent transcription by CREB. The experiment presented in figure 3.1D revealed that Tax and pCREB are both required for CBP recruitment to the Tax-responsive promoter fragment, and again that pCREB alone is insufficient for recruitment of the eponymous coactivator. As a control, we tested both CBP and p300 histone acetyltransferase activity and found that both proteins were similarly active, indicating that they were functionally competent in our assays (data not shown). These data show that recombinant full-length CBP and p300 require both Tax and pCREB to bind the vCRE-containing promoter template in our recruitment assays.

3.3b CRE-BOUND PCREB RECRUITS THE ISOLATED KIX DOMAIN BUT NOT FULL-LENGTH P300

In the experiments presented in figure 3.1 we used a promoter fragment carrying four copies of the Tax-responsive vCRE cloned upstream of a minimal promoter. Because of the significance of the data obtained with this fragment, we were interested in determining whether Tax and pCREB similarly supported the recruitment of p300 to the natural HTLV-1 promoter fragment (pLTR-G-less) (176). Figure 3.2A shows that essentially identical p300 recruitment was achieved with the natural HTLV-1 promoter compared with the synthetic promoter (see figure 3.1B). As expected, we did not observe Tax binding or p300 recruitment in the absence of CREB (Fig. 3.2A, lane 3).

D.

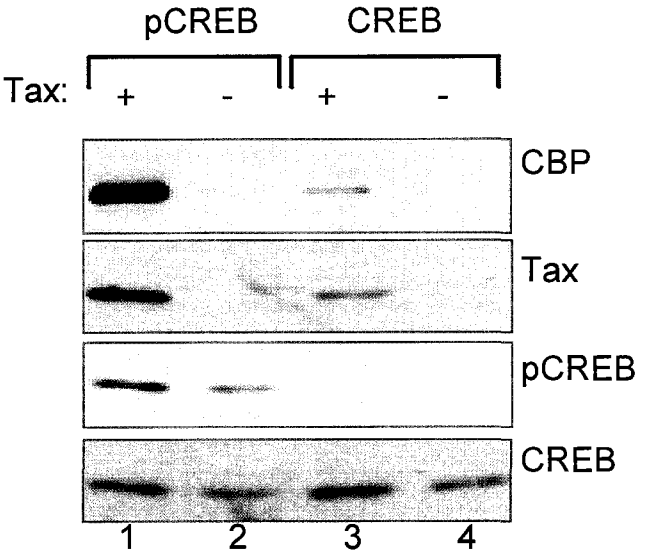


Figure 3.1D. Analysis of recombinant, full-length CBP recruitment the immobilized 4xTRE template in the presence of CREB, CREB+Tax, pCREB, and pCREB+ Tax (8 pmol each), as indicated. The experiment was performed exactly as described in panel B, except purified, recombinant full-length CBP (2 pmol) was used in the binding reaction in place of p300. Experiment performed by T. R. Geiger.

A.

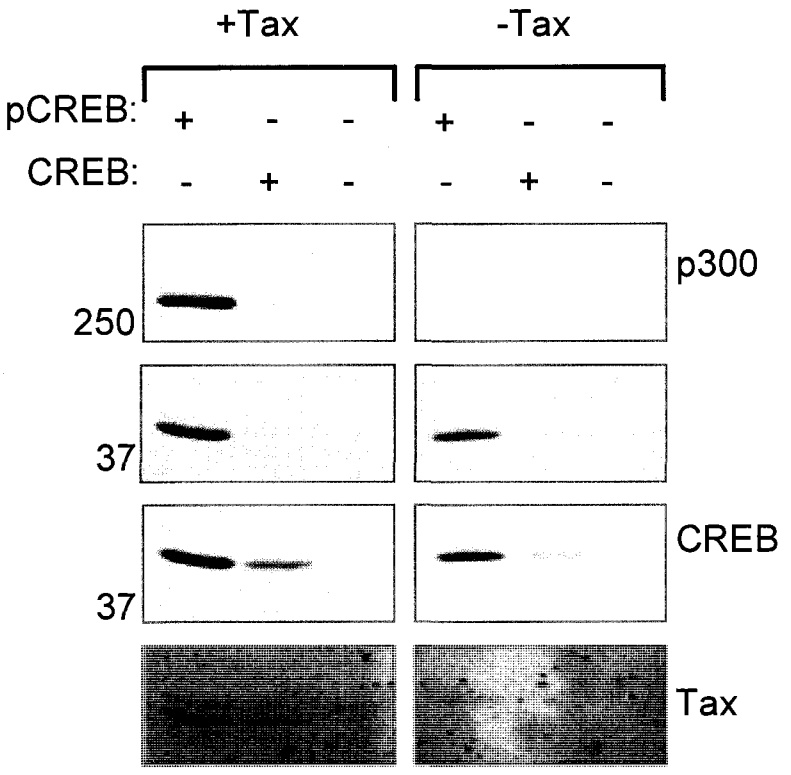


Figure 3.2. Individual viral and cellular CRE sequences support Tax/pCREB recruitment of p300. A. The immobilized template assay was performed with the natural HTLV-1 promoter. Binding reactions and analyses were performed as described in figure 1B. Experiment performed by T. R. Geiger.

We next analyzed the recruitment of p300 to complexes formed on immobilized double-stranded oligonucleotide templates carrying various individual binding. We were interested in comparing pCREB and Tax/pCREB recruitment of p300 to an off-consensus versus a consensus CRE. CREB binds the consensus CRE with approximately 10-fold higher affinity than the offf-consensus CREs present in the viral promoter (179). In parallel binding assays, we compared recruitment of p300 by Tax and pCREB to the single, promoter proximal viral CRE and a consensus cellular CRE, which does not carry the GC-rich sequences necessary for Tax binding (Fig. 3.2B). Each oligonucleotide was designed with the core CRE sequence starting 23 base pairs from the upstream biotinylated end. As expected, Tax was not recruited to the cellular CRE (Fig. 3.2C, lanes 1 and 3). However, we reasoned that since this oligonucleotide contains the optimal CREB binding site, it may allow pCREB alone to recruit p300. Instead, recruitment of p300 by pCREB to the consensus CRE was nearly undetectable (Fig. 3.2C, lanes 1-4). We also designed a binding site carrying a consensus CRE and the GC-rich flanking sequences necessary for Tax recruitment (Fig. 3.2B). Inclusion of the GC-rich sequences adjacent to the consensus CRE recovered Tax binding and restored p300 recruitment by the Tax/pCREB complex (Fig. 3.2C, lanes 5-8). Together, these data reveal a strict requirement for Tax in complex with pCREB in the recruitment of p300 to the viral promoter.

Our inability to detect pCREB recruitment of the coactivators is significant. Therefore, we wanted to confirm that the full-length coactivators possessed

B.

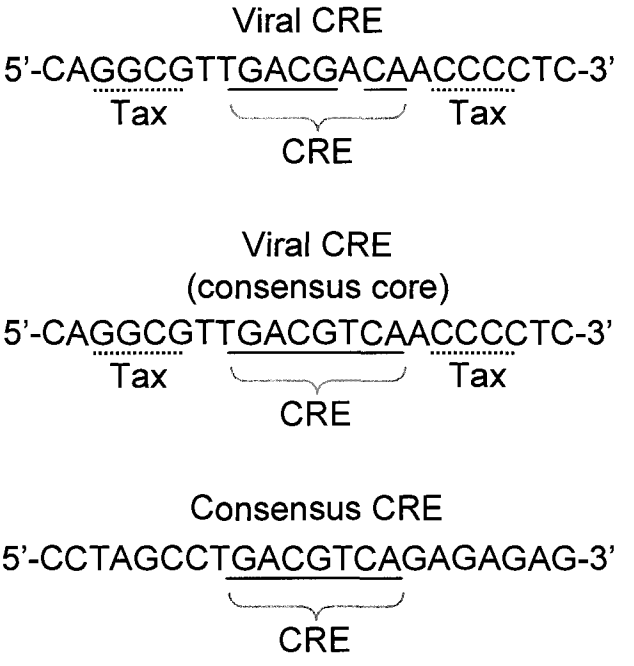


Figure 3.2B. Partial top strand sequence of the double-stranded oligonucleotides used in the immobilized template assays shown in C and D (see materials and methods for the complete sequences). The conserved Tax-binding sequences are indicated with a dashed underline. Nucleotides conforming to the consensus CRE are underlined.

C.

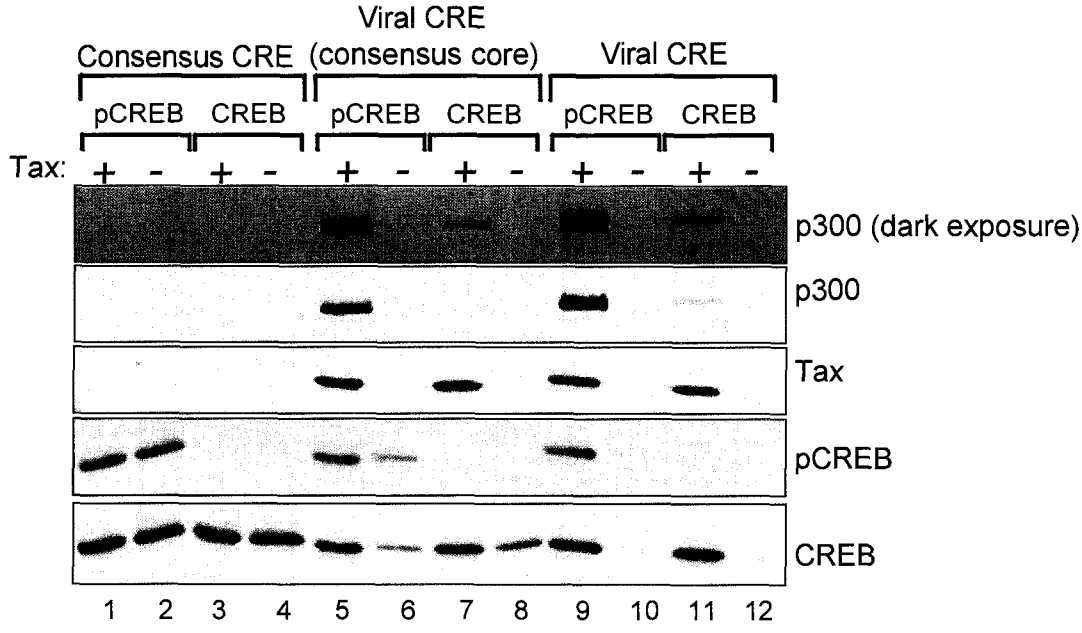


Figure 3.2C. The immobilized template assay was performed with the individual 45 bp doubled-stranded CRE sequences shown in B. Binding reactions and analyses were performed as described in figure 1B. The upper panel is a darker and higher contrast version of the same p300 blot shown directly below and shows weak detection of p300 in lanes 1 and 2. Experiment performed by T. R. Geiger.

regulatory properties lost in the isolated KIX domain. We next directly analyzed the binding of the various DNA-bound complexes to the KIX domain of CBP. The experiment shown in figure 3.2D was performed in parallel with the experiment shown in figure 3.2C, enabling direct comparison of KIX and p300 recruitment. Importantly, in the absence of Tax, we observed distinct patterns of KIX and p300 recruitment by pCREB (Fig. 3.2D). Paradoxically, pCREB strongly recruited KIX, despite being defective for p300 recruitment (compare Fig. 3.2C and D, lanes 1 and 2). As previously observed (36, 37), Tax in complex with unphosphorylated CREB also bound KIX (Fig. 3.2D, lanes 7 and 11), in contrast to our observations with full-length p300. Together, these data indicate that recruitment studies with the KIX domain may not represent the physiological interaction, and that strong recruitment of the full-length coactivators requires additional regulators, such as Tax.

3.3c TAX/PCREB RECRUITMENT OF P300 TO THE VIRAL PROMOTER CORRELATES WITH STRONG TRANSCRIPTIONAL ACTIVATION

Because of the relevance of our data to Tax and pCREB regulatory function, we tested the recruitment of native p300 to physiologically-relevant chromatin templates. For these studies, we assembled the Tax-responsive promoter fragment used in figure 3.1 into chromatin using purified *Drosophila* core histones and the recombinant assembly proteins Acf1/ISWI and *Drosophila* NAP-1 (180-182). Micrococcal nuclease assays were performed to verify the assembly of nucleosomes onto the immobilized promoter fragment. The

D.

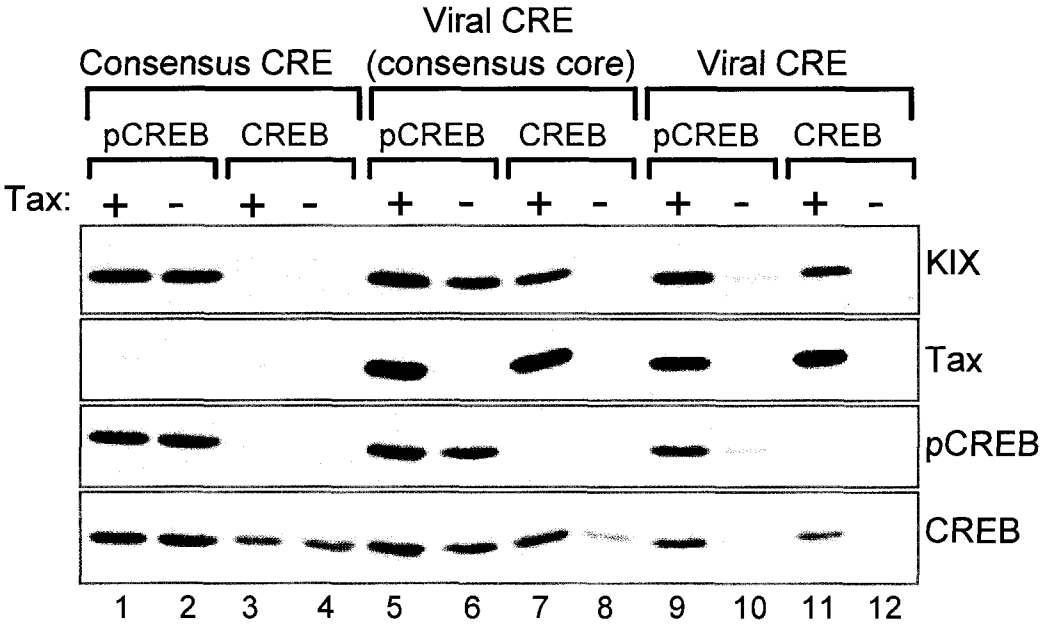


Figure 3.2D. The immobilized template assay was performed with the individual 45 bp doubled-stranded CRE sequences shown in B. Binding reactions and analyses were performed as described in figure 1B. The upper panel is a darker and higher contrast version of the same p300 blot shown directly below and shows weak detection of p300 in lanes 1 and 2. Experiment performed by T. R. Geiger.

chromatin template was preincubated with pCREB alone or pCREB in the presence of increasing concentrations of Tax, followed by incubation with T-cell nuclear extract (HTLV-1-negative CEM cells). The immobilized template was washed and bound proteins were subjected to SDS-PAGE and visualized by western blot. To assess the binding of nucleosomes to the washed templates, histones were visualized by coomassie brilliant blue staining (Fig. 3.3A, lower panel). Tax in complex with pCREB, but not pCREB alone, was required for efficient recruitment of endogenous p300 to the promoter template (Fig. 3.3A, upper panel). We next assayed a portion of each binding reaction shown in figure 3.3A by *in vitro* transcription. As expected, transcription from the 4TxRE/G-less template was fully repressed in the presence of chromatin (Fig. 3.3B, lanes 1,2) (40, 43). The addition of pCREB had no detectable effect on transcriptional output, despite the strong binding to the template shown in figure 3.3A (lane 2). The absence of a transcription signal with pCREB correlated directly with the weak recruitment of p300 observed in the immobilized template assay. The addition of Tax and pCREB together, however, resulted in potent activation of transcription. We were also interested in testing whether Tax and unphosphorylated CREB could activate vCRE-dependent transcription. However, CREB becomes phosphorylated in the presence of the ATP, which is required for the *in vitro* transcription reaction (data not shown). We therefore analyzed the CREB Ser^{133} to Ala mutant (Ser^{133}→A CREB), which cannot be phosphorylated. Tax and Ser^{133}→A CREB were unable to activate transcription, confirming that Tax and pCREB are both necessary for viral transcription (Fig. 3.3C). We next

A.

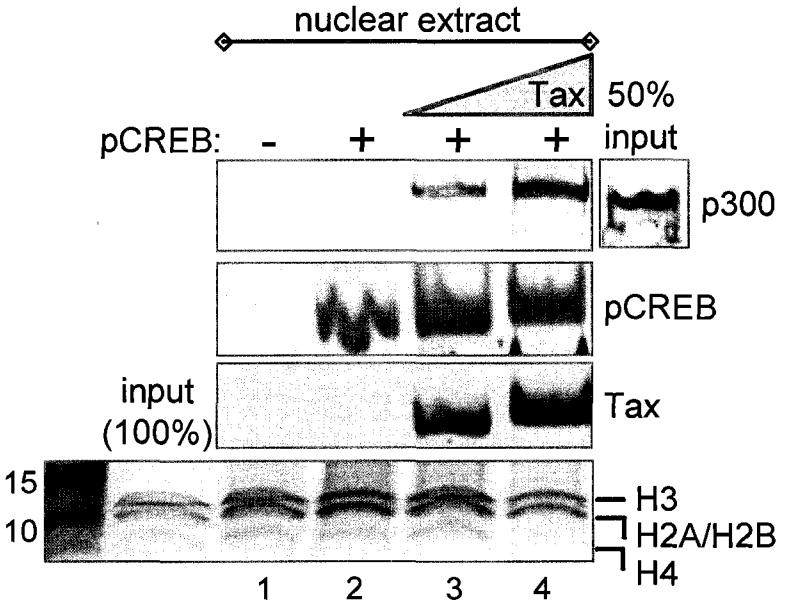
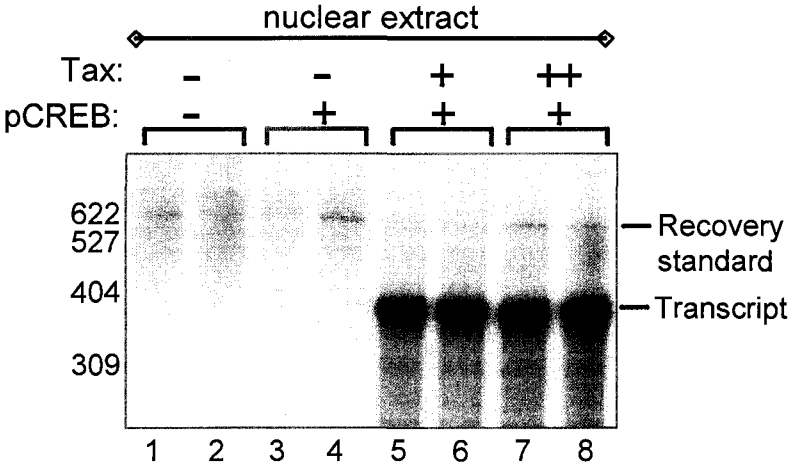


Figure 3.3. Recruitment of endogenous p300 correlates with transcriptional activation from chromatin templates. A. Immobilized, chromatin-assembled 4TxRE promoter DNA (1 pmol) was incubated in the absence (lane 1) or presence of pCREB (2.5 pmol) (lane 2) and increasing amounts of Tax (2.5 or 10 pmol) (lane 3, 4), as indicated. Following the pre-incubation step, CEM nuclear extract was added and samples were incubated for 60 min at 4°C. Immobilized complexes were washed, and analyzed by 4-20% SDS-polyacrylamide gradient gel and western blot (upper three panels). A portion of each binding reaction was resolved on an 18% SDS polyacrylamide gel and stained with coomassie to detect bound histone proteins (lower panel). Western blot of p300 present in the nuclear extract input (50%), and a coomassie stained gel of input histone (100%) are shown. Experiment performed by N. Sharma.

B.



C.

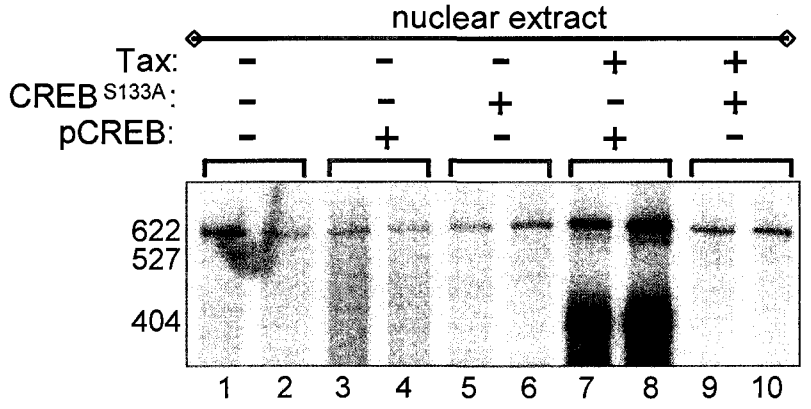


Figure 3.3B. Small aliquots from each binding reaction above (8% of the total) were incubated with acetyl CoA and nucleoside triphosphates, and analyzed in duplicate by *in vitro* transcription. The position of molecular weight size markers, recovery standard and full-length G-less transcripts are indicated. **C.** Transcription reactions were performed with the unphosphorylatable mutant Ser^{133}→A CREB ($CREB^{S133A}$) compared with pCREB. (All samples were analyzed on a single gel but lanes 7-8 and 9-10 were rearranged for this figure to maintain consistency with other experiments). Experiment performed by N. Sharma.

assayed p300 recruitment together with Tax-pCREB-dependent transcription in the presence of increasing concentrations of GST-KIX. We have previously shown that the KIX binding to the Tax/pCREB/vCRE complex blocks recruitment of full-length p300 (43). GST-KIX (but not GST) simultaneously blocked p300 recruitment and potently inhibited Tax/pCREB-mediated transcription (Fig. 3.3D). These data directly correlate p300 recruitment with Tax-dependent transactivation.

The inability of pCREB to produce a transcriptional response from the vCRE promoter *in vitro* prompted us to explore the effect of CREB phosphorylation at a comparable promoter *in vivo*. We performed transient co-transfection assays using a Tax-expression plasmid and a luciferase reporter plasmid. The vCRE-luciferase reporter plasmid carried three tandem copies of the vCRE immediately upstream of a core promoter (36). We used this Tax-responsive reporter construct to examine the effect of CREB phosphorylation mediated through the vCREs, without the influence of other promoter elements. Transfection assays were performed in HTLV-1 negative Jurkat T-cells in the absence or presence of forskolin, a cAMP agonist that induces Ser^{133}CREB phosphorylation. Figure 3.3E shows that forskolin treatment had no effect on vCRE-dependent transcription in the absence of Tax, consistent with the *in vitro* transcription assays performed with pCREB. Forskolin also had no effect on Tax transactivation, as Tax expression is sufficient to induce CREB phosphorylation *in vivo* (Fig. 3.3E, lane 3) (38, 156, 157). Similar results were obtained using the natural HTLV-1 promoter (data now shown). We confirmed that forskolin was

active, as treatment with the agonist increased the levels of intracellular CREB phosphorylation [Fig. 3.3E, see (38)]. These data are consistent with chromatin immunoprecipitation (ChIP) studies that demonstrated the *in vivo* recruitment of p300 by CREB and Tax (175). These data provide further supporting evidence indicating that CREB phosphorylation alone is insufficient for transcriptional activation.

D.

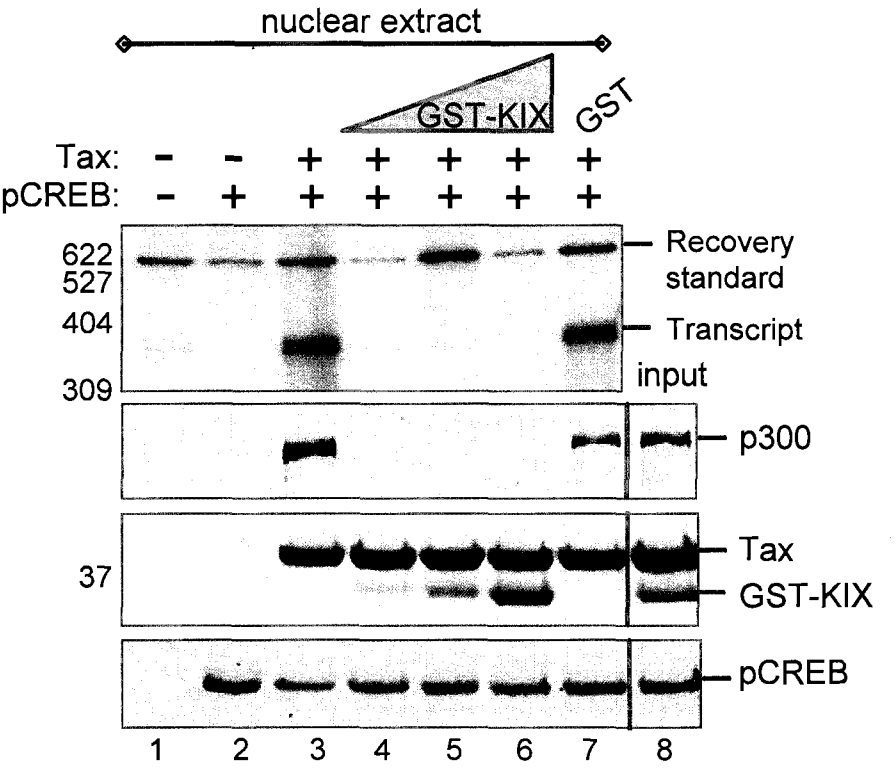


Figure 3.3D. Protein binding and transcription reactions were performed as described in A and B, but in the presence of increasing amounts of purified GST-KIX (2.5, 5 and 25 pmol) (lanes 4-6), as indicated. GST alone (25 pmol) was used as a negative control (lane 7). Results from the transcription assay are shown in the top panel, and western blots of bound p300, Tax, GST-KIX, and pCREB are shown in the lower three panels. Input Tax, pCREB, GST-KIX (2.5 pmol purified protein), and p300 (50% of nuclear extract) is shown in lane 8. Experiment performed by N. Sharma.

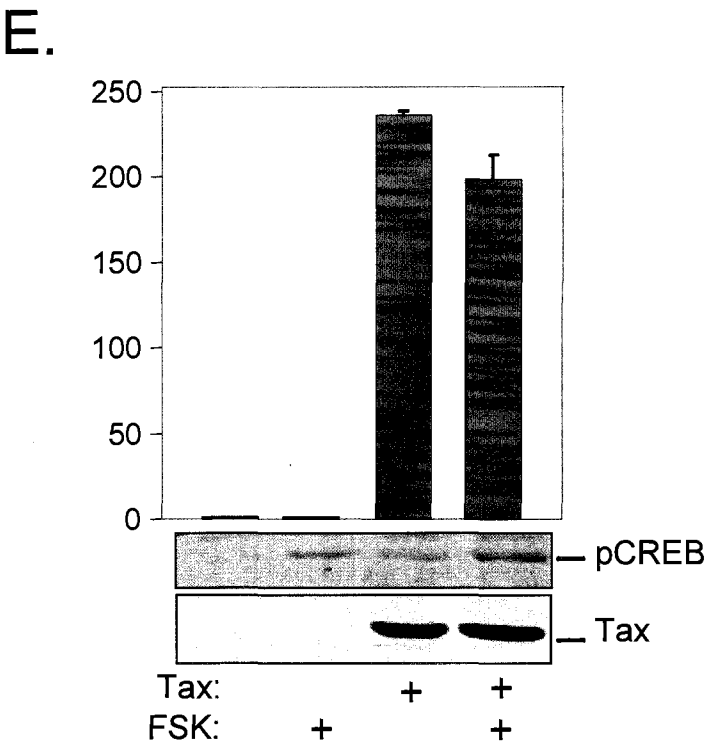


Figure 3.3E. HTLV-1-negative Jurkat T-cells were serum-starved (0.5% FBS) and then co-transfected with the vCRE-luc reporter plasmid (100 ng) , and a Tax expression plasmid (pSG-Tax, 100 ng) . Eight hours prior to harvest, cells were treated with forskolin (FSK) (10 μM) or the DMSO vector then analyzed by luciferase assay. Transient transfections were performed in triplicate, and each experiment was repeated three times. Corresponding western blots probed against pCREB and Tax are shown for the samples analyzed in the luciferase assay.

3.4 DISCUSSION

Since the early 1990s, the physical interaction between Ser^{133}-phosphorylated CREB and the KIX domain of CBP/p300 has been viewed as the sole obligate event for the recruitment of the coactivators and the activation of signal-dependent target genes. The original benchmark *in vitro* observations have been corroborated in a number of biochemical and structural studies using the KIX domain (47, 147, 166-168). This view, however, is beginning to change, as a number of recent studies have found that signal-induced CREB phosphorylation does not lead to CBP/p300 recruitment at the majority of CREB target promoters *in vivo* (164, 169, 170). These data suggest that additional mechanisms are required for efficient recruitment of CBP/p300 to phosphorylated CREB bound at promoters *in vivo*.

The HTLV-1 Tax oncoprotein functions together with CREB to potently activate transcription mediated through the three viral CREs present in the viral promoter. Previous studies demonstrated that Tax had little effect on the otherwise strong recruitment of KIX by pCREB (36, 37). Instead, Tax significantly enhanced the recruitment of KIX to unphosphorylated CREB (36, 37). This led to the hypothesis that Tax bypasses the requirement for CREB phosphorylation in CBP/p300 recruitment to the viral promoter. However, several recent studies show that Tax stimulates CREB phosphorylation *in vivo* (38, 156, 157). This observation is consonant with the fact that Tax robustly activates HTLV-1 transcription independent of inducers of CREB phosphorylation. Together, these data indicate that Tax functions in concert with pCREB to activate HTLV-1

transcription. However, the precise role of pCREB in Tax function remains enigmatic.

In this study, we initiated an investigation to better elucidate the interplay between Tax and pCREB in CBP/p300 recruitment and Tax transactivation. Since Tax has been shown to bind the coactivators at multiple sites (36, 44, 45, 161, 183), we analyzed Tax/pCREB recruitment of full-length p300 to immobilized CRE-containing DNA templates. Unexpectedly, we found that promoter-bound pCREB alone only very weakly recruited full-length p300 or its paralogue CBP. Tax in complex with phosphorylated CREB, however, strongly recruited both full-length coactivators. Tax in complex with unphosphorylated CREB only weakly recruited the coactivators. Because these results diverge significantly from existing models of pCREB and Tax/CREB recruitment of CBP/p300, we directly compared recruitment of full-length p300 and the isolated KIX domain. We found that CRE-bound pCREB alone strongly recruited KIX, despite only very weak recruitment of full-length p300. The failure of pCREB to recruit full-length p300 was highly reproducible on various CRE sequences, including the consensus CRE derived from the somatostatin promoter, and on both naked and chromatin-assembled promoter templates. Consistent with our *in vitro* observations, stimuli-induced CREB phosphorylation had no effect on vCRE-dependent transcription *in vivo*. Finally, we observed a direct correlation between recruitment of native p300 to chromatin-bound Tax/pCREB complexes and transcriptional activation *in vitro*. Based on these findings, we conclude that CRE-bound pCREB is not sufficient for CBP/p300 recruitment and transcriptional

activation, and that additional regulators, such as Tax, serve to facilitate pCREB recruitment of the coactivators and subsequent transcriptional activation.

The conserved KIX domain of CBP/p300 is composed of three α-helices that form a compact hydrophobic core with two discrete transcription factor binding surfaces (46, 47). These binding sites reside on opposite faces of KIX, and each has been shown to bind several unrelated transcription factors (46, 47, 184-188). We recently demonstrated that full-length pCREB and Tax interact simultaneously at each of the two distinct binding sites on KIX, forming a very stable quaternary complex with the vCRE DNA (39). The simultaneous contacts made by Tax and pCREB within KIX likely occur as a consequence of their close proximity when bound to the vCRE, as well as intimate protein-protein interactions between the two activators (Fig. 3.4). This observation may provide insight into the mechanism by which Tax and pCREB cooperate to recruit CBP/p300. We hypothesize that the pCREB binding site on KIX is inaccessible in the context of the full-length coactivator. Tax binding on one surface of KIX induces a conformational change that exposes the high-affinity pCREB binding site. Since this site is obscured in the context of the full-length coactivators, other regions of CBP/p300 must regulate the accessibility or confirmation of the hydrophobic groove where pCREB binds (Fig. 3.4).

Our observation that Tax is required for recruitment of full-length CBP/p300 by pCREB suggests a role for cellular factors that are functionally analogous to Tax. For example, cellular KIX-interacting transcription factors may also bind immediately adjacent to pCREB, exposing the pCREB interaction site.

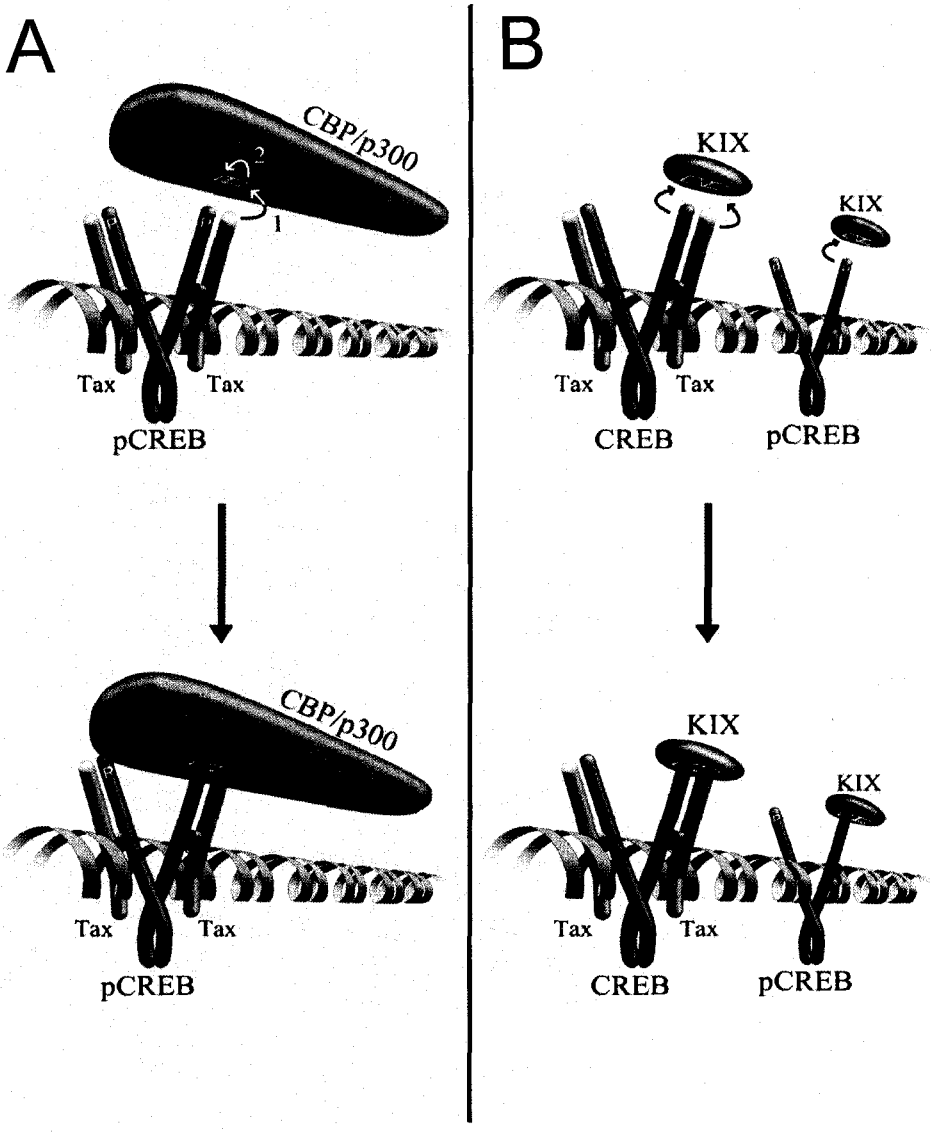


Figure 3.4. Model comparing the recruitment of full-length CBP/p300 and the KIX domain by CRE-bound Tax and pCREB. A. Full-length CBP/p300 recruitment by the DNA-bound Tax/pCREB complex. Model depicts initial KIX contact by Tax promoting a conformational change that enables pCREB binding and stable coactivator recruitment. **B.** Model depicts distinct mechanisms of KIX recruitment by pCREB and the Tax/CREB complex. Tax recruits KIX in the presence of unphosphorylated CREB (left). The pCREB binding site on KIX is accessible, allowing recruitment in the absence of Tax (right).

MLL and c-jun each bind KIX at the same site as Tax, which is distinct from the pCREB binding site (46, 186-188). Recently, Goto et al. showed that binding of the MLL activation domain to KIX enhanced binding of the phosphorylated KID region of CREB, providing direct evidence for cooperative binding of transcription factors within the KIX domain (46). These data provide additional support for the hypothesis that additional KIX-binding transcription factors likely participate in phosphorylation-dependent recruitment of full-length CBP/p300 by CREB *in vivo*.

There are over 200 transcription factors that interact at multiple domains within CBP/p300 (189). It is possible that one or more of these transcription factors bound at distal cis-acting elements within CREB target gene promoters may induce a conformational change elsewhere in full-length CBP/p300 that is transmitted into KIX, facilitating stable binding of pCREB. Alternatively, the presence of multiple trans-acting factors at target promoters, each with relatively low affinity for CBP/p300, may together increase the local concentration of the coactivators sufficiently to enable pCREB binding. This idea is supported by the fact that we observe weak phosphorylation-dependent CREB binding to p300 at very high concentrations of the coactivator (0.4 μM, data not shown). Since CBP and p300 appear to be limiting in cells (190), the unique combinations of transcription factors, with varying affinities for the coactivators, may promote distinct programs of transcriptional response to signal-dependent phosphorylation of CREB.

Finally, the family of proteins called transducers of regulated CREB (TORCs) have been recognized as important co-factors in phosphorylated

CREB-mediated transcriptional activation *in vivo* (191). Two recent studies explored transcriptional activation by phosphorylated CREB and found that TORC2 was required for CBP/p300 recruitment and activation of selective genes *in vivo* (169, 170). Reduction of TORC2 activity produced a decline in the occupancy of promoter-bound CBP/p300 and diminished transcription in response to CREB phosphorylation (169, 170). Notably, the TORC proteins facilitated coactivator recruitment by pCREB via interactions with both CBP/p300 and the bZIP domain of DNA-bound CREB (169, 170, 192). Therefore, the mechanism of TORC coactivator recruitment to pCREB is likely functionally analogous to Tax.

In summary, we found that pCREB alone is not sufficient for recruitment of full-length CBP/p300. Instead, other cofactors, like Tax, are obligate regulators in pCREB recruitment of the coactivators. The data presented herein provides compelling evidence for a conformational change in full-length CBP/p300 upon Tax binding that uncovers the pCREB binding site in KIX. However, the precise molecular mechanism that facilitates phosphorylation-dependent binding of CREB to the full-length coactivators remains elusive. By characterizing the mechanisms underlying Tax-mediated co-recruitment of CBP/p300, and by identifying and characterizing cellular proteins that stabilize the assembly of coactivator complexes with phosphorylated CREB, we may better understand how signal-dependent phosphorylation of CREB can lead to selective modulation of cellular gene expression.

3.5 MATERIALS AND METHODS

3.5a NUCLEAR EXTRACT

CEM cells, an HTLV-1 negative human T-cell line, were cultured in IMDM supplemented with fetal bovine serum. Nuclear extracts were prepared as previously described (193).

3.5b EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

Bacterially-expressed Tax-His_6, Ser^{133}→A CREB (158), and GST-KIX (CBP amino acids 588-683) (36) proteins were purified to >98% homogeneity as previously described (36). The KIX domain of CBP used in this study is 85% identical to the p300 KIX domain. $CREB_{327}$ was purified to apparent homogeneity, and free of contaminating nucleic acids, as recently described (194). $CREB_{327}$ is a naturally occurring splice variant where serine 119 corresponds to serine 133 in human $CREB_{341}$ (160). To avoid confusion, we used the serine 133 nomenclature throughout this work. Both $CREB_{327}$ and $CREB_{341}$ gave identical results in the CBP/p300 recruitment (data not shown). Full-length His_6-tagged p300 and flag-tagged CBP were expressed from recombinant baculovirus in Sf9 cells and purified as previously described (159). All proteins were dialyzed against TM buffer (50 mM Tris pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 20% v/v glycerol, 0.025% v/v 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.

3.5c PROMOTER FRAGMENTS AND OLIGONUCLEOTIDES

A 643 bp promoter fragment was amplified by PCR from the 4TxRE/G-less plasmid template that carries 4 reiterated copies of the HTLV-1 promoter proximal vCRE cloned upstream of the HTLV-1 core promoter (176). Primers were designed to amplify the sequence between −187 and +456 and generate a fragment carrying a biotin group at the upstream end of the promoter. The native HTLV-1 promoter fragment, carrying sequences upstream to –306, was similarly prepared from the plasmid pHTLV-1/G-less. Primers for PCR amplification of 4TxRE/G-less, top strand: 5′-Bio/CATCGATAAGCTTCTAG, bottom strand: 5′-CATGATTACGCCAGGC; primers for pHTLV-1/G-less, top strand: 5′-Bio/TGCCTGCAGGTCGAC, bottom strand: 5′-GCCTCAGGTAGGGCGGCGGG. The top strand, 5′-biotinylated sequences of the complimentary oligonucleotides are as follows (CRE sequences underlined). Viral CRE: 5′-Bio/TTGTCAAGCCGTCCTCAGGCGTTGACGACAACCCCTCACCTCAAA; viral CRE with consensus octanucleotide core 5′-Bio/TTGTCAAGCCGTCCTCAGGCGTTGACGTCAACCCCTCACCTCAAA; cellular CRE (somatostatin promoter) 5′-Bio/ATCAGGCTTCCTCCTCCTAGCCTGACGTCAGAGAGAGAGGTCGCC. The promoter fragments and the biotinylated double-stranded oligonucleotides and were coupled to streptavidin Dynabeads® (Dynal Biotech USA, cat #112.06) at

0.08 pmol of DNA/ml of beads and 0.4 pmol of DNA/ml of beads, respectively. Binding was performed according to the manufacturer's instruction.

3.5d ANTIBODIES

The following antibodies were used in western blots: anti-CREB (sc-186), anti-phospho-Ser^{133}CREB (sc-7978-R), anti-p300 (sc-584), anti-CBP (sc-583), and anti-GST (sc-138). All were purchased from Santa Cruz Biotechnology. Alexa Fluor IR700 and IR800 goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Molecular Probes. A monoclonal Tax antibody (Hybridoma 168B17-46-92) was obtained from the National Institutes of Health Aids Research and Reference Reagent Program.

3.5e IMMOBILIZED TEMPLATE ASSAY USING PURIFIED PROTEINS

Tax, CREB, or pCREB (8 pmol each) were added to 20 µl reactions containing TM buffer with 10% glycerol, 10 µM $ZnSO_4$, 50 µM ATP, 80 µM acetyl CoA, 40 ng/µl of poly(dA-dT)-poly(dA-dT), and Dynabead-bound DNA (1 pmol for PCR fragments and 2 pmol for double-stranded oligonucleotides). Binding reactions were pre-incubated for 15 minutes at 30°C. Recombinant p300, CBP, or GST-KIX (2 pmol each) were then added to each sample in a final volume of 40 µl. Samples were mixed at 4°C for 1 hour. Beads were magnetically isolated, washed 3X with TM buffer, resuspended in 40 µl of Laemmli sample buffer (LSB), and boiled 5 minutes immediately prior to protein fractionation by SDS-PAGE.

3.5f COUPLED IMMOBILIZED TEMPLATE ASSAY AND *IN VITRO* TRANSCRIPTION

Following chromatin assembly, preinitiation complexes were formed on 1 pmol of the bead-bound promoter fragment in the absence or presence of Tax (2.5 or 10 pmol) and/or CREB (2.5 pmol) in a 50 μl reaction. The transcription factors were incubated with TM buffer, 50 μM ATP and 50 μM acetyl CoA for 15 minutes at 30°C. CEM nuclear extract (100 μg) was added to each binding reaction and incubated an additional 60 minutes at 4°C. To analyze bound proteins, 46 μl of the total binding reaction were removed, washed three times with TM buffer, resuspended in LSB, and analyzed by SDS PAGE and western blot. The remaining 4μl were processed for transcription analysis. The reaction was brought to 30 μl with TM buffer and 50 μM acetyl CoA, and RNA synthesis was initiated with the addition of 250 μM ATP, 250 μM CTP, 12 μM UTP, and 0.8 μM [α-^{32}P] UTP (3,000 Ci/mmol). Transcription reactions were processed and analyzed as previously described (144). Molecular weight markers (radiolabeled HpaII-digested pBR322) were used to estimate the size of the RNA products. A labeled 622 bp DNA fragment was added to each reaction mixture as a recovery standard.

3.5g CHROMATIN ASSEMBLY

Recombinant *Drosophila* histones were assembled into chromatin as previously described (40, 181, 182), except that the assembly was performed on the linear, bead-bound promoter fragments. Assembly was performed at a 4:7

histone:DNA mass ratio. Degree of assembly was confirmed by micrococcal nuclease digestion (181). Micrococcal nuclease digestion of the chromatin assembled 4TxRE promoter template is shown in Sharma et al. (195).

3.5h PROTEIN ANALYSIS BY SDS-PAGE

Proteins were resolved by SDS polyacrylamide linear gradient gels and either silver stained or transferred to nitrocellulose and probed with the indicated antibodies. For the chromatin-based binding reactions, two-thirds of each sample was also resolved on an 18% polyacrylamide gel and histone proteins were visualized by coomassie brilliant blue stain.

3.5i TRANSIENT-TRANSFECTION ASSAYS

Human Jurkat T-cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin. Cells were serum-starved (0.5% FBS) and transfected with a constant amount of DNA using Lipofectamine reagent (Invitrogen). After 24 hr, the cells were harvested and lysed, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) with a Turner Designs model TD 20-e luminometer. Firefly luciferase activity was normalized to *Renilla* luciferase activity from the herpes simplex virus thymidine kinase (TK) promoter (pRL-TK; Promega). The expression plasmid for Tax (pSG-Tax) (162) and the vCRE-Luc reporter plasmid (36) have been previously described. The transient

transfection assay was performed in triplicate and repeated in three independent experiments.

3.5j IMAGE PROCESSING

The ImageQuant program (Molecular Dynamics) was used to quantify results. Images were processed in Adobe Photoshop, with minor adjustments 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.

3.6 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)

CHAPTER 4
THE HTLV-1 ENCODED TAX PROTEIN INDUCES CREB PHOSPHORYLATION

4.1 ABSTRACT

HTLV-1 Tax utilizes the cellular transcriptional machinery to mediate high-level transcription of the provirus. The viral CRE (vCRE) enhancer elements are bound by the complex formed between Tax and the cellular transcription factor CREB. The coactivators CBP and p300 also bind to this ternary complex, which facilitates initiation of HTLV-1 transcription. PKA-phosphorylation of CREB is well known to enhance transcriptional activity at cellular promoters. We have previously shown that pCREB (but not CREB) is essential for stable complex formation composed of the vCRE, Tax, CREB, and the KIX domain of CBP (38). Consonant with these observations, we found that HTLV-1 infected T-cell lines have high levels of Ser133 phosphorylated CREB, relative to uninfected T-cell lines, and that elevation in pCREB levels is directly due to Tax expression. This effect is inhibited by either staurosporine, a broad-spectrum inhibitor of various protein kinases, or W7, a Ca^{2+}/calmodulin antagonist. More specifically, STO-609, an inhibitor of Ca^{2+}/calmodulin kinase kinase (CaMKK), reduces Tax-mediated CREB phosphorylation and viral transcription. These data uncover a new function for Tax in the context of HTLV-1 transcription – stimulation of CREB phosphorylation via the CaMK pathway. This function of Tax may be key in the development of ATL, as CREB phosphorylation has been implicated in leukemogenesis.

4.2 INTRODUCTION

The human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus etiologically linked to an aggressive and ultimately fatal cancer known as adult T-cell leukemia (ATL). Only a small percentage of infected individuals develop ATL after infection following a prolonged latency period of up to 30 years (5). The primary mechanism of virus transmission in an infected individual is through clonal expansion of HTLV-1 infected T-cells. The virally-encoded Tax protein plays a critical role in promoting cellular proliferation, and is directly linked to the development of cellular transformation (22, 23).

Tax is a potent transcriptional factor that stimulates HTLV-1 gene expression and deregulates cellular pathways in the infected cell. Three 21 base pair repeat enhancer elements, called viral cyclic AMP response elements (vCREs), are located in the HTLV-1 transcriptional control region and are critical for Tax-activated transcription. Tax associates with the vCREs through protein-DNA interactions and protein-protein interactions with the cellular transcription factor CREB (32-35). This promoter-bound complex recruits the cellular coactivators CBP/p300 (36, 37). The role of Ser133 phosphorylation of CREB in Tax-mediated viral transcription has been controversial. However, our recent studies reveal that CREB phosphorylation is absolutely required for Tax-dependent viral transcription (38). Consistent with this observation, Tax induces constitutively elevated levels of CREB phosphorylation *in vivo* (38).

CREB has been identified as a substrate for approximately 20 different protein kinases (154). Many of these kinases, including protein kinase A (PKA),

protein kinase C (PKC), protein kinase B (PKB), Ca^{2+}/calmodulin-dependent kinases (CaMK) and mitogen activated protein kinase (MAPK) are known to phosphorylate CREB at serine 133. This, in part, leads to increase CREB-dependent transcription. The CaMK are a family of structurally related multifunctional serine/threonine protein kinases including CaMKI, CaMKII, and CaMKIV (196). CaMKI and CaMKIV are phosphorylated and activated by CaMKK (197) . CaMKI is broadly expressed in mammalian cells and is cytosolic. CaMKIV is mainly expressed in neurons, T-cells and testis and predominantly localized in the nucleus. CaMKII is composed of at least 10 subunits and is expressed in a variety of tissues (198). CaMKI and CaMKIV directly induce CREB phosphorylation at Ser133, leading to stimulation of CREB-mediated transcription (199-201). However, CaMKII induces CREB phosphorylation at both Ser133 and Ser142, leading to inhibition of CREB-mediated transcription (202).

In this study, we sought to identify and characterize the kinase responsible for Tax-mediated CREB phosphorylation. We found that Tax-dependent phosphorylation of CREB at Ser133 was inhibited by a Ca^{2+}/calmodulin antagonist but not by a protein kinase A inhibitor. Additionally, a specific inhibitor of CaMKK, STO-609, suppressed CREB phosphorylation induced by Tax. This suggests that the CaMKK-dependent CaMK family members such as CaMKI and CaMKIV are involved in Tax-mediated CREB phosphorylation. Furthermore, STO-609 specifically inhibited Tax-mediated viral transcription. Therefore, we propose a model in which Tax stimulates the CaMK pathway and enhances

phosphorylation of CREB at Ser133 to ensure pCREB availability for Tax transactivation.

4.3 RESULTS

4.3a TAX INDUCES CREB PHOSPHORYLATION STATE VIA THE ACTIVATION OF SPECIFIC KINASES

Previous studies have shown that Ser133 CREB phosphorylation is elevated in HTLV-1 infected T-cells and this increased phosphorylation is Tax-dependent (38, 156, 157). CREB can be phosphorylated at Ser133 by many kinases including PKA, PKB, PKC, MAPK, and CaMK. Phosphorylation is in response to diverse signaling pathways including growth factor, cytokine, and steroid hormone stimuli (154). CREB also can be dephosphorylated by several cellular phosphatases such as protein phosphatase PP1 and PP2A (203, 204). Therefore, the mechanism of Tax-mediated CREB phosphorylation may involve either stimulating a kinase or inhibiting a phosphatase. To distinguish between these two possibilities, we performed *in vitro* kinase assays as well as *in vitro* phosphatase assays in parallel. Uninfected Jurkat T-cells were serum-starved for 24 hrs and whole cell extracts were prepared to provide kinase activity or phosphatase activity. Kinase reactions were carried out by adding purified recombinant CREB as a substrate, ATP and whole cell extracts in the absence or presence of purified recombinant Tax protein at 30°C for 30 min. Phosphatase reactions were carried out by adding purified recombinant PKA phosphorylated

CREB as a substrate and whole cell extracts in the absence or presence of purified recombinant Tax protein ar 30°C for 30 min. pCREB was detected by immunoblot analysis using an anti-Ser133 phospho-CREB-specific antibody. Interestingly, the addition of purified recombinant Tax dramatically enhanced CREB phosphorylation *in vitro* in a dose-dependent manner (Fig. 4.1A). However, the addition of purified recombinant Tax did not affect dephosphorylation of pCREB by phosphatases present in Jurkat cell whole cell extract (Fig. 4.1B). These findings demonstrate that Tax mediates CREB phosphorylation via stimulation of a kinase, not via inhibition of a phosphatase.

4.3b TAX STIMULATES CREB PHOSPHORYLATION THROUGH A CALMODULIN SIGNALING PATHWAY

Based on the observed Tax induced CREB phosphorylation *in vivo* and *in vitro*, we were interested in the identification of the responsible kinase. Since Tax is not known to possess intrinsic kinase activity, Tax must therefore directly stimulate a kinase present in the cell-free extract. To identify the Tax-targeted kinase, we used various pharmacologic inhibitors of kinases that have been shown to block specific kinase pathways (Fig. 4.2). These inhibitors included staurosporine, a broad-spectrum kinase inhibitor, H-89, a protein kinase A (PKA) inhibitor, wortmannin, a PI-3 kinase inhibitor, W7, a calmodulin antagonist, Ro-31-8220, a PKC inhibitor, SB203580, a p38-MAP (mitogen-activated protein) kinase inhibitor, and PD98059, a p42/44 MAP kinase inhibitor. *In vitro* kinase assays were carried out in the presence of each inhibitor as indicated in figure

A.

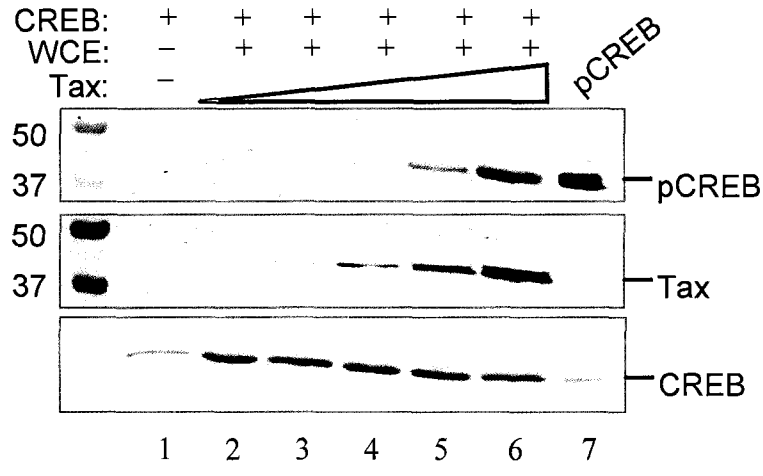


Figure 4.1 Tax induces CREB phosphorylation state via the activation of specific kinases. A. *Tax induces CREB phosphorylation in vitro.* CREB (33 nM) was incubated with Jurkat whole cell extracts (prepared following serum starvation) for 30 min in the presence of ATP (1 mM) and increasing concentrations of purified recombinant Tax protein (12.5-100 nM), as indicated. Reactions were terminated by addition of 4X SDS sample buffer and analyzed by western blot. Antibodies used for detection were anti-Ser133 phosphoCREB, a monoclonal anti-Tax, and anti-CREB antibody.

B.

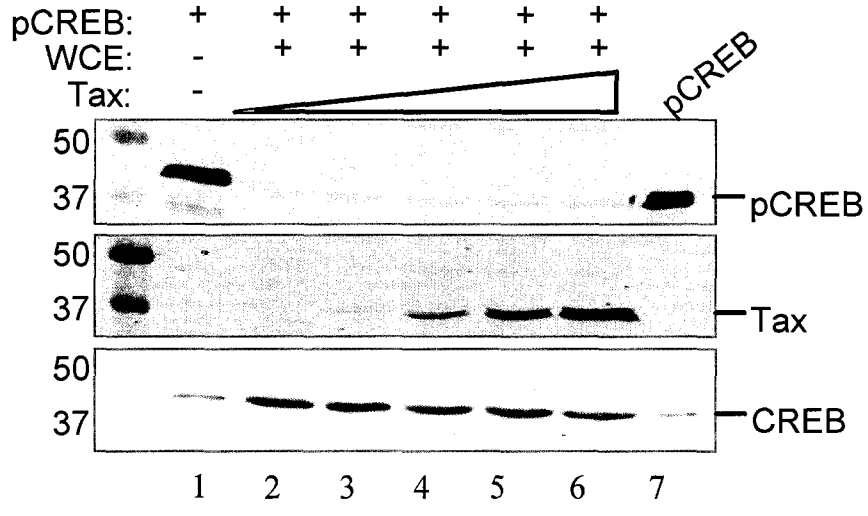


Figure 4.1B. *Tax did not inhibit dephosphorylation of pCREB.* pCREB (30 nM) was incubated with Jurkat whole cell extracts for 30 min in the presence of increasing concentrations of purified recombinant Tax protein, as indicated. Reactions were terminated by addition of 4X SDS sample buffer and analyzed by western blot.

Compound	Protein kinase
Staurosporine	A potent, cell-permeable broad spectrum inhibitor of kinases
H-89	A cell-permeable selective and potent inhibitor of PKA
Wortmannin	A cell-permeable and irreversible inhibitor of PI3-kinase
W7	A calmodulin antagonist
Ro-31-8220	A competitive, selective inhibitor of PKC
SB203580	A highly specific and potent inhibitor of p38 MAP kinase
KN-93	A CaM kinase II inhibitor
PD98059	A potent and selective MEK inhibitor
STO-609	A cell-permeable, highly, selective, potent, ATP-competitive inhibitor of CaM kinase kinase (CaMKK)

Figure 4.2. The compounds for kinase inhibition in this study. These inhibitors target distinct kinases which have been implicated in the regulation of CREB phosphorylation.

4.3A. As we expected, staurosporine blocked Tax-dependent CREB phosphorylation. Interestingly, the inhibition of PKA by H-89 had no effect on CREB phosphorylation. However, inhibition of the Ca^{2+}/ calmodulin (CaM) pathway by W7 significantly reduced Tax-dependent CREB phosphorylation (Fig. 4.3A). These data were corroborated using the inhibitors in HTLV-1-infected/transformed cells. After 24hr of serum starvation, HTLV-1 infected cells (C8166) were treated with the inhibitors and CREB phosphorylation was analyzed by immunoblot analysis using an anti-Ser133 phospho-CREB-specific antibody. Again, staurosporine and W7 each reduced the Tax-dependent elevation in pCREB levels (Fig. 4.3B). Taken together these data suggest that Tax utilizes the Ca^{2+}/calmodulin pathway to induce CREB phosphorylation.

4.3c A SPECIFIC CAM KINASE KINASE INHIBITOR, STO-609, INHIBITS TAX-MEDIATED CREB PHOSPHORYLATION *IN VITRO* AND *IN VIVO*

Multiple kinases including CaM Kinase IV, CaM Kinase I, and CaM Kinase II in the Ca^{2+}/calmodulin pathway have shown to phosphorylate CREB at Ser133 (154). A schematic diagram of the Ca^{2+}/calmodulin pathway with inhibitors used in this study is shown in figure 4.4. To further distinguish between CaMKII and CaM kinase kinase (CaMKK), two more kinase inhibitors, KN-93 and STO-609, were used. CaMKII phosphorylation (at both Ser133 and Ser142) is inhibitory to CREB function (202). Addition of the CaMKII inhibitor KN-93 had no effect on Tax-dependent CREB phosphorylation in the *in vivo* and *in vitro* kinase assays (Fig. 4.5 A&B), eliminating CaMKII as a possible target of Tax. In contrast, STO-

A.

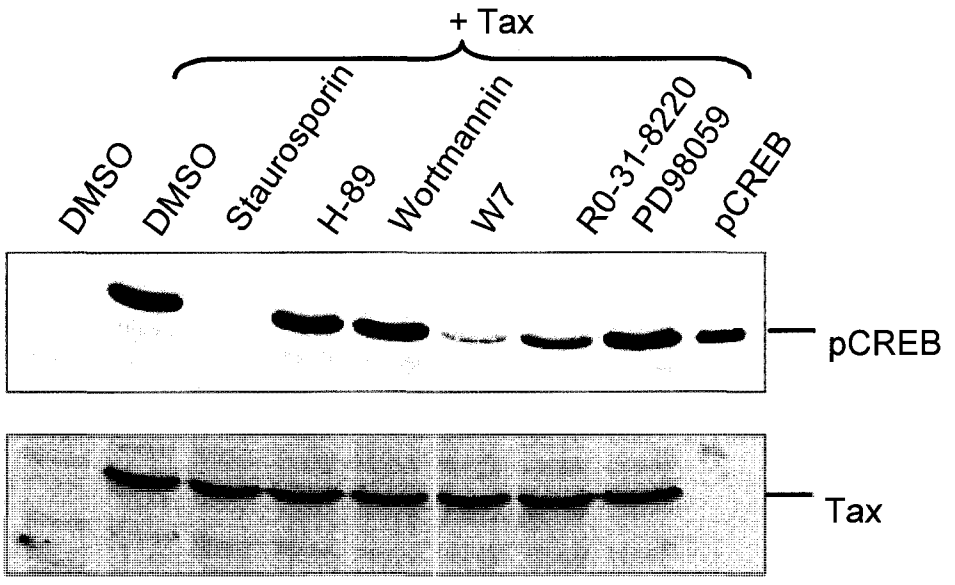


Figure 4.3 Tax stimulates CREB phosphorylation through a calmodulin signaling pathway. A. *Both Staurosporine and W7 decrease Tax-mediated CREB phosphorylation in vitro.* Recombinant purified CREB (33 nM) was incubated with Jurkat whole cell extract and Tax (100 nM) in the presence of DMSO or the following inhibitors: staurosporin (1μM), H-89 (1μM), wortmannin (1μM), W7 (100 μM), Ro-31-8220 (5 μM), PD98059 (25μM) for 30 min. Reactions were terminated by addition of 4X SDS sample buffer and analyzed by western blot.

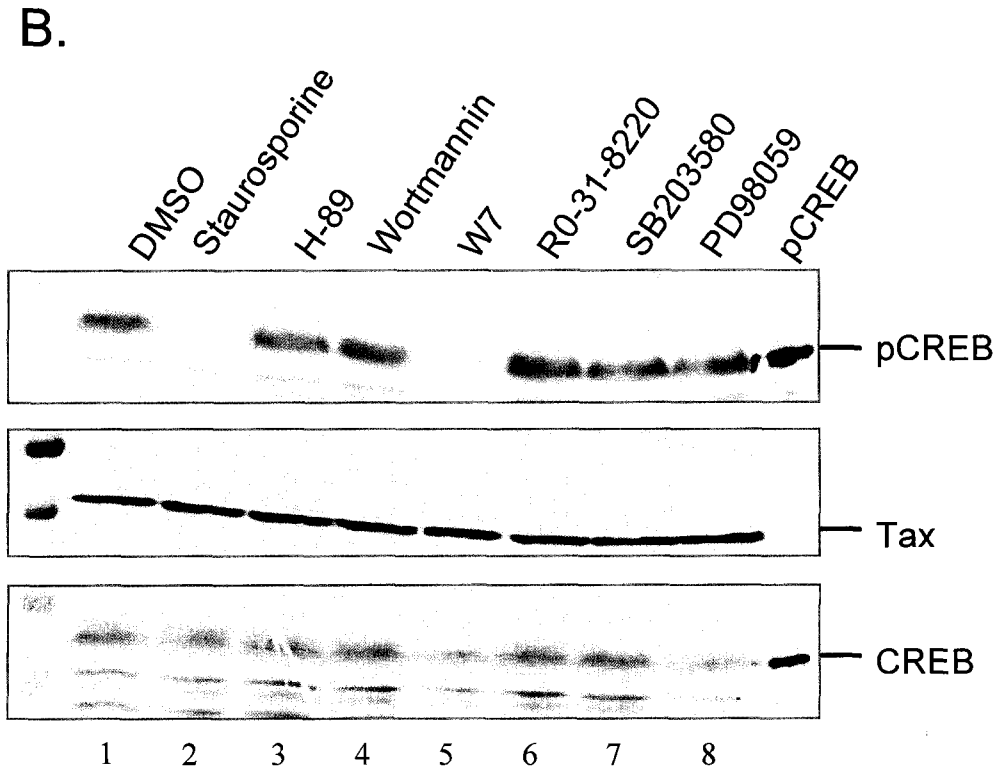


Figure 4.3B. *Both Staurosporine and W7 decrease phosphorylation of CREB in HTLV-1 infected C8166 cells.* HTLV-1-infected/ transformed C8166 cells were serum starved for 24 hrs and then treated with one of the following pharmacologic kinase inhibitors; staurosporine (1μM), H-89 (1μM), wortmannin (1μM), W7 (100 μM), Ro-31-8220(5 μM), SB203580(10μM) or PD98059(25μM) for 2 hrs. Cells were harvested, whole cell extracts were prepared and analyzed by western blot analysis.

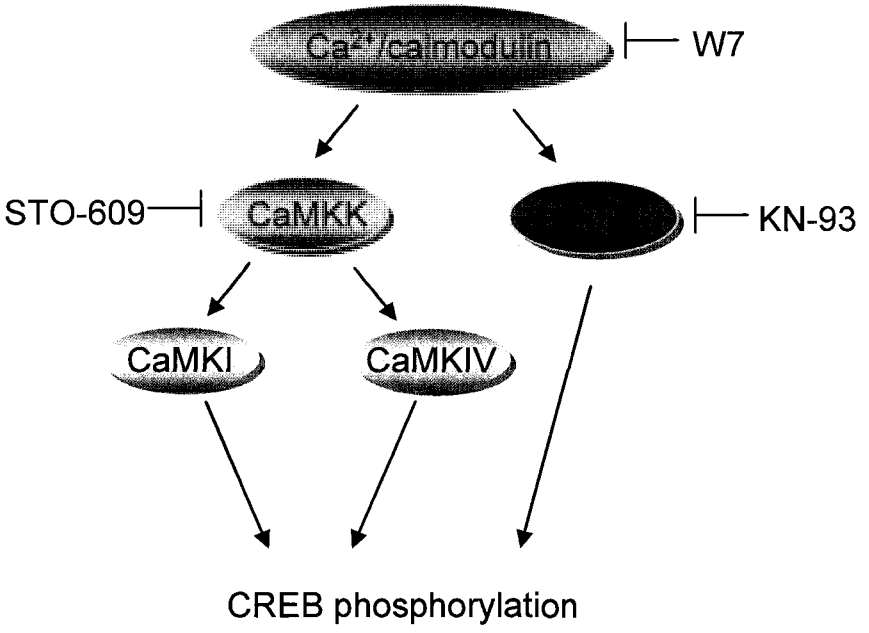


Figure 4.4. Schematic diagram of Ca^{2+}/ calmodulin signaling pathways involved in the CREB phosphorylation. Inhibitors which block specific kinase were indicated.

A.

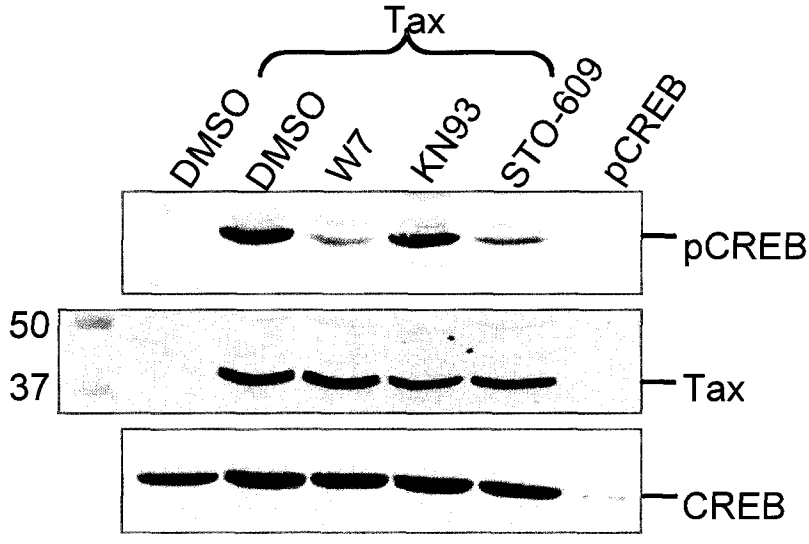


Figure 4.5. A specific CaM kinase inhibitor, STO-609, inhibits Tax-mediated CREB phosphorylation *in vitro* and *in vivo*. A. *Both W7 and STO-609 suppress Tax-mediated CREB phosphorylation in vitro. In vitro* kinase assays were performed as in figure 4.3A in the presence of DMSO or the following inhibitors; W7 (100 μM), KN-93 (10 μM), and STO-609 (1mg/ml), as indicated. Reactions were terminated by addition of 4X SDS sample buffer and analyzed by western blot.

B.

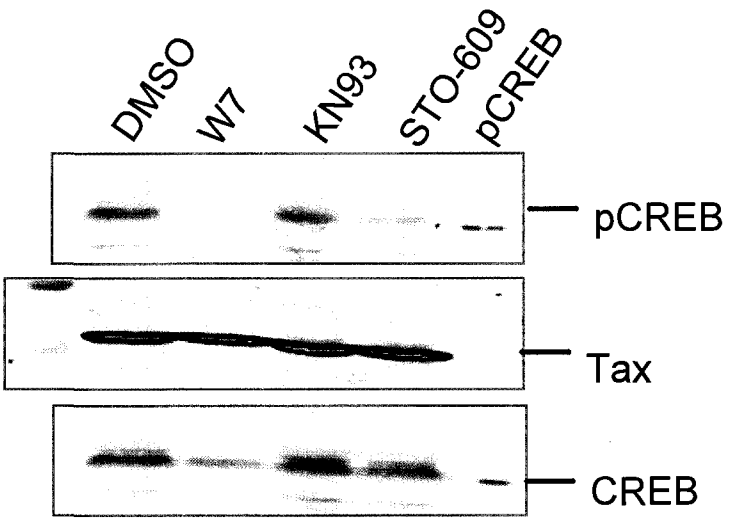


Figure 4.5B. *Both W7 and STO-609 suppress Tax- mediated CREB phosphorylation C8166 cells.* HTLV-1 infected C8166 cells were serum starved for 24 h and then treated with W7 (100 μM), KN-93 (10 μM), and STO-609 (1mg/ml) for 2hrs. CREB phosphorylation was measured by western blot analysis.

609, an inhibitor of CaMKK, significantly abrogated CREB phosphorylation in both assays. These results indicate that CaMKI and CaMKIV, and not CaMKII, are the CaMKK-dependent CaM kinase family members primarily responsible for Tax-mediated CREB phosphorylation.

4.3d STO-609, A SPECIFIC CAMKK INHIBITOR, INHIBITS TAX-MEDIATED VIRAL TRANSCRIPTION

CREB phosphorylation is required for quaternary complex formation at the HTLV-1 promoter as well as for Tax-mediated viral transcription (38). CaMKK is a part of the CaM kinase cascade and uniquely phosphorylates and activates CaMKI and CaMKIV, each of which directly phosphorylates CREB at Ser133. To confirm the importance of this pathway in Tax-stimulated CREB phosphorylation, we examined the effect of STO-609 on Tax activation of HTLV-1 transcription and NF-κB-dependent transcription. Jurkat cells were pretreated with STO-609 for 1h and then transfected with a reporter plasmid, in the absence or presence of Tax. After 24 hrs, cells were harvested and luciferase activity was measured. This CaMKK inhibitor dramatically reduced Tax transactivation from the HTLV-1 promoter (Fig. 4.6A). To examine whether the effect of STO-609 occurred mainly through the Tax-responsive elements, we used the vCRE-luciferase reporter plasmid, which contains three tandem copies of the vCRE immediately upstream of a core promoter (36) and obtained similar results (Fig. 4.6B). However, STO-609 did not affect Tax activation of the NF-κB pathway (Fig. 4.6C). These data

strongly suggest that Tax directly stimulates the CaM kinase pathway leading to CREB phosphorylation resulting in activation of HTLV-1 transcription.

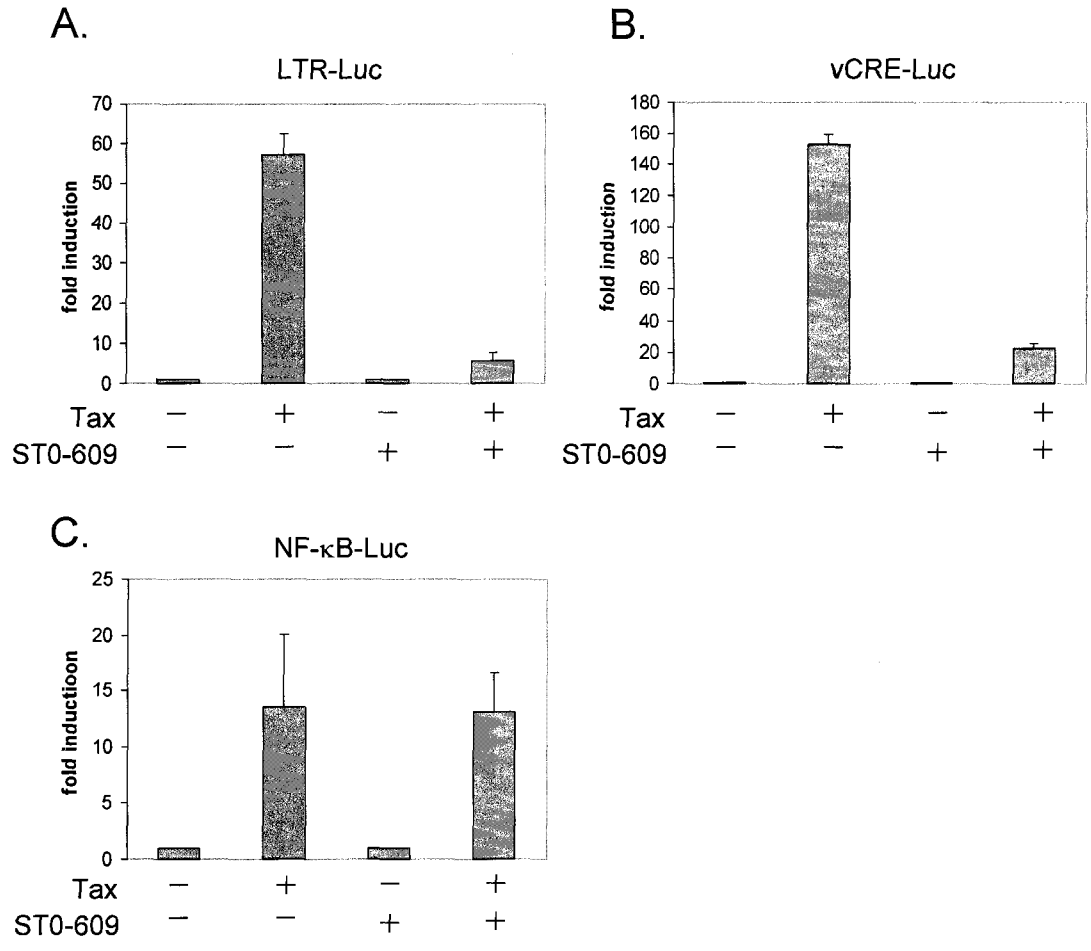


Figure 4.6. Transient transfection assays reveal that Tax transactivation of HTLV-1 requires the CaMKK pathway. Jurkat cells were serum-starved for 24 hr, treated with DMSO or STO-609 (10 mg/ml where indicated) 1hr prior to transfection, and transiently transfected using lipofectamine and 100 ng of one of the following reporter constructs: HTLV-1LTR (**A**), viral CRE (**B**), or NF-κB (**C**) in the absence or presence of Tax expression plasmid (100 ng). Cells were harvested 24 h after transfection and luciferase activity was measured.

4.4 DISCUSSION

Our studies indicated that the virus has evolved a Tax-dependent mechanism to promote CREB phosphorylation *in vivo*. The CREB phosphorylation is an essential partner for Tax-dependent CBP/p300 recruitment and transcriptional activation of HTLV-1. In this study, we confirmed that Tax is directly responsible for Ser133 CREB phosphorylation by performing *in vitro* kinase assays. Using these assays, we have also demonstrated that Tax stimulates a CREB kinase and does not inhibit a CREB phosphatase. This result indicates that Tax activates a cellular kinase pathway(s) that converge on Ser-133 phosphorylation of CREB.

To elucidate the kinase targeted by Tax, we used a variety of inhibitors that have been shown to block specific kinase pathways. We identified two inhibitors that reduced Tax-dependent stimulation of CREB phosphorylation. Surprisingly, these inhibitors, W7 and STO-609, both strongly implicate the Ca^{2+}/calmodulin kinase (CaMK) pathway. Both *in vivo* and *in vitro* kinase assays support this conclusion (Fig. 4.3A and B). Remarkably, the *in vitro* kinase inhibition assay fully mirrored the in vivo assay: i.) establishing a role for Tax in promoting elevated Ser133-phophorylated CREB levels, and importantly, ii.) implicating the Ca^{2+}/calmodulin pathway as the target of Tax in mediating CREB phosphorylation. Although the precise role of Ca^{2+} in Tax stimulation of the target CaM kinase is unknown at present, it is noteworthy that another HTLV-1-encoded protein, p12 has been shown to produce a sustained release of Ca^{2+} that leads to deregulated expression of a number of Ca^{2+} regulated genes. Our *in*

vitro data suggests that Tax is directly targeting the CaM kinase pathway. p12-induced Ca^{2+} release may contribute to the activity of the Tax-targeted kinase in HTLV-1 infected cells. Some other viral proteins also have been reported to induce CREB phosphorylation through activation of cellular signaling pathways. Hepatitis B virus (HBV) X protein mediates CREB phosphorylation by activating the RAS-RAF-MAPK pathway (205). Additionally, HIV Tat protein also stimulates Ser-133 CREB phosphorylation through a signaling cascade involving the MAPK pathway (206). It therefore appears that stimulation of cellular pathways leading to CREB phosphorylation is a common viral mechanism. Importantly, elevated levels of pCREB have been associated with enhanced tumorigenic potential (109, 150). Furthermore, CREB phosphorylation has been implicated in lymphocyte proliferation (207, 208).

CaMKK phosphorylates, and thus activates, the closely related kinases CaMKI and CaMKIV. These two downstream kinases directly phosphorylate CREB at Ser133. Each of these kinases is a candidate target for Tax stimulation of CREB phosphorylation. We have ruled out a role of the related CaMKII, as KN-93 had no effect on Tax-dependent phosphorylation. This is not surprising because CaMKII phosphorylates CREB at both Ser133 and Ser142, and Ser142 phosphorylation is inhibitory to CBP/p300 recruitment and transcription function (202).

Our studies suggest that the CaMKK cascade is important in Tax-stimulated HTLV-1 transcription. We found that the specific CaMKK inhibitor dramatically reduced Tax transactivation function specifically through the vCRE

of the viral promoter (Fig. 3.6). These data indicate that i) CREB phosphorylation is necessary for Tax to activate viral transcription, and ii) Tax stimulates constitutively elevated CREB phosphorylation through activation of the CaMKK cascade. Interestingly, CaMKIV has also been shown to activate serum response factor (SRF) via phosphorylation, inducing the release of HDAC4 (209). SRF has been shown to bind the HTLV1 promoter and upregulate vial expression (210), suggesting that stimulation of the CaM kinase pathway by Tax may result in synergistic stimulation of HTLV-1 transcription. Also, it is very plausible that Tax may affect cell function through enhanced phosphorylation of other CaM kinase substrates, such as CBP/p300, ATF-1, and SRF (211, 212).

In summary, these studies reveal a new function for Tax, the stimulation of CREB phosphorylation, leading to increased HTLV-1 transcription. Tax is also known to constitutively activate PI3 kinase, which may lead to enhanced cellular proliferation (143). Tax-mediated deregulation of multiple cellular signaling cascades may contribute to proliferation and transformation. Further characterization of the mechanism of Tax stimulation the CaM kinase pathway will provide much more comprehensive understanding of the molecular basis of Tax function as a transcriptional activator and deregulator of cell function.

4.5 MATERIALS AND METHODS

4.5a. CELL CULTURE, REAGENTS, AND ANTIBODIES

Jurkat T-cells and HTLV-1-transformed C8166 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin–streptomycin. Where indicated, C8166 cells were serum-starved by cultivation in the presence of 0.5% serum for 24 hrs and then incubated for 1 h with a variety of protein kinase inhibitors. The inhibitors staurosporine, H-89, wortmannin, W7, Ro-31-8220, SB203580, PD98059, KN-93, and STO-609, were all purchased from Calbiochem and were prepared in DMSO. Cells were lysed and resuspended in SDS sample dyes. Proteins were separated by 10% SDS-PAGE and analyzed by western blot. The antibodies used in the western blots presented herein were as follows: anti-CREB (C-21) and anti-phospho-Ser133 CREB (Santa Cruz Biotechnologies). An anti-Tax monoclonal antibody (National Institutes of Health AIDS Research and Reference Reagent Program) was also used for detecting Tax.

4.5b. TRANSIENT TRANSFECTION ASSAYS

Jurkat cells were co-transfected with Lipofectamine reagent (Invitrogen) and a constant amount of DNA. DMSO or STO-609 (10 μg/ml) was applied 2h prior to transfection and cells were harvested 24 h after transfection. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega) with a Turner Designs model TD 20-eluminometer. Luciferase activity was normalized to *Renilla* luciferase activity from the herpes simplex virus thymidine kinase (TK) promoter (pRL-TK; Promega). HTLV-1-Luc carries the HTLV-1

promoter driving the luciferase gene. NF-kB-Luc contains three tandem copies of the NF-kB response element immediately upstream of a core promoter The expression plasmid for Tax (pSG-Tax) (162) and the pminLuc–vCRE-Luc reporter plasmid (36) have been previously described. The transient transfection assay was performed in triplicate and repeated in three independent experiments.

4.5c. EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

Bacterially-expressed CREB (32) and $TaxHis_6$ (158) proteins were purified as previously described. 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. All proteins were purified to >95% homogeneity. 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.

4.5d. *IN VITRO* ASSAYS

Jurkat cells were starved by culturing in IMDM with 0.5 % FBS for 24 h before lysis for kinase assay. Cells were washed in cold PBS and lysed in 50 mM Tris buffer, pH7.5, 1 mM EDTA, 1mM EGTA, 0.5 mM Na3VO4, 50 mM sodium fluoride, 1 % Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride. The lysate was immediately submerged in liquid nitrogen for 2 minutes and stored at –80°C.

Before use, the lysate was thawed and clarified by centrifuging at 14,000 rpm for 2min. In our *in vitro* assay system, purified CREB protein (33 nM) was incubated with 30 μg of Jurkat whole cell extracts for 30 minutes at 30°C in the absence or presence of 1mM ATP and purified recombinant Tax-His_6 (12.5-100 nM). For *in vitro* phosphatase assay, purified recombinant PKA phosphorylated CREB (33 nM) was incubated with Jurkat whole cell extract prepared in lysis buffer (50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 % Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin) in the absence or presence of purified recombinant Tax-His_6 (12.5-100 nM) for 30 minutes at 30°C. The reactions were terminated by addition of 4x SDS sample buffer and analyzed by western blot with anti-Ser133-phospho-CREB antibody.

CHAPTER 5
THE PROTO-ONCOGENE BCL-3, INDUCED BY TAX, REPRESSES VIRAL TRANSCRIPTION VIA p300 DISPLACEMENT FROM THE HTLV-I PROMOTER

Chapter 5 describes a study of the mechanism of Tax transactivation of Bcl-3 and the effect of Bcl-3 on Tax-dependent HTLV-1 transcription. I performed the experiments shown in figures 5.1, 5.2A-D, 5.3A-B and 5.4A. This work has been submitted to the *Journal of Biological Chemistry* for publication. The citation for the manuscript is:

Kim, Y.M., Sharma, N, & Nyborg, J.K. (2008). The proto-oncogene Bcl-3, induced by Tax, represses viral transcription via p300 displacement from the HTLV-I promoter. submitted

5.1 ABSTRACT

The etiology of human T-cell leukemia virus type 1 (HTLV-1)-induced adult T-cell leukemia (ATL) is linked to the expression of the viral oncoprotein Tax. Although the mechanism of retroviral transformation is unknown, Tax interferes with fundamental cellular processes including proliferation and apoptosis, and these events may directly link Tax to early steps in malignant progression. In this study, we examined the potential interplay between Tax and the potent proto-oncogene B-cell chronic leukemia protein 3 (Bcl-3). Bcl-3 is a critical regulator of cell survival and proliferation. We detected elevated Bcl-3 transcript levels in HTLV-1 infected cells, and found that Tax induced Bcl-3 expression primarily through up-regulation of the NF-kB pathway. An intronic NF-kB binding site within the Bcl-3 gene served as the primary target of Tax-induced NF-kB activation. Unexpectedly, we found that the Bcl-3 protein forms a stable complex with Tax *in vivo* and *in vitro*. We further found that this complex represses Tax-dependent HTLV-1 transcription. To assess the mechanism of repression, we characterized transcription factor binding to an immobilized HTLV-1 promoter template. We found that Bcl-3 associates with the HTLV-1 promoter in a Tax-dependent manner, and inhibits the binding of the critical cellular coactivator p300. Together, these data suggest that Tax-induced Bcl-3 overexpression benefits the virus in two important ways. First, Bcl-3 may promote cell division and thus clonal proliferation of the virus. Second, Bcl-3 may attenuate virion production, facilitating immune evasion. A consequence of this regulatory loop may be Bcl-3-induced malignant transformation of the host cell.

5.2 INTRODUCTION

Human T-cell leukemia virus type-1 (HTLV-1) is a complex retrovirus etiologically linked to an unusually aggressive and ultimately fatal malignancy called adult T- cell leukemia (ATL). ATL is characterized by the presence of a chromosomally-integrated HTLV-1 provirus in highly aneuploid T-cells (5). However, the majority of HTLV-1 infected individuals remain lifelong asymptomatic carriers despite the fact that up to 70% of their CD4+ T-cells carry integrated provirus (30, 31). The molecular events that lead to Tax-dependent transformation of the HTLV-1 infected T-cell remain enigmatic.

The HTLV-1-encoded oncoprotein Tax is directly linked to malignant transformation by the virus. Tax is a strong transcriptional activator required for efficient expression of the viral genome. Tax activates HTLV-1 transcription through three conserved cyclic AMP response elements (CRE) located in the transcriptional control region of the virus. These elements, called viral CREs (vCRE) serve as the binding site for a complex composed of Tax and the cellular transcription factor CREB, which together recruit the cellular coactivators CBP/p300 (34, 37, 144, 213). Serine 133 phosphorylation of CREB (pCREB) is essential for strong p300 recruitment by the Tax/CREB complex, which is required for transcriptional activation by Tax (213). Interestingly, Tax promotes CREB phosphorylation in the infected cell, apparently to ensure sufficient pCREB availability for Tax activation of viral gene expression (38, 156, 157).

Mitotic replication has been established as a major vehicle of viral transmission within an infected individual. The Tax protein is required for the

promotion of clonal proliferation, and is directly linked to malignant transformation (19). Tax triggers changes in a variety of intracellular signal transduction pathways and deregulates gene expression though interaction with many different cellular proteins (214). For example, Tax-mediated activation of the NF-κB pathway results in the upregulation of a large number of genes implicated in proliferation and survival (52). Tax achieves constitutive activation of the NF-κB pathway via several mechanisms, including activation of IκB kinase (IKK) and inactivation of NF-κB inhibitor, IκB members such as IκBα and IκBβ (52).

Bcl-3 (B-cell lymphatic leukemia protein 3) is a member of the IκB family of NF-κB inhibitors and was initially identified as a putative proto-oncogene from chronic B-cell lymphocytic leukemia (215). Typically, increased Bcl-3 expression levels result from chromosomal translocations (216, 217), and lead to increased cell survival, proliferation, and malignant potential (218). For example, high levels of Bcl-3 were observed in over 90% of nuclei from hepatocarcinoma cells (219). There is compelling evidence that Bcl-3 overexpression is sufficient to enhance tumorigenic potential in many cell types, including T-cells (220, 221). Unlike other IκB family proteins, Bcl-3 is a predominantly nuclear protein and contains a transactivation domain. Studies have shown that Bcl-3 preferentially binds to p50 or p52 NF-κB homodimers and functions as a transcriptional activator or repressor via NF-κB elements (222-229). Additionally, Bcl-3 physically interacts with coactivators such as CBP/p300, Tip60, and TORC3, as well as transcriptional repressors such as the histone deacetylase complexes (230-233). These data are consistent with a model of malignant transformation whereby

overexpressed Bcl-3 inappropriately activates specific target genes that promote cell proliferation and survival.

The strong correlation between elevated Bcl-3 expression and leukemias, lymphomas, and other types of cancers led us to investigate whether Tax may deregulate this important proto-oncogene. Previous studies have demonstrated a correlation between Tax expression and elevated Bcl-3 transcript levels (132, 234). In this study, we found that Tax transcriptionally upregulates Bcl-3 expression, mediated primarily through the NF-kB site located in the second intron of the Bcl-3 gene. Unexpectedly, Bcl-3 formed a stable complex with Tax both *in vivo* and *in vitro*. Consistent with this observation, we found Bcl-3-dependent repression of Tax transactivation of HTLV-1, specifically mediated through the vCREs. Using the immobilized template assay to specifically address the mechanism of Bcl-3 repression, we found that Bcl-3 stably associated with the Tax/pCREB complex on the viral promoter. Unexpectedly, Bcl-3 binding correlated with significantly reduced recruitment of full-length p300, providing a molecular mechanism for the observed Bcl-3 repression of Tax-dependent HTLV-1 transcription. Notably, these data reveal a tightly-regulated interrelationship between Tax and the proto-oncogene Bcl-3 that may have implications for both survival of the retrovirus and transformation of the infected host T-cell.

5.3 RESULTS

5.3a BCL-3 TRANSCRIPT LEVELS ARE ELEVATED IN HTLV-1-INFECTED CELL LINES

We began our study by examining Bcl-3 expression levels in HTLV-1-infected, Tax expressing cell lines. Two previous studies have correlated elevated Bcl-3 RNA levels in the presence of Tax (132, 234). We performed reverse transcriptase-polymerase chain reaction (RT-PCR) and compared Bcl-3 transcript levels in HTLV-1 infected SLB-1 and C8166 cells to transcript levels in uninfected Jurkat and CEM human T-cells. Figure 5.1 shows elevated Bcl-3 RNA levels in HTLV-1 infected cells, relative to the uninfected cells. These data correlated with elevated Bcl-3 protein levels in these cells (data not shown).

5.3b TAX INDUCES BCL-3 EXPRESSION THROUGH AN INTRONIC ENHANCER ELEMENT

To determine the regions of the Bcl-3 gene that confer Tax responsiveness, we performed transient transfection assays using luciferase reporter plasmid constructs carrying the Bcl-3 promoter region (-1700 to +212, relative to the transcription start site), or the promoter region together with two previously defined enhancer sequences (235) (Fig. 5.2A). The HS3 and HS4 enhancer elements were originally defined based on their conservation between species and hypersensitivity to DNase I (235). Both the HS3 and HS4 hypersensitive sites are located in the second intron of the Bcl-3 gene. Jurkat T-cells were cotransfected with each of the Bcl-3 constructs in the absence or presence of an expression plasmid for Tax (pGS-Tax). Figure 5.2B shows that

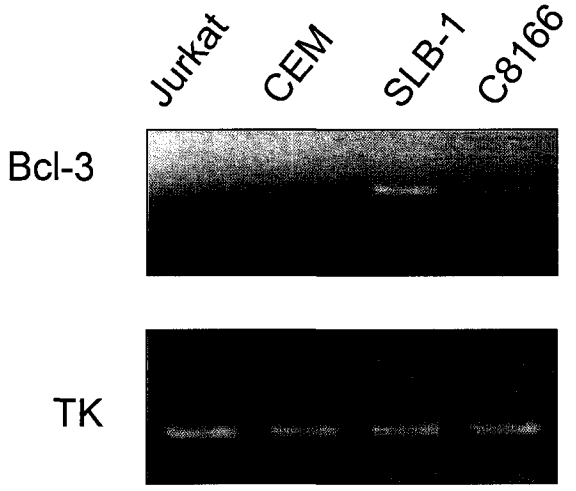


Figure 5.1. Bcl-3 transcript levels are elevated in HTLV-1 infected T-cell lines. Bcl-3 RNA from uninfected (CEM, Jurkat) and HTLV-1 infected (SLB-1, C816645) cells was amplified by RT-PCR and subjected to agarose gel electrophoresis to determine relative transcript levels. Thymidine kinase (TK) levels are shown as an internal control.

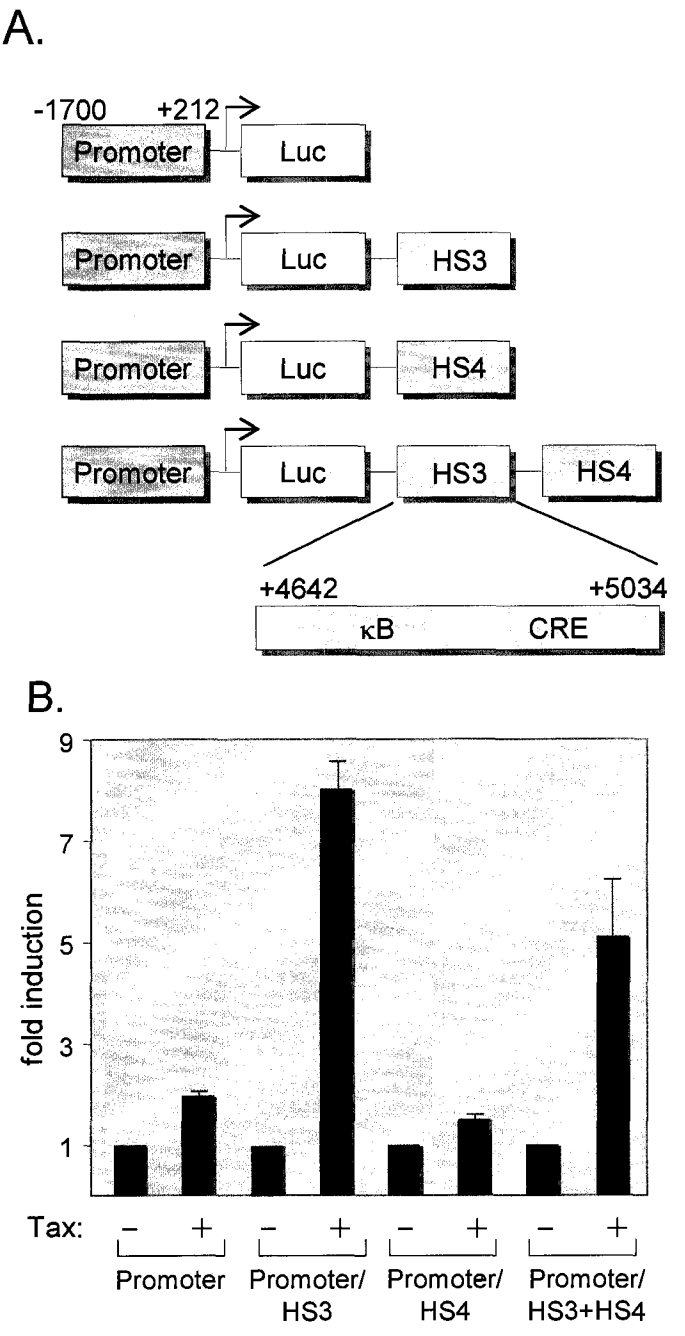


Figure 5.2. Tax mediates Bcl-3 induction by intronic enhancer. **A.** *Schematic diagram of the luciferase reporter containing the Bcl-3 promoter either alone or in combination with enhancer HS3, HS4 and HS3/4.* The presence of NF-kB and CREB binding site in HS3 is indicated. **B.** *Tax transactivates Bcl-3 through the enhancer HS3.* Jurkat cells were cotransfected with the indicated reporter constructs in the absence or presence of a Tax expression plasmid (pSG-Tax).

Tax only modestly activated Bcl-3 expression through the upstream promoter, however, inclusion of the HS3 region from the second intron conferred Tax-responsiveness to the Bcl-3 reporter construct.

5.3c TAX INDUCES BCL-3 EXPRESSION PRIMARILY VIA ACTIVATION OF THE NF-KB PATHWAY.

The Tax-responsive Bcl-3 HS3 region carries both conserved NF-kB and ATF/CREB recognition elements (Fig. 5.2A). Tax has previously been shown to activate transcription through both of these transcription factor pathways, although Tax activation of cellular genes through the NF-κB pathway is the most prevalent (236). To determine which pathway was utilized by Tax in the activation of Bcl-3 transcription, we performed transient transfection assays using the mutant Tax expression plasmids M22 and M47. M22 Tax is defective for activation through the NF-κB pathway, whereas M47 Tax is defective for activation of HTLV-1 transcription via the vCREs (237). Figure 5.2C shows that Tax mutant M22 was defective for the activation of Bcl-3 transcription through the HS3 region (and to a lesser extent through the upstream Bcl-3 promoter). Tax mutant M47 had no effect on expression through the upstream promoter or the promoter/intronic enhancer construct. These data indicate that Tax stimulation of the NF-kB pathway is the primary route for upregulation of Bcl-3 transcription. To further confirm that the NF-kB pathway was responsible for Tax induced expression of Bcl-3, we performed a parallel experiment testing Bcl-3 reporter constructs carrying mutations in the kB and CRE transcription factor recognition

C.

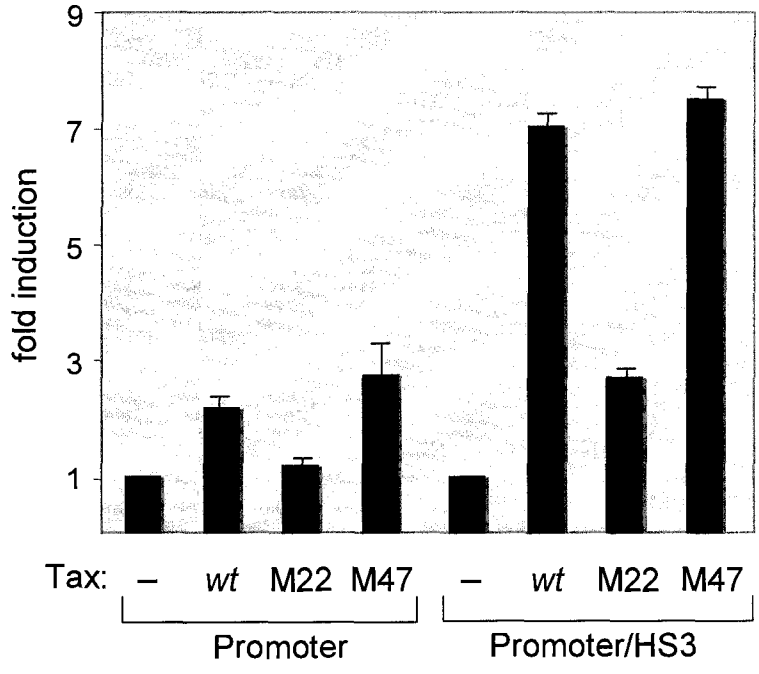


Figure 5.2C. *M22 is defective for Tax transactivation of Bcl-3.* Jurkat cells were transiently transfected with the reporter constructs in the absence or presence of expression plasmids for wild-type Tax (pSG-Tax), or the M22 or M47 mutants (pSG-M22, pSG-M47), as indicated. Luciferase activity was measured 24 h after transfection and fold-induction was quantified. The graph represents the average of three independent experiments.

sites (235). Figure 5.2D shows that the NF-kB site in the second intron is the primary element responsible for Tax induction of the Bcl-3 gene. Mutation of the CRE had an approximate two-fold repressive effect on Bcl-3 expression.

5.3d TAX AND BCL-3 FORM A STABLE COMPLEX BOTH *IN VITRO* AND *IN VIVO*

As a member of the IκB family, the Bcl-3 protein contains seven repeats of the ankyrin domain. The ~33 amino acid repeat is a tandem array of α-helices responsible for mediating protein-protein interactions. Since Tax has been previously shown to bind the ankyrin repeats of IκBα (238), we were interested in testing whether Tax interacts directly with Bcl-3. To investigate this possibility, we first performed GST pull-down assays using bacterially-expressed GST-Bcl-3 (230). Purified GST-Bcl-3 was bound to glutathione-agarose beads and incubated with purified recombinant Tax. Figure 5.3A shows that Tax bound to GST-Bcl-3 but not to GST alone. This result indicates that Tax and Bcl-3 directly interact *in vitro*. To determine whether Bcl-3 interacts with Tax *in vivo*, we performed co-immunoprecipitation assays. Because the commercially available antibody against Bcl-3 was inadequate for most analyses, we co-transfected 293T cells with expression plasmids for Tax and flag-tagged Bcl-3. Whole-cell extracts prepared from transfected 293T cells were immunoprecipitated with an anti-flag antibody, and the presence of Tax in the complex was analyzed by western blot. Figure 5.3B show that Tax was co-immunoprecipitated with Bcl-3,

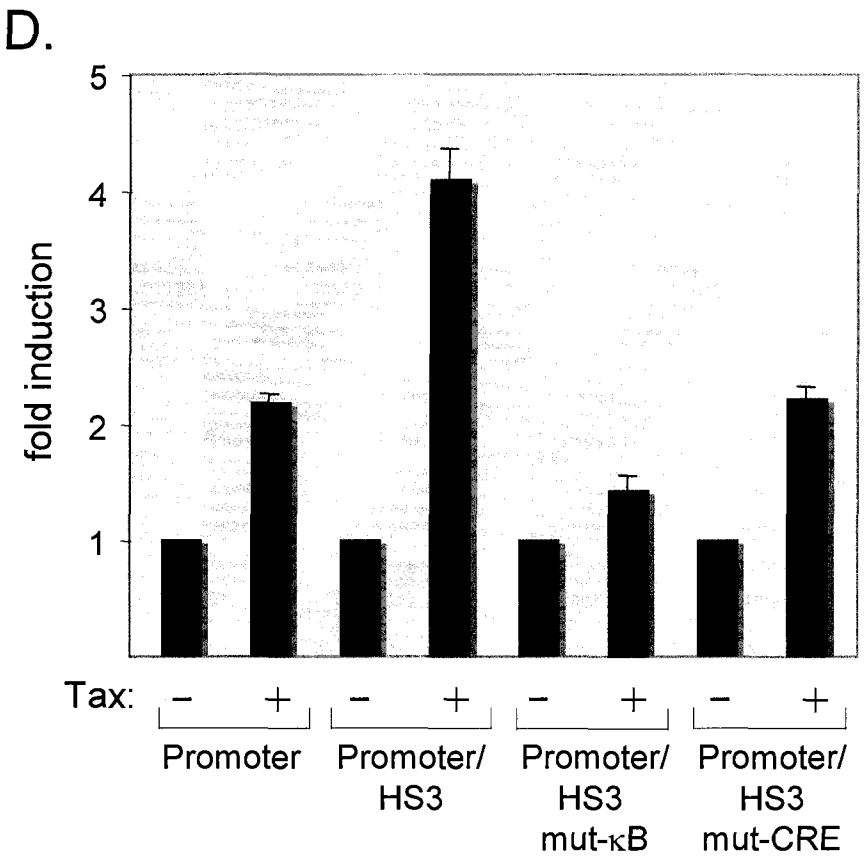


Figure 5.2D. *Tax requires NF-κB binding site in enhancer HS3 for transactivation of Bcl-3.* Jurkat cells were cotransfected, in the absence or presence of a Tax expression plasmid, with the Bcl-3 promoter/HS3-Luc constructs carrying mutations in the κB or CRE sites, as indicated. Luciferase activity was measured 24 h after transfection and fold-induction was quantified. Averages from three independent experiments are presented.

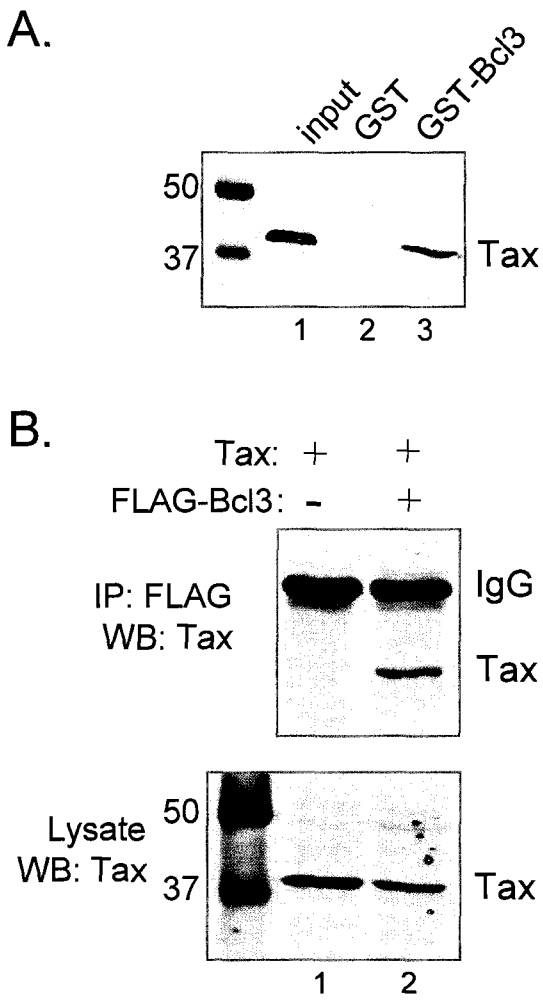


Figure 5.3. Tax and Bcl-3 interact in a stable complex. A. *GST pull-down assay reveals that Tax and Bcl-3 interact in vitro.* Recombinant purified Tax (40 pmol) was incubated with GST (10 pmol) or GST-Bcl-3 (10 pmol) immobilized on glutathione agarose beads. Bound complexes were washed and analyzed by western blot. Tax binding was detected using an anti-Tax antibody (upper panel) and GST-Bcl-3 was detected using an anti Bcl-3 antibody (lower panel). **B.** *Co-immunoprecipitation assay reveals that Tax and Bcl-3 are present in a complex in vivo.* 293T cells were transfected with pSG-Tax (3μg) in the absence or presence of pCMV2-FLAG-Bcl-3 (3μg). Twenty-four hours after transfection, proteins were immunoprecipitated from whole cell lysates with an anti FLAG antibody and analyzed by western blotting with an anti-Tax antibody.

indicating that Tax and Bcl-3 interact *in vivo*. The reciprocal co-immunoprecipitation experiment confirmed the interaction (data not shown).

5.3e BCL-3 EXPRESSION REDUCES TAX-TRANSACTIVATION THROUGH THE VCRE

The observation that Tax and Bcl-3 interact both *in vitro* and *in vivo* raised the question as to whether Bcl-3 binding to Tax might affect Tax transcription function. A recent study demonstrated that Bcl-3 expression reduced HTLV-1 transcription through interaction with the CREB coactivator TORC3 (234). We performed luciferase assays that measured Tax-dependent transcription from a chimeric promoter carrying the three tandem copies of the viral CREs upstream of a core promoter (36). This construct enabled analysis of transcriptional effects mediated through the vCREs (and thus Tax), without contribution from other regions of the HTLV-1 promoter. Jurkat cells were co-transfected with expression plasmids encoding Tax and Bcl-3, together with the vCRE-luciferase reporter plasmid. After 24 hrs, cells were harvested and luciferase activity was measured. Figure 5.4A shows that Bcl-3 potently suppressed Tax-dependent transcription from the viral CRE promoter in a dose-dependent manner. We obtained similar results with the natural HTLV-1 promoter/luciferase construct (data not shown). To investigate the effect of Bcl-3 on Tax transactivation *in vitro*, we utilized a fragment carrying the natural HTLV-1 promoter (Fig. 5.4B). A biotin group was incorporated at the upstream end of the DNA, and the fragment was immobilized on magnetic streptavidin-agarose beads. The HTLV-1 promoter template was

A.

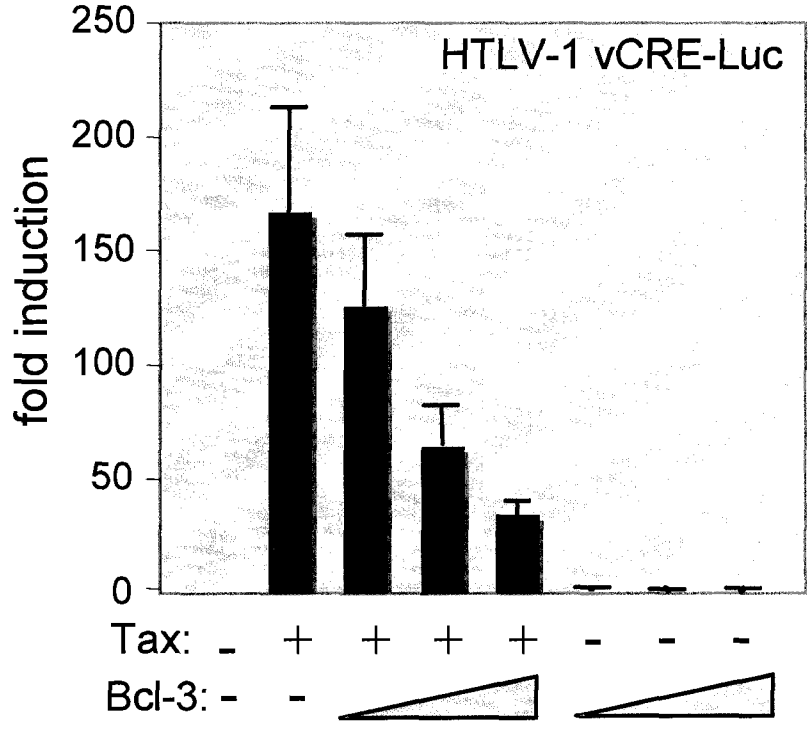


Figure 5.4. Bcl-3 inhibits Tax transactivation *in vivo* and *in vitro*. **A**. HTLV-1-negative Jurkat T-cells were co-transfected with the vCRE-luc reporter plasmid (100 ng), and a Tax expression plasmid (pSG-Tax, 100 ng), or Bcl-3 expression plasmid, as indicated. Transient transfections were performed in triplicate, and each experiment was repeated three times.

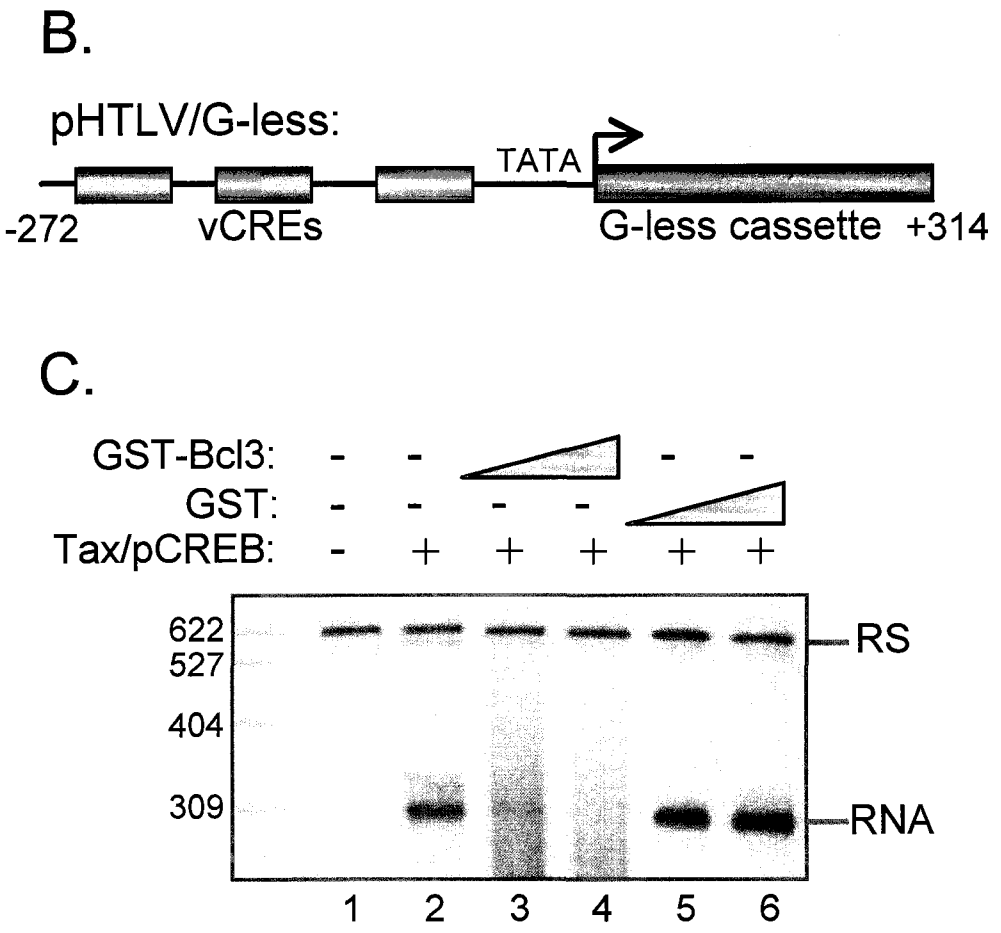


Figure 5.4B. *Schematic representation of the HTLV-1 promoter templates used in the in vitro transcription reaction.* **C.** *In vitro transcription assay showing Bcl-3 inhibition of Tax/pCREB-dependent activation.* The immobilized HTLV-I template fragment was assembled into chromatin, then preincubated with purified, recombinant pCREB, Tax, GST-Bcl-3, or GST, followed by incubation with nuclear extract (30 µg) from the HTLV-I-negative human T-lymphocyte cell line CEM, as indicated. The positions of the recovery standard, the transcript, and radiolabeled DNA markers are indicated. This experiment was performed by N. Sharma.

assembled into chromatin, followed by preincubation with pCREB, Tax, and increasing concentrations of GST-Bcl-3 (or GST alone as a control). The reactions were then supplemented with T-cell nuclear extract (HTLV-1-negative CEM cells) and assayed by *in vitro* transcription. Figure 5.4C shows that the addition of Bcl-3, as compared to GST alone, strongly repressed Tax/pCREB activation in a dose-dependent manner. These data corroborate the findings from the luciferase assays.

5.3f. BCL-3 INHIBITS TAX TRANSACTIVATION THROUGH BLOCKED RECRUITMENT OF p300

To investigate the molecular basis for Bcl-3 inhibition of Tax transcription function, we analyzed the interactions of purified proteins on an immobilized promoter template carrying four tandem copies of the vCREs (p4TxRE/G-less) (176). Purified, recombinant Tax, pCREB and Bcl-3 proteins were pre-incubated with the DNA template, followed by incubation with purified full-length p300 (213). The templates were washed, and the bound proteins were analyzed by western blot. Figure 5A shown that purified p300 associated with the Tax/pCREB complex bound to the template (lane 5). This observation is in agreement with the requirement for both Tax and pCREB for efficient recruitment of p300 and strong Tax transactivation (213). Consistent with the binding data presented in figure 3, Bcl-3 associated with the vCRE template only in the presence of Tax (Fig. 5.5A, lane 4). Unexpectedly, titration of Bcl-3 into the reactions containing Tax and pCREB significantly reduced the binding of p300 to the immobilized

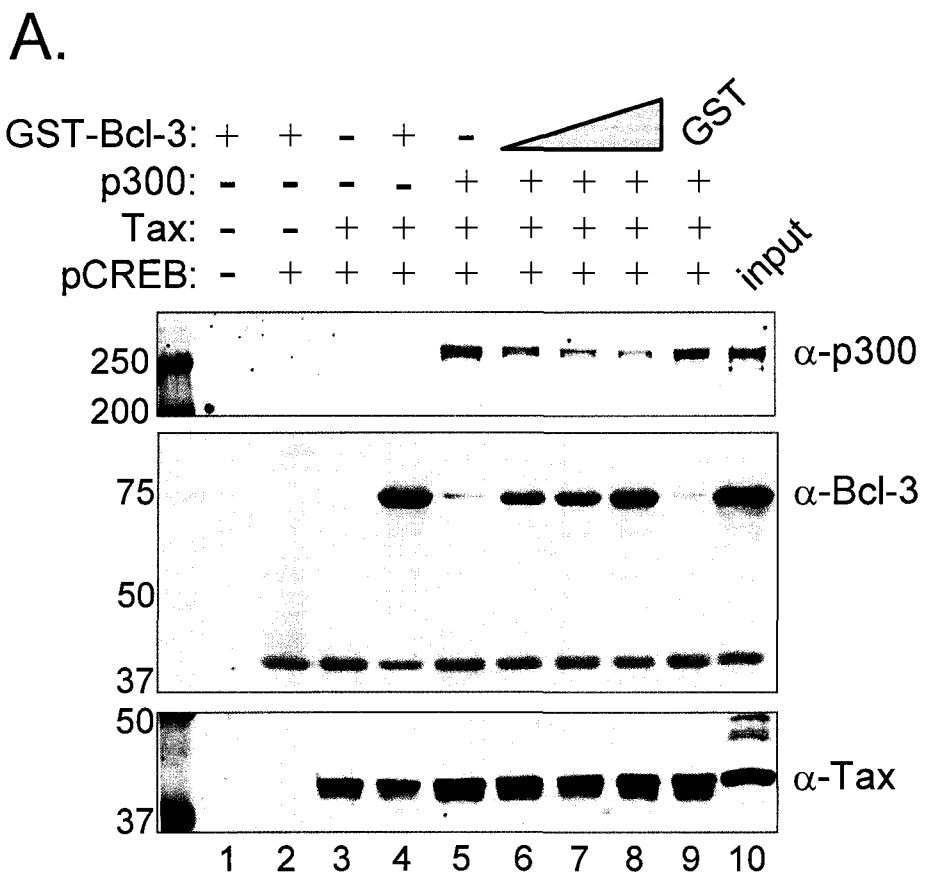


Figure 5.5. Tax and Bcl-3 form a complex on the vCREs and inhibit p300 recruitment. A. A 643 bp promoter fragment amplified from p4TxRE/G-less template was incubated with increasing amount of GST-Bcl-3 in the presence of pCREB, Tax, and p300 as indicated. GST alone was used as a negative control. Bound complexes were washed, and DNA-bound proteins were separated on a 10% SDS-polyacrylamide gel and analyzed by western blot. The band that appears to comigrate with GST-Bcl-3 is a truncation product of p300. This experiment was performed by N. Sharma.

template, with a concomitant increase in Bcl-3 binding (Fig. 5.5A, lanes 6-8). Bcl-3 binding also reduced Tax association with the template, however, to a lesser extent than that observed for p300 (Fig. 5.5A, lane 4). This observation suggests that Bcl-3 may concomitantly reduce the affinity of Tax at the vCRE. Essentially identical results were obtained using an immobilized template carrying the natural HTLV-1 promoter (data now shown). We next examined whether Bcl-3 similarly displaced native p300 from the HTLV-1 promoter template. We performed the DNA pull-down experiment as described above, except that the immobilized fragment was incubation with T-cell nuclear extract. Figure 5.5B shows that Bcl-3 also reduced native p300 binding similar to that observed with the full-length recombinant protein.

5.4 DISCUSSION

Expression of the Tax protein is directly responsible for transformation of HTLV-1-infected cells. Compelling evidence suggests that the oncogenicity of Tax is linked to its ability to promote cell survival and proliferation. This is accomplished through the deregulation of cellular gene expression and through protein-protein interactions with critical host-cell regulatory molecules. In this study, we detected elevated expression of the proto-oncogene Bcl-3 in HTLV-1-infected cells, consistent with previous studies (132, 234). We found that Tax activates Bcl-3 transcription primarily through the NF-kB site located in the intronic enhancer. Bcl-3 is expressed in many different tissue types, most notably the spleen and other lymphoid organs (239). In both B-cells and T-cells,

B.

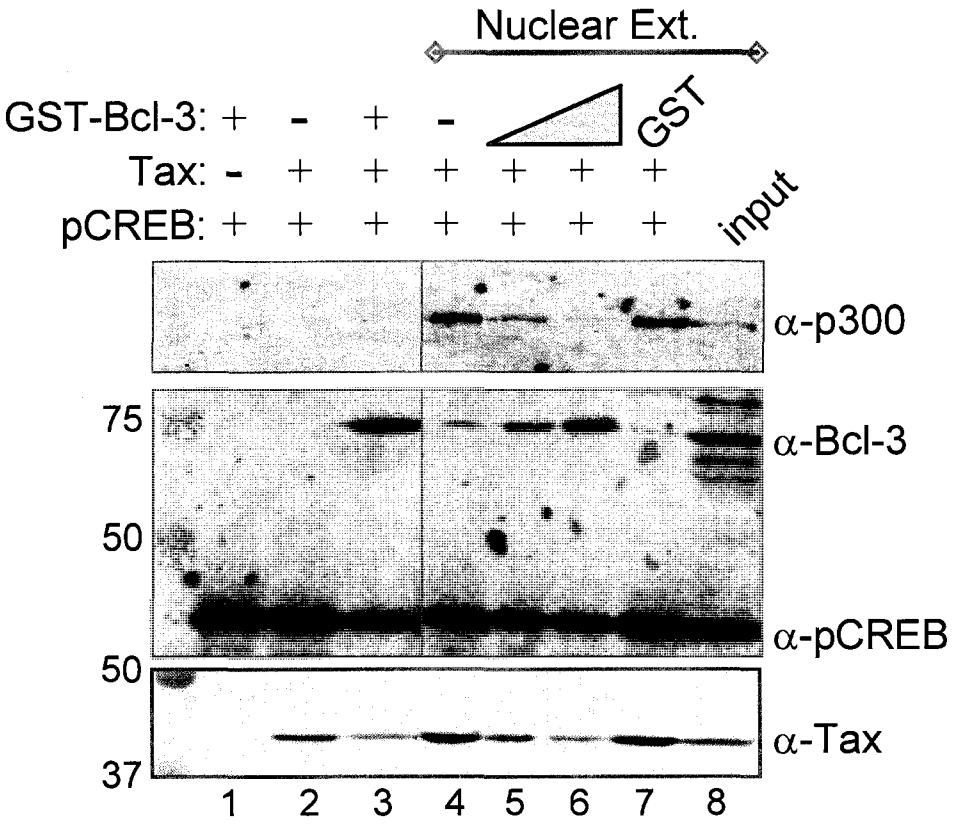


Figure 5.5B. The immobilized template assay was performed as describe in panel A, except that CEM nuclear extract was used in place of p300. This experiment was performed by N. Sharma.

mitogenic stimuli induces Bcl-3 expression (216). Evidence supporting a role for Bcl-3 in human T-cell malignancies includes the findings that Bcl-3 overexpression enhances the survival of activated T-cells and that elevated levels of Bcl-3 have been detected in many lymphoid cancers (220, 221, 240). The link between Bcl-3 overexpression and malignant transformation may stem from the transcriptional activation properties of Bcl-3 on the cell cycle regulator cyclin D1 (224). Cyclin D1 expression is strongly correlated in the development and progression of many cancers. For example, a recent study demonstrated that enforced overexpression of cyclin D1 directly facilitates malignant transformation by promoting genomic instability (241). Bcl-3 also suppresses p53 activation in response to DNA damage (242). It is noteworthy that Tax has also been shown to increase the expression of cyclin D1 as well as inhibit the function of p53 (79, 116). These observations suggest that together, Tax and Bcl-3 may contribute to T-cell transformation and the development of ATL.

We also made the unexpected observation that Tax interacts with Bcl-3 both *in vivo* and *in vitro*. We hypothesized that the existence of a stable Tax-Bcl-3 complex may alter Tax function. Consistent with this, we found that expression of Bcl-3 strongly inhibited Tax activation of HTLV-1 transcription, in agreement with a recently published study (234). We further investigated the potential mechanism of Bcl-3 inhibition of Tax transcription function using the immobilized template assay. Unexpectedly, we found that Bcl-3 bound to the Tax/pCREB complex, and blocked recruitment of the critical coactivator p300. A model depicting this form of regulation is shown in figure 5.6. The dramatic reduction in

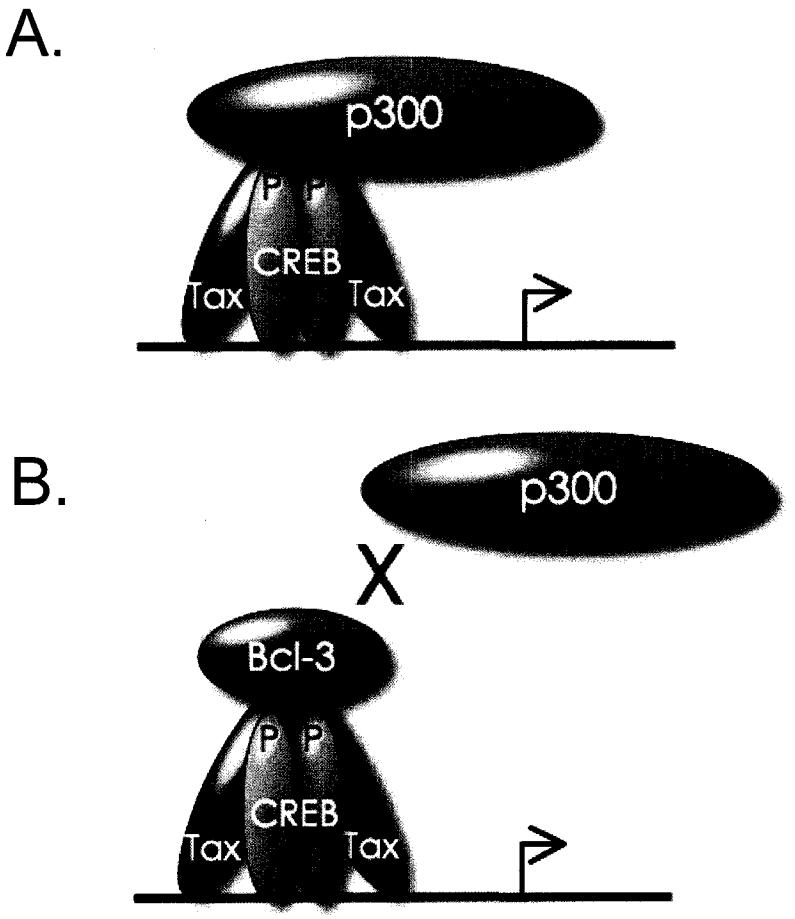


Figure 5.6. Schematic showing Bcl-3 inhibition of p300 recruitment to the HTLV-1 promoter. For simplicity, only one of the three vCREs is shown.

p300 binding likely contributes to the observed Bcl-3 inhibition of Tax transactivation of HTLV-1. This is consistent with a study from our laboratory that demonstrated a vital role for CBP/p300 in Tax-mediated HTLV-1 transcription (213). Interestingly, a recent study demonstrated Bcl-3 repression of TORC3-dependent HTLV-1 transcription (234). TORC3 is a regulator of CREB transcriptional activity (191). Models in which Bcl-3 interacts with both Tax and TORC proteins are not mutually exclusive, and suggest that Bcl-3 may target both CREB and Tax to ensure potent repression of transcription from the viral promoter. We speculate that the Tax/Bcl-3 complex may alter the transcription of additional Tax or Bcl-3 regulated cellular genes.

In summary, these data establish that Tax expression leads to Bcl-3 overexpression via activation of the NF-kB pathway. The Bcl-3 protein then associates with the promoter-bound Tax/pCREB complex to block recruitment of the essential coactivator p300, thus repressing viral transcription. We propose that this interrelationship has the following consequences in an HTLV-1 infected cell: First, Tax-induced overexpression of Bcl-3 promotes cell proliferation via the transcriptional up-regulation of cyclin D1 and/or other key regulators of cell division. Second, Tax-induced overexpression of Bcl-3 results in down-regulation of HTLV-1 gene expression via inhibition of CBP/p300 recruitment to the promoter. While initially these observations are seemingly incongruous, it is conceivable that these two Bcl-3-dependent events endow a net evolutionary benefit to the virus: enhanced mitotic replication of the provirus concomitant with immune evasion that contributes to survival of the retrovirus.

5.5 MATERIALS AND METHODS

5.5a CELL CULTURE

Both HTLV-1 infected (SLB-1, C8166) and uninfected (Jurkat, CEM) human T-cell lines were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin. CEM Nuclear extracts were prepared as previously described (193). Immortalized human 293T kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin.

5.5b ANTIBODIES

The following antibodies were used in western blots: anti-Bcl-3 (C-14), anti-phospho ser^{133}CREB (sc-7978-R), anti-p300 (N-15). All were purchased from Santa Cruz Biotechnology. A monoclonal Tax antibody (Hybridoma 168B17-46-92) was obtained from the National Institutes of Health Aids Research and Reference Reagent Program. Antibody against FLAG (M2, F9291) was purchased from Sigma. Alexa Fluor IR700 and IR800 goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Molecular Probes.

5.5c EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

Bacterially-expressed Tax-His_6 (158) was purified to >98% homogeneity as previously described (36). $CREB_{327}$ was purified to apparent homogeneity,

and free of contaminating nucleic acids, as recently described (194). Full-length His_6-tagged p300 was expressed from recombinant baculovirus in Sf9 cells and purified as previously described (159, 213). GST-Bcl-3 (230) was expressed in *E. coli* and purified by glutathione-agarose chromatography. 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.

5.5d RNA ISOLATION AND RT-PCR

Cytoplasmic RNA was isolated from cultured cells using a cytoplasmic RNA reagent (Invitrogen) to avoid DNA contaminants, according to the manufacturer's instructions. The mRNA (2 μg) was reverse-transcribed using the Superscript II reverse transcriptase (Invitrogen) with oligo (dT) and dNTP for 2hrs at 42°C. The cDNA was used as a template to amplify a 560-bp Bcl-3 fragment using the upstream primer 5'-TCA AGA ACT GCC ACA ACG ACA C-3' and the downstream primer, 5'-CTG GGG TCA GAG TCC TGG GAG-3'. As an internal control, a 220-bp thymidine kinase (TK) cDNA fragment was amplified using the upstream primer 5'-GAG TAC TCG GGT TCG TGA AC-3' and the downstream primer, 5'-GGT CAT GTG TGC AGA AGC TG-3'.

5.5e TRANSIENT-TRANSFECTION ASSAYS

Human Jurkat T-cells were transfected with a constant amount of DNA using Lipofectamine reagent (Invitrogen). After 24 hr, the cells were harvested and lysed, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) with a Turner Designs model TD 20-e luminometer. Firefly luciferase activity was normalized to *Renilla* luciferase activity from the herpes simplex virus thymidine kinase (TK) promoter (pRL-TK; Promega). The Bcl-3-luciferase constructs carrying various Bcl-3 promoter and enhancers regions (235) were gifts from Dr. McKeithan (University of Nebraska Medical School). The expression plasmid for Tax (pSG-Tax) (162), Bcl-3 (CMV-FLAG-Bcl-3) (224), and the vCRE-Luc reporter plasmid (36) have been previously described. The transient transfection assay was performed in triplicate and repeated in three independent experiments.

5.5f GST PULL-DOWN ASSAYS

GST pull-down experiments were performed as previously described (161). Briefly, 20 µl of glutathione-agarose beads were equilibrated in 0.5X Superdex buffer (25 mM HEPES [pH 7.9], 12.5 mM $MgCl_2$, 10 µM $ZnSO_4$, 150 mM KCl, 20% [vol/vol] glycerol, 0.1% Nonidet P-40, and 1 mM EDTA). Purified GST (10 pmol) or GST-Bcl-3 (10 pmol) were incubated with the beads for 1 to 2 h at 4°C and then washed with 0.5X Superdex buffer. His-tagged purified Tax (40 pmol) protein was then added to the washed beads and incubated for overnight at 4°C. The beads were washed as before, and bound proteins were

eluted with SDS sample dyes. Bound proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose for subsequent Western blot analysis.

5.5g CO-IMMUNOPRECIPITATION ASSAYS

293T cells were transfected with a constant amount of DNA using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Cells were transfected with expression plasmids for Tax (pSG-Tax) and Bcl-3 (CMV-FLAG-Bcl-3)(224) as indicated in the experiment. Total cell lysates were prepared in 1% Triton X-100/phosphate buffered saline (PBS) buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4), and protease inhibitors (2 mM benzamidine, 2 μg/mL leupeptin, 2 μg aprotinin, and 1 mM PMSF). Proteins were immunoprecipitated using anti-FLAG antibodies with protein A/G agarose beads (Santa Cruz Biotechnology) overnight at 4°C, and washed several times in 1X PBS buffer. The bound proteins were analyzed on a 10% SDS-polyacrylamide gel and detected by Western blotting with anti-Tax (hybridoma 168B17–46-92) antibody.

5.5h *IN VITRO* TRANSCRIPTION ASSAY

The promoter fragment was amplified from the plasmid, pHTLV-1/G-less, which carries the natural HTLV-1 promoter driving expression of a G-less cassette (176). Primers for PCR amplification of HTLV-1/G-less, top strand: 5'-Bio/GTC TGA AAA GGT CAG GGC C-3' and 5'-GGA TAT ATG AGA TGA GTA

GG-3'. This PCR-amplified fragment carried the HTLV-1 promoter from -272 through the transcription start site and 314 bp into the G-less cassette. The promoter fragment was immobilized on streptavidin-agarose beads (Dynabeads® Dynal Biotech USA) via a biotin group incorporated at the upstream end of the promoter. The immobilized template was assembled into chromatin using native *Drosophila* histones as previously described (213). The template (75 fmol) was pre-incubated for 20 min at 30°C in the absence or presence of Tax and/or pCREB (600 fmol), and GST-Bcl-3, or GST alone (approximately 1.25 and 2.5 pmol) and in a 30 μl reaction. CEM nuclear extract (30 μg) and acetyl CoA (100μM) was added to each reaction and incubated an additional 30 minutes at 30°C. The transcription reactions were carried out as previously described (213). Molecular weight markers (radiolabeled HpaII-digested pBR322) were used to estimate the size of the RNA products. A labeled 622 bp DNA fragment was added to each reaction as a recovery standard.

5.5i IMMOBILIZED TEMPLATE ASSAY

A 643 bp promoter fragment was amplified by PCR from the 4TxRE/G-less plasmid template that carries 4 reiterated copies of the HTLV-1 promoter proximal vCRE cloned upstream of the HTLV-1 core promoter (176). The promoter fragment was immobilized on Dynabeads®. Binding reactions were pre-incubated for 20 minutes at 30°C in the presence of Tax and pCREB (1 pmol each). Recombinant GST-Bcl-3 (2.5, 5, 10 pmol), GST alone (10 pmol), and p300 (1 pmol) were added for an additional 30 min. Binding reactions were

carried out as previously described (213) for details. Nuclear extract (100 μg), prepared from the uninfected human T-cell line CEM, was added in the indicated experiments following pre-incubation, and incubated an additional 1h at 4°C.

5.5j IMAGE PROCESSING

Images were processed in Adobe Photoshop, with minor adjustments to brightness/contrast as needed (gamma was kept at 1). No bands were obscured or altered. Images were annotated in PowerPoint.

5.6 ACKNOWLEDGEMENTS

We are most grateful to Patrick Viatour and Albert Baldwin for the Bcl-3 plasmids and Timothy McKeithan for the Bcl-3 reporter constructs. We thank Dinaida Lopez and Jason Rivest for purified proteins, and Julita Ramìrez and Holli Giebler for critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health (CA55035).

CHAPTER 6
THE HTLV-1 TAX PROTEIN INDUCES CYCLIN D1 EXPRESSION THROUGH BOTH THE CREB AND NF-κB PATHWAYS

Chapter 6 describes a study of the mechanism of Tax transactivation of cyclin D1. 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 Virology* for publication. The citation for the manuscript is:

Kim, Y.M., & Nyborg, J.K. (2008). The HTLV-1 Tax protein induces cyclin D1 expression through both the CREB and NF-kB pathways. Submitted.

6.1 ABSTRACT

Adult T-cell leukemia/lymphoma is a fatal malignancy etiologically linked to infection with the human T-cell leukemia virus (HTLV-1). The viral protein Tax activates HTLV-1 and cellular genes by cooperating with cellular transcription factors. Cyclin D1 is a critical regulator of cell cycle progression, and Tax deregulation may be linked to viral oncogenesis. Here, we characterize the mechanism of Tax transactivation of cyclin D1. We find that Tax utilizes both the CREB/ATF and NF-κB pathways, but physically associates with the cyclin D1 gene via the promoter proximal CREB binding site. These data advance our understanding of cyclin D1 deregulation by Tax.

6.2 INTRODUCTION

The human T-cell leukemia virus type 1 (HTLV-1)-encoded oncoprotein Tax promotes oncogenesis through disruption of diverse host-cell growth control pathways, resulting in aberrant cell division. Tax exerts opposing influences on cellular homeostasis through diverse mechanisms of deregulation, including the physical interaction with cell cycle regulators and transcriptional activation of cell cycle control genes, events that ultimately lead to enhanced cell division (26, 78, 96, 243). For example, Tax has been shown to activate the genes encoding cyclin D1 and D2 (79, 244). Cyclin D1, the ubiquitously expressed mammalian nuclear protein, regulates G1 to S phase progression through formation of active complexes that phosphorylate and inactivate the RB protein. As such, cyclin D1 deregulation is strongly correlated in the development and progression of many cancers. A recent study demonstrated that enforced overexpression of cyclin D1 directly facilitates malignant transformation by promoting genomic instability via centrosome amplification, mitotic spindle abnormalities, and aneuploidy (241).

The expression of the cyclin D1 gene is regulated by several transcription factors, including NF-κB and CREB (245-247). A previous study demonstrated that Tax stimulates cyclin D1 expression primarily, but not exclusively, through the promoter proximal NF-κB binding site (79). These data are consistent with a significant number of studies showing that the prominent mechanism of cellular gene activation by Tax is through persistent stimulation of the NF-kB pathway (236). However, a full understanding of Tax activation of cyclin D1 is lacking.

6.3 RESULTS AND DISCUSSION

6.3a CYCLIN D1 TRANSCRIPT LEVELS ARE ELEVATED IN HTLV-1-INFECTED T-CELL LINES

To evaluate cyclin D1 RNA levels in HTLV-1 infected cell, we performed reverse transcriptase-polymerase chain (RT-PCR). We compared cyclin D1 transcript levels in HTLV-1 infected SLB-1 and C8166 cells to levels in uninfected Jurkat and CEM human T-cells. Figure 6.1A shows elevated cyclin D1 levels in HTLV-1 infected cells, relative to the uninfected cells, consistent with a previous study that reported enhanced cyclin D1 expression upon Tax induction in JPX-9 cells (79).

6.3b TAX ACTIVATES CYCLIN D1 TRANSCRIPTION THROUGH THE PROXIMAL PROMOTER

Many transcription factors have been shown to regulate cyclin D1 transcription (Fig. 6.1B). To determine which promoter regions mediate Tax transactivation of the cyclin D1 gene, we performed transient transfection assays using a collection cyclin D1 promoter deletion constructs driving luciferase expression (248) (Fig. 6.1B). Jurkat T-cells were cotransfected with each of the deletion constructs in the absence or presence of an expression plasmid for Tax (pGS-Tax). Figure 6.1C demonstrates that deletion of the cyclin D1 promoter to –66 (–66/Luc), relative to the start site, retained Tax responsiveness.

A.

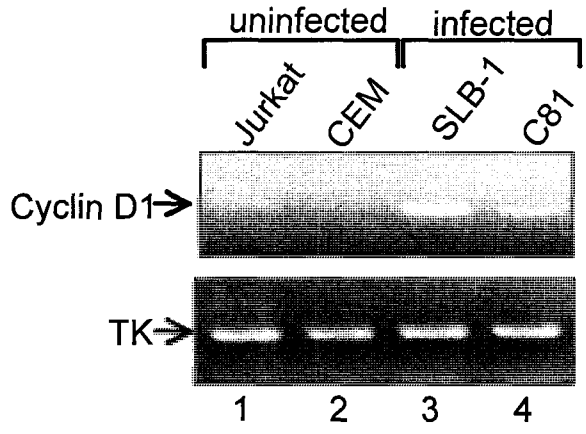
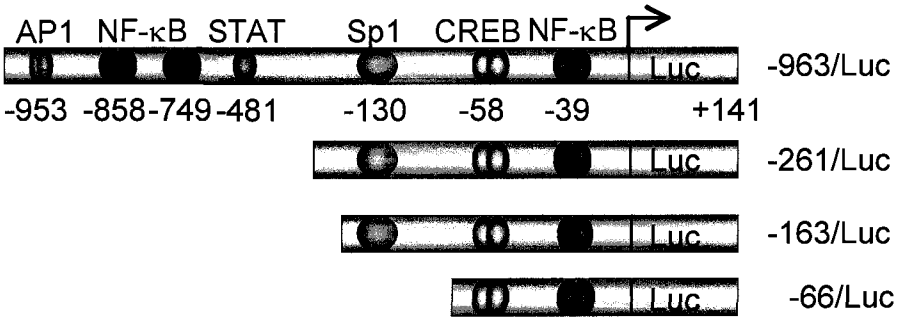


Figure 6.1A. *Cyclin D1 transcript levels are elevated in HTLV-1 infected T-cell lines.* Cyclin D1 RNA from uninfected (CEM, Jurkat) and HTLV-1 infected (SLB-1, C816645) cells was amplified by RT-PCR and subjected to agarose gel electrophoresis to determine relative transcript levels. Thymidine kinase (TK) levels are shown as an internal control.

B.



C.

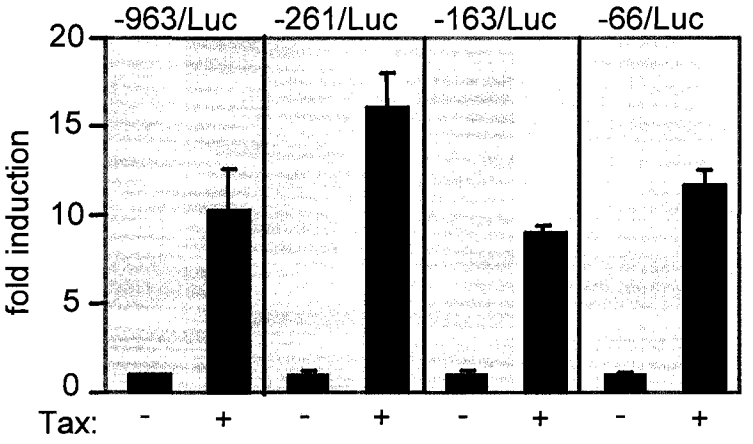


Figure 6.1B. *Schematic diagram of cyclin D1 promoter.* Relevant cis-acting elements and the coordinates of the deletion constructs used in the assay are shown. **C.** *Tax transactivates cyclin D1 through the proximal promoter.* Jurkat cells were cotransfected with the indicated deletion constructs of the human cyclin D1 promoter/Luc, in the absence or presence of a Tax expression plasmid (pSG-Tax).

6.3c BOTH THE M22 AND M47 TAX MUTANTS ARE MODERATELY DEFECTIVE FOR TAX TRANSACTIVATION OF CYCLIN D1

The Tax responsive cyclin D1 –66/Luc deletion construct carries a single NF-κB site and cyclic AMP response element (CRE). Although Tax activation through the NF-κB pathway is well established (236), Tax activation through cellular CREs has been infrequently reported and is not well understood (244, 249-251). However, recent studies demonstrate that Tax expression induces constitutively elevated levels of serine 133 phosphorylated CREB (pCREB), suggesting a mechanism of Tax-mediated transcriptional activation through cellular CREs (38, 156, 157). To establish the pathway utilized by Tax in the activation of cyclin D1, we performed transient transfection assays using the mutant Tax expression plasmids M22 and M47. M22 Tax is defective for activation of the NF-κB pathway, whereas M47 Tax is defective for activation of HTLV-1 via the vCREs (237). Interestingly, both Tax mutants were reduced in their potential to activate cyclin D1, and cotransfection of both mutants recovered the activity of Tax (Fig. 6.1D). These data suggest that both NF-κB and CREB mediate Tax transactivation of cyclin D1.

6.3d TAX ASSOCIATES WITH THE CYCLIN D1 PROMOTER IN HTLV-1 INFECTED CELLS

The Tax protein activates the NF-κB pathway via phosphorylation and degradation of IκB in the cytoplasm, leading to nuclear translocation of the transcriptionally competent NF-kB proteins (236). The preponderance of data

D.

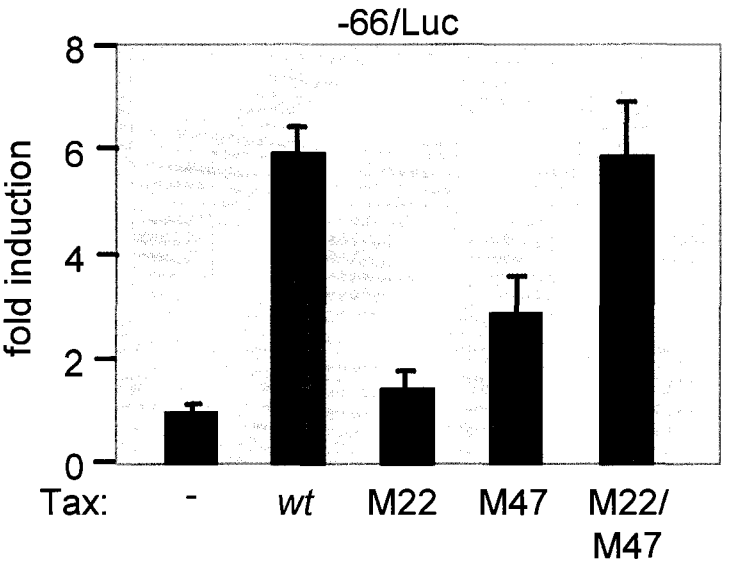


Figure 6.1D. *Both M22 and M47 are moderately defective for Tax transactivation of cyclin D1.* Jurkat cells were transiently transfected with the cyclin D1 □66/Luc reporter in the absence or presence of expression plasmids for wild-type Tax (pSG-Tax), or the M22 or M47 mutants (pSG-M22, pSG-M47) , as indicated. Luciferase activity was measured 24 h after transfection and fold-induction was quantified. The graph represents the average of three independent experiments.

supporting this pathway is consistent with the absence of evidence for a physical association of Tax with promoter-bound NF-kB. Similarly, *in vitro* studies indicate that cellular CREs are not competent for Tax binding [see (34, 35, 213)]. This is because CREs within cellular promoters generally do not carry the highly conserved and functionally relevant G/C-rich flanking sequences that are found at the 21 bp viral CREs (vCRE) located in the HTLV-1 promoter. At the specialized vCRE sequences, Tax, in complex with CRE-bound CREB, interacts with the G/C-rich flanking sequences immediately adjacent to the CRE, forming a stable, promoter-bound complex (34, 35, 145). However, a recent study reported Tax association with specific CRE-containing cellular gene promoters using the ChIP assay (251), suggesting that additional mechanisms of Tax-association with cellular CREs may exist. We therefore investigated whether Tax binds at the cyclin D1 promoter *in vivo* using the chromatin immunoprecipitation (ChIP) assay. Figure 6.2 shows elevated Tax binding at the cyclin D1 promoter in HTLV-1 infected SLB-1 cells, relative to uninfected Jurkat T-cells. Consistent with the binding of Tax, we also observed elevated RNA polymerase II association at the cyclin D1 promoter in the HTLV-1 infected cell line. These findings reveal that Tax associates with the endogenous cyclin D1 promoter.

6.3e TAX BINDS TO THE CYCLIN D1 PROMOTER *IN VITRO*

To more closely examine the physical interaction between Tax and the cyclin D1 promoter, we performed *in vitro* DNA pull-down assays using cyclin D1 proximal promoter constructs carrying either the natural sequence (*wt*) or

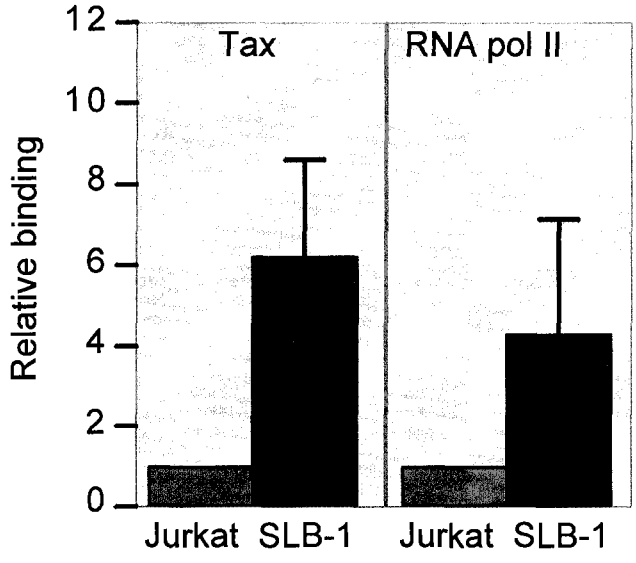


Figure 6.2. Tax is associated with the cyclin D1 promoter in SLB-1 cells. Graphical representation of the ChIP analysis following real-time PCR. Chromatin from Jurkat and SLB-1 cells was immunoprecipitated using antibodies against the indicated proteins. Real-time PCR was used to examine occupancy of the indicated proteins at the cyclin D1 promoter following immunoprecipitation. Tax binding at the IL-2 promoter in SLB-1 cells was used as a negative control. Data shown represents the average of three independent experiments.

deletion of both the CRE and κB sites (ΔCRE/ΔkB) (Fig. 6.3A). The 60 bp promoter templates were immobilized on streptavidin-coated magnetic Dynal beads via incorporation of a biotin group on the upstream end of each template, as previously described (213). The immobilized templates were incubated with CEM nuclear extract (an HTLV-1-negative T-cell line) in the absence or presence of highly purified Tax protein. The templates were extensively washed and promoter-bound proteins were separated on SDS-PAGE and analyzed by western blot. Figure 6.3B shows that CREB, present in the nuclear extract, bound to the wild type cyclin D1 promoter, but not to the mutant promoter carrying the CRE/kB deletions. Similarly, we detected a physical association of Tax with the wild type cyclin D1 promoter, but not with the mutant promoter carrying the κB/CRE binding site deletions (Figure 6.3B). We did not observe Tax binding to the template in the absence of nuclear extract, indicating that the association is dependent upon cellular factors (data not shown). These data corroborate the *in vivo* ChIP assay and further show that Tax specifically associates with the functionally relevant promoter proximal region of cyclin D1.

6.3f THE CRE IS REQUIRED FOR TAX BINDING AT THE CYCLIN D1 PROMOTER

We next sought to differentiate between the CRE and the κB binding sites in the association of Tax with the cyclin D1 promoter. We designed two additional cyclin D1 promoter constructs carrying targeted deletions of the individual CRE

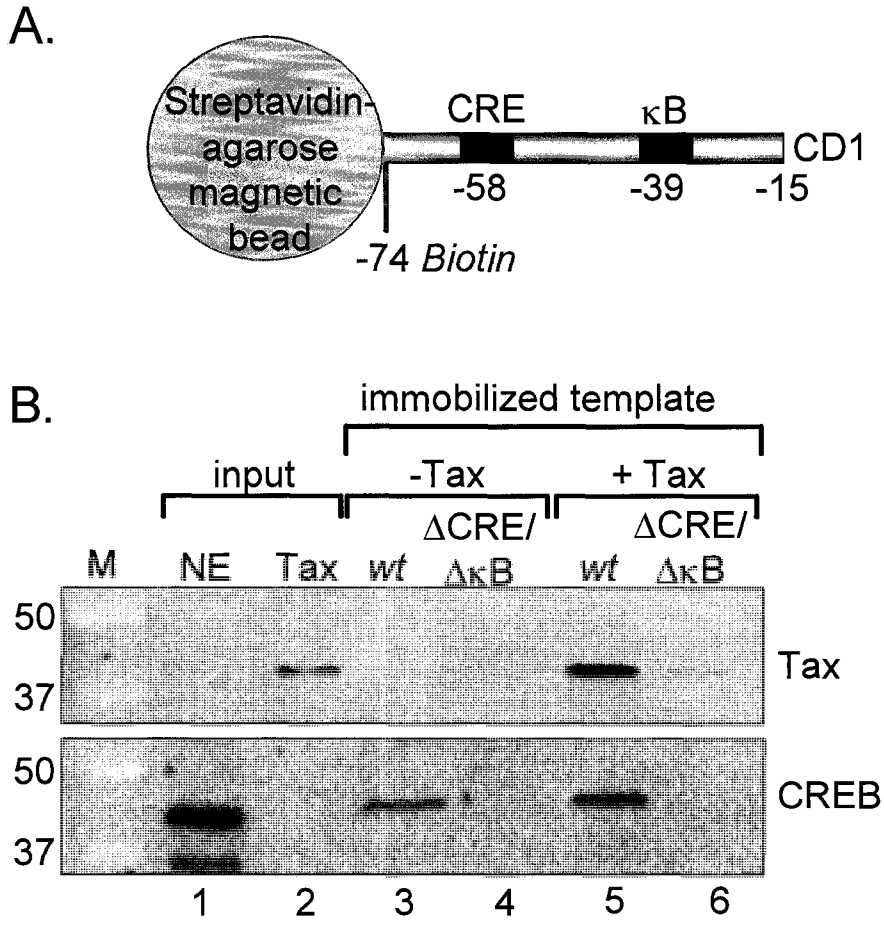


Figure 6.3A. *Schematic diagram of DNA pull-down assay.* Cyclin D1 proximal promoter constructs were immobilized into streptavidin-coated magnetic beads. **B.***Tax interacts with the cyclin D1 promoter through the CRE and/or κB sites.* The immobilized template assay was performed with a wild-type (*wt*) cyclin D1 promoter fragment and a mutant fragment carrying substitutions in both the κB and CRE binding sites (ΔCRE/ΔκB). Binding reactions were performed with CEM nuclear extract (NE) in the absence or presence of purified Tax. Samples were analyzed by western blot.

C.

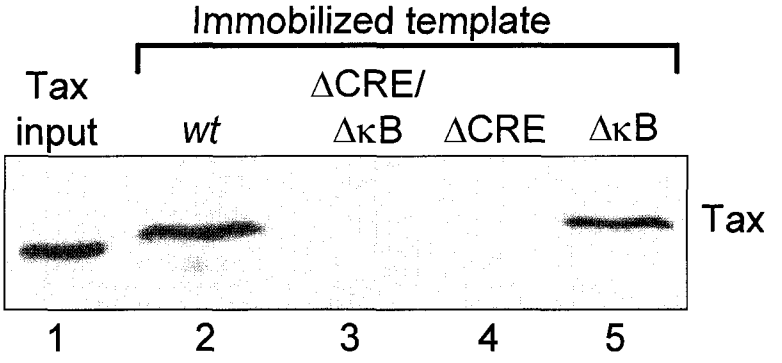


Figure 6.3C. *The CRE is required for Tax binding at the cyclin D1 promoter* in vitro. The immobilized template assay was performed with four cyclin D1 promoter fragments: wild-type (*wt*), CRE deletion (ΔCRE), κB deletion (ΔκB), or both (ΔCRE/ΔκB). Binding reactions were performed and analyzed as described in panel A.

or κB recognition elements. Figure 6.3C shows that Tax binding was abolished upon deletion of the CRE (ΔCRE), whereas deletion of the κB site (ΔκB) had no effect. These data indicate that the physical association of Tax with the cyclin D1 promoter in vitro requires the presence of CREB, or a CREB-like factor. The mechanism of Tax association with the cyclin D1 CRE is unknown, as the cyclin D1 CRE lacks the canonical G/C-rich flanking sequences required for high affinity association of Tax with CREB/DNA.

To assess the functional relevance of the CRE and κB sites in Tax transactivation of cyclin D1, we introduced κB and CREB point mutations into –66/Luc, and performed transient transfection assays in the absence or presence of the Tax expression plasmid. The data presented in figure 6.3D shows that both the κB and CRE elements contribute to Tax transactivation of cyclin D1, corroborating both the binding data and functional assays performed with the Tax mutants M22 and M47 (Fig. 6.1D).

In summary, the data presented herein establish cyclin D1 as a target of Tax transactivation in vivo. We observe elevated cyclin D1 RNA levels in HTLV-1 infected cells, consistent with a previous study (79). We further show that Tax activates cyclin D1 through the proximal promoter region (–66), and that the CRE and NF-κB sites within this region are both critical to mediating Tax stimulation. Targeted deletion of these individual sties, coupled with data using of specific Tax mutants, further support this conclusion. While it was expected that Tax stimulation of the NF-kB pathway would lead to elevated cyclin D1 expression,

D.

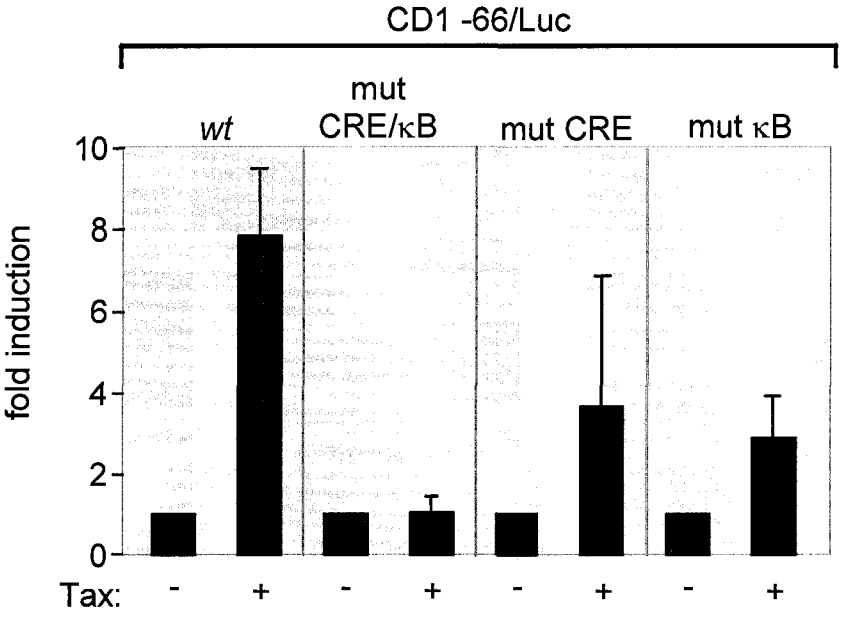


Figure 6.3D. *Tax requires both NF-κB and CREB binding sites for activation of the cyclin D1 promoter* in vivo. Jurkat cells were cotransfected, in the absence or presence of a Tax expression plasmid, with the cyclin D1 promoter-Luc constructs carrying mutations in the κB or CRE sites, as indicated. Luciferase activity was measured 24 h after transfection and fold-induction was quantified. Averages from three independent experiments are presented.

our observation that the CRE participates both physically and functionally in mediating Tax transactivation was unexpected. Notably, we detected Tax binding at the cyclin D1 promoter in vivo and in vitro, and biochemical experiments defined the cyclin D1 promoter proximal CRE as essential for the association of Tax. Because Tax alone does not bind DNA, and nuclear extract is required for Tax association with the CRE, it is likely that Tax binding to the cyclin D1 promoter is facilitated by CREB. Recent studies have shown that Tax induces CREB phosphorylation in infected cells, and that phosphorylated CREB is critical to Tax transactivation mediated through the vCREs at the HTLV-1 promoter (38, 156, 157). We therefore hypothesize that the physical interaction between Tax and CREB, together with enhanced Tax-dependent CREB phosphorylation, may facilitate Tax activation of cyclin D1.

6.4 MATERIALS AND METHODS

6.4a CELL CULTURE

Both HTLV-1 infected (SLB-1, C8166) and uninfected (Jurkat, CEM) human T-cell lines were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin.

6.4b RNA ISOLATION AND RT-PCR ANALYSIS

Cytoplasmic RNA was isolated from cultured cells using the Cytoplasmic

RNA Reagent (Invitrogen) to avoid DNA contaminaion according to the manufacturer's instructions. mRNA (2 μg) was reverse transcribed using the Superscript II reverse transcriptase (Invitrogen) with oligo (dT), and dNTPs for 2hrs at 42°C. The cDNA was used as a template to amplify a 560-bp cyclin D1 cDNA fragment using forward primer 5′-GAC CAT CCC CCT GAC GGC CGA G-3′ and reverse primer, 5′-CGC ACG TCG GTG GGT GTG C-3′. Cycling conditions are denaturation at 94°C, 30 s, annealing at 55°C, 30 s, and extension at 72°C, 30 s for 30 cycles and final extension at 72°C for 10 min. As an internal control, a 220-bp thymidine kinase (TK) cDNA fragment was amplified using forward primer 5′-GAG TAC TCG GGT TCG TGA AC-3′ and reverse primer, 5′-GGT CAT GTG TGC AGA AGC TG-3′. Cycling conditions are denaturation at 94°C, 30 s, annealing at 55°C, 30 s, and extension at 72°C, 30 s for 25 cycles and final extension at 72°C for 10 min.

6.4c TRANSIENT TRANSFECTION ASSAYS

Jurkat cells were grown to a density of 10^6 cells/ml and transfected with a constant amount of DNA using Lipofectamine reagent (Invitrogen). After 24 hr of transfection, the cells were harvested and lysed, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) with a Turner Designs model TD 20-eluminometer. Reporter plasmids containing the cyclin D1 promoter deletion constructs driving firefly luciferase expression were gifts from Dr. R. Pestell (Thomas Jefferson University, Philadelphia, PA). Firefly luciferase activity was normalized to *Renilla* luciferase activity from the herpes

simplex virus thymidine kinase (TK) promoter (pRL-TK; Promega). The expression plasmids for Bcl-3 (pCMV2-Flag-Bcl-3) and p52 (pCMV2-Flag-p52) were gifts from Dr. A. Baldwin (University of North Carolina School of Medicine, Chapel Hill, North Carolina). The expression plasmid for Tax (pSG-Tax) and Tax mutants M22 (pSG-M22) and M47 (pSG-M47) (162) have been previously described. The transient transfection assays were performed in triplicate and repeated in three independent experiments.

6.4d CHROMATIN IMMUNOPRECIPITATION ASSAY

SLB-1 or Jurkat cells were cross-linked with formaldehyde and sonicated to produce average 500 bp chromatin fragments on average. These chromatin fragments were immunoprecipitated with antibodies against Tax, CBP, or RNA polymerase II. Preimmune serum (rabbit IgG) was used as a control. The Immunoprecipitated chromatin was recovered, the cross-links reversed and the DNA was purified and used as a template for real-time PCR using iQ SYBR Green Supermix (Bio-Rad). The primers used to amplify the cyclin D1 promoter (–83 to +42 from the initiation start site) in the ChIP assays were as follows: forward 5′-CTG CCG GGC TTT GAT CTT TGC TTA-3′ and reverse 5′-ACT CTG CTG CTC GCT GCT ACT-3′. The amount of immunoprecipitated cyclin D1 promoter DNA relative to that present in total input chromatin was calculated as described previously (252, 253). The Cycle threshold (CT) (the cycle number at which each PCR reaction reaches a fluorescence threshold) was set within the linear range for all reactions. Input was 10% of total chromatin. ΔCT = CT (input)

–CT (sample). ChIP analysis using IgG was used for background subtraction ($2^{\Delta CT\ sample} - 2^{\Delta CT\ IgG}$). The Correlation coefficient was 0.993 and the PCR efficiency was 90-110%.

6.4e ANTIBODIES

Antibodies against CBP (A22), RNA polymerase II (H-224) and CREB (C-21) were purchased from Santa Cruz Biotechnology. Tax monoclonal antibody (hybridoma 168B17–46-92) was obtained from the National Institutes of Health Aids Research and Reference Reagent Program.

6.4f OLIGONUCLEOTIDES

The top strand sequences of the complimentary 5′ biotinylated oligonucleotides are used for the immobilized template assays were as follows. CD1 WT: 5′-Bio/GGA ATT CCT TTG ATC TTT GCT TAA CAA CAG TAA CGT CAC ACG GAC TAC AGG GGA GTT TTG TTG AAG TT; CD1 mut1 5′-Bio/GGA ATT CCT TTG ATC TTT GCT TAA CAA CAG CAC GGA CTA CAG TTG AAG TTG CAA AGT CCT GGA GCC TC; CD1 mut2 5′- Bio/GGA ATT CCT TTG ATC TTT GCT TAA CAA CAG CAC GGA CTA CAG GGG AGT TTT GTT GAA GTT GCA AAG TC; CD1 mut3 5′-Bio/GGA ATT CCT TTG ATC TTT GCT TAA CAA CAG TAA CGT CAC ACG GAC TAC AGT TGA AGT TGC AAA GTC CT. The biotinylated double-stranded oligonucleotides were coupled to streptavidin

Dynabeads® (Dynal Biotech USA, cat #112.06) at 1 pmol of DNA/μl of beads. Binding was performed according to the manufacturer's instruction.

6.4g DNA PULL-DOWN ASSAYS

Tax (10 pmol) and CEM nuclear extracts (300 μg) were added to 50 μl reactions containing TM buffer with 10% glycerol, 10 μM $ZnSO_4$, 40 ng/μl of poly(dA-dT)-poly(dA-dT), and Dynabead-bound DNA (2 pmol double-stranded oligonucleotides). Binding reactions were incubated for 30 minutes at 25°C. Beads were magnetically isolated, washed 3 times with TM buffer, resuspended in 40 μl of 1X SDS sample buffer, and boiled 5 minutes immediately prior to protein fractionation by SDS-PAGE.

6.4h NUCLEAR EXTRACT

CEM cells, an HTLV-1 negative human T-cell line, were cultured in IMDM supplemented with fetal bovine serum as described in 6.5a. Nuclear extracts were prepared as previously described (193).

6.4i. EXPRESSION AND PURIFICATION OF RECOMBINANT TAX

Bacterially-expressed $TaxHis_6$ (158) protein was purified as previously described and was 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. The protein was purified to >95% homogeneity.

6.5 ACKNOWLEDGMENTS

We thank Drs. Pestell and Baldwin for the cyclin D1 luciferase constructs, and Julita Ramirez and Holli Giebler for their assistance with the manuscript. This work was supported by a grant from the National Institutes of Health (CA55035).

SUPPLEMENTAL FIGURES

The supplemental figures contained in this section include experiments I performed that did not appear in the previous chapter "Tax induces cyclin D1 expression". These experiments present complementary data that expands on the results shown in chapter 6.

Figure 6.4 Tax and Bcl-3/p52 does not synergize to stimulate transcription of cyclin D1.

It is well established that Bcl-3 upregulates cyclin D1 expression by acting as a cofactor by interaction with p52 homodimers bound at the –39 NF-κB *cis*-acting element (224). We have evidences that Tax increases Bcl-3 transcription (132) and Tax requires the promoter proximal NF-κB binding site to stimulate cyclin D1 expression (79). Thus, there might be cooperation between Tax and Bcl-3 for Tax-mediated transactivation of the cyclin D1 gene through the NF-κB binding site. To test this possibility, Jurkat cells were co-transfected with CD1 963-Luc or CD1 66-Luc reporter in the presence or absence pSG-Tax and expression plasmids for Bcl-3 (CMV-FLAG-Bcl-3) and p52 (CMV-FLAG-p52). Bcl-3 and p52 activated cyclin D1 transcription; however, we did not observe cooperation between Tax and Bcl-3 in the activation of transcription from the cyclin D1 promoter (Fig. 6.4) .

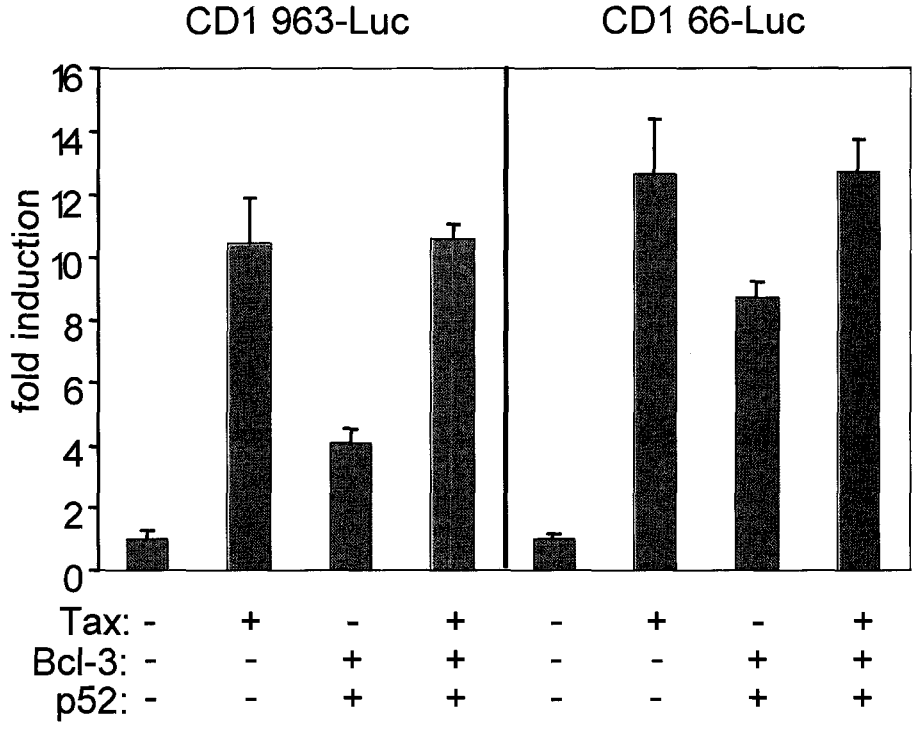


Figure 6.4. Tax and Bcl-3/p52 does not synergize to stimulate transcription of cyclin D1. Jurkat cells were cotransfected with the CD1 963-Luc or CD1 66-Luc reporter (100 ng) in the presence or absence of pSG-Tax and expression plasmids for Bcl-3 (CMV-FLAG-Bcl-3) (100 ng) and p52 (CMV-FLAG-p52) (100 ng) as indicated. Luciferase activity was measured 24 h after transfection and fold-induction quantified.

Figure 6.5 The Tax responsive region of the cyclin D1 promoter was cloned into G-less cassette

By performing DNA immobilization assays, we detected Tax binding at the cyclin D1 promoter. To examine the function of this interaction, *in vitro* transcription assays were performed. We have previously utilized a G-less cassette plasmid (176) to clone four tandem copies of the HTLV-1 promoter-proximal viral CRE upstream of the HTLV-1 core promoter (p4TxRE/G-less) (Fig. 6.5A) for *in vitro* transcription assays (40). We therefore used this system to make a cyclin D1 promoter G-less cassette. Forward (F) and reverse (R) PCR primers were designed to amplify the Tax responsive region of cyclin D1 using the cyclin D1 luciferases construct as a template (Fig. 6.5B). The HTLV-1 promoter was then removed from the p4TxRE/G-less cassette, and the PCR product containing the Tax responsive region of the cyclin D1 promoter was cloned upstream of the 380-bp G-less cassette (Fig.6.5C).

Figure 6.6 Tax was unable to increase transcription from the cyclin D1 promoter

Previous studies have shown that the HTLV-1 viral CREs mediate CREB-dependent Tax transactivation (34). To determine whether the single cyclin D1 CRE can mediate CREB-dependent Tax transactivation in a similar fashion, *in vitro* transcription assays were performed by adding increasing amounts of Tax to reaction mixtures containing CREB. The addition of 1 pmol of CREB to the preincubation reaction mixtures did not significantly increase the amount of

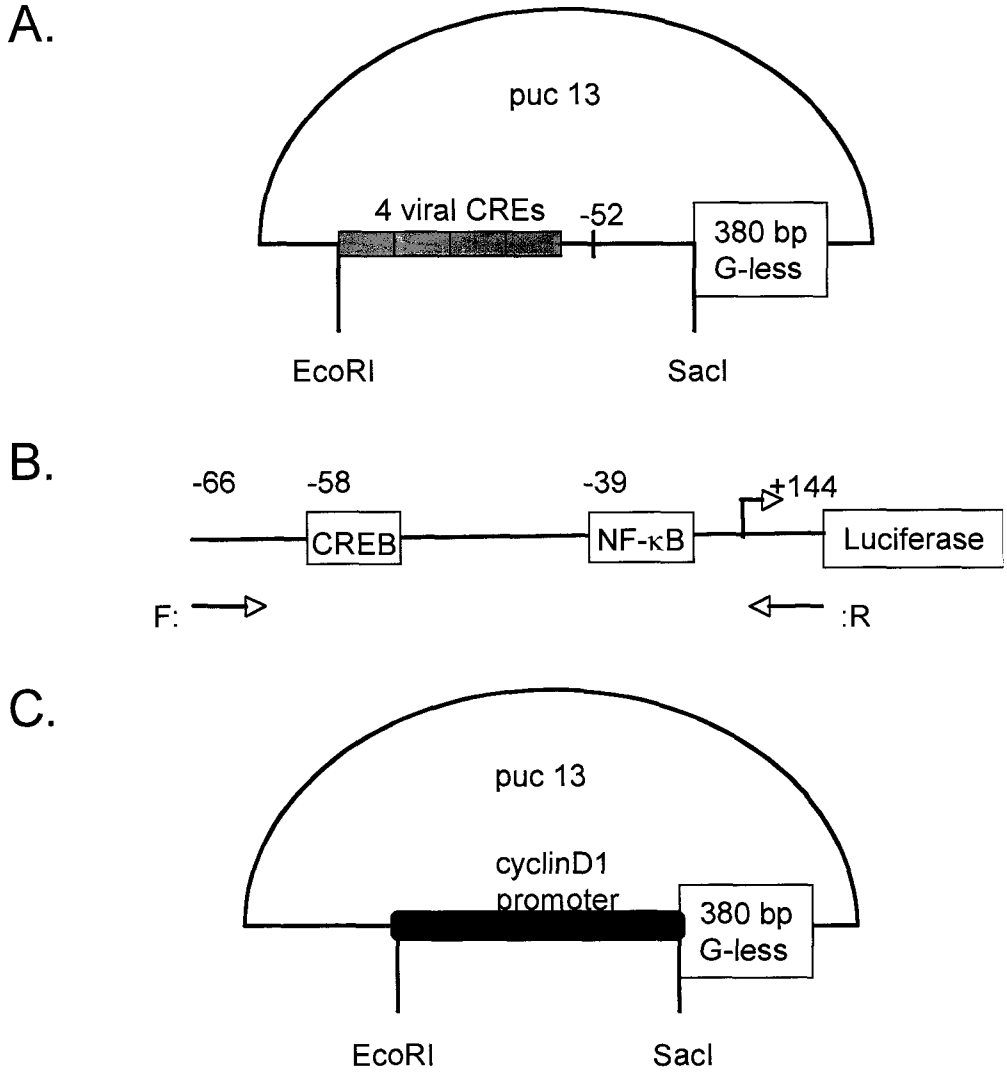


Figure 6.5. The Tax responsive region of the cyclin D1 promoter was cloned into G-less cassette.
A. Schematic diagram of p4TxRE/G-less cassette.
B. Design of primers for the cyclin D1 Tax responsive region
C. Schematic diagram of pcyclin D1/G-less cassette.

transcription from the 4TxRE viral promoter (Fig. 6.6A compare lanes 1 and 2). However, the addition of Tax to the reaction mixtures containing 1 pmol of CREB increased the level of RNA synthesis from the 4TxRE viral promoter (Fig. 6.6A compare lanes 1 and 4). In contrast, the addition of 1 pmol of CREB to the preincubation reaction mixtures significantly stimulated transcription from the cyclin D1 promoter (Fig. 6.6B compare lanes 1 and 2). However, the addition of Tax was unable to increase transcription from the cyclin D1 promoter (Fig. 6.6B compare lanes 1 and 4).

Although we did not observe Tax transactivation of cyclin D1 in the *in vitro* transcription assay, several points need to be considered. Since CEM nuclear extract contains little or no NF-kB without stimulation, exogenous NF-kB such as p65 may be required to mediate Tax effect on the activation of cyclin D1 promoter. Moreover, the DNA templates may need to be assembled into chromatin, in order to see the Tax effect on cyclin D1 transcription.

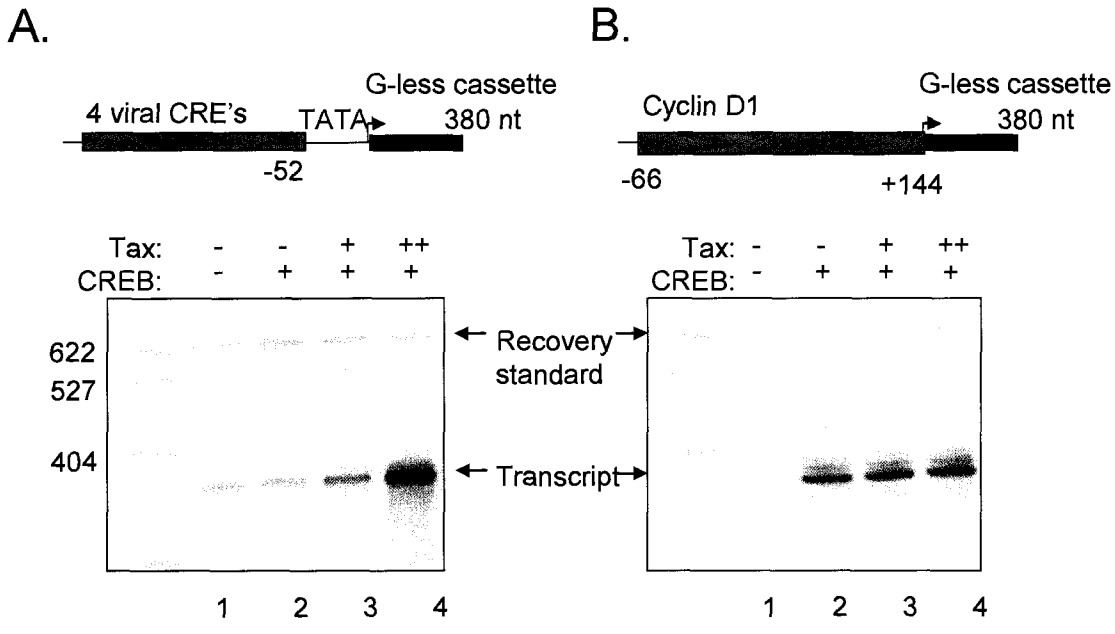


Figure 6.6. Tax was unable to increase transcription from the cyclin D1 promoter. Schematic representation of the 4TxRE promoter DNA (A) and cyclin D1 promoter DNA (B) used in the *in vitro* transcription assays was shown. Transcription from these supercoiled templates (150 ng) was analyzed in the presence of increasing amounts of Tax (1 pmol and 2 pmol), as indicated. All reactions contained acetyl CoA (100 μM). Molecular weight size markers, recovery standard and full-length transcripts are indicated. The fold activation was calculated relative to basal transcription.

CHAPTER 7
FUTURE DIRECTIONS

Serine 133 phosphorylated CREB is an essential partner in Tax-dependent CBP/p300 recruitment and transcriptional activation of HTLV-1. The importance of pCREB in mediating Tax transcription function is evidenced by the observation that the virus has evolved a Tax-dependent mechanism to promote CREB phosphorylation *in vivo*. To further our understanding of this process, we have examined the cellular kinase pathways that converge on Ser133 phosphorylation of CREB and found that enhanced CREB phosphorylation is achieved by Tax stimulation of the CaM kinase. These observations raise several questions. Which specific kinase among the CaM kinase is stimulated by Tax? How does Tax activate the specific kinase? Does the kinase contribute to Tax-dependent cellular proliferation?

The Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) pathway encompasses three known kinases, CaM kinase kinase (CaMKK), CaMKI, and CaMKIV. All of our evidence points to a direct effect of Tax on the CaM kinase pathway, as we detect no difference in the protein levels of these enzymes between uninfected and HTLV-1 infected cells. To test whether Tax may stably interacts with any of the kinases, co-immunoprecipitation assays or GST-pull down assays can be used. CaMKIV and CaMKI, closely related kinases, directly phoshorylate CREB and regulated by Ca^{2+}/CaM binding, which relieves steric autoinhibition of the active site. In addition to Ca^{2+}/CaM binding, CaMKIV and CaMKI are further activated by CaMKKα/β, which phosphorylate these kinases at

a single Thr residue in the activation loop. *In vitro* kinase assays using purified, recombinant CaMKK, CaMKI, and CaMKIV, in place of the whole cell extract, may determine the identity of the Tax-stimulated kinase. Inhibitory studies (Fig. 4.5) suggested that CaMKK is a cadidite of Tax targeted kinase. To directly test whether Tax stimulates recombinant, purified GST-CaMKKα/β, we can perform in vitro kinase assays and directly monitor the phosphorylation of CaMKIV (and CaMKI) at Thr by western blot or by autoradiography. Since CaMKIV directly phosphorylates CREB, CaMKIV stimulation by Tax or Tax and CaMKKs can be also measured. Knock-down of the kinases via siRNA would identify the Tax-stimulated CREB kinase in a biologically relevant context. Successful knock-down can be verified by RT-PCR and western blot analysis and the effect of each kinase knock-down on CREB phosphorylation can then be examined.

Once the Tax targeted kinase is identified, the mechanism by which Tax promotes stimulation of the kinase should be determined. Ca^{2+}/CaM interacts with a carboxyl-terminal region within CaMKK, CaMKI and CaMKIV, which relieves autoinhibition. Importantly, Ca^{2+}/CaM binding to CaMKI/IV is a prerequisite to phosphorylation by the CaMKKs. Tax may bind to the autoinhibitiory loop, conferring constitutive activation to the kinase. To test this hypothesis, we can measure the effect of Tax in kinase assays using unactivated kinases or kinases carrying mutations at key sites of regulation.

Phosphorylated CREB affects Tax-mediated viral transcription and may therefore affect cellular proliferation, which can be directly measured. It is

plausible that Tax, via activation of the CaM kinase pathway and elevated CREB phosphorylation, may promote cell proliferation.

We have demonstrated that Tax expression in HTLV-1 infected T-cells correlates with elevated Bcl-3 mRNA and protein levels. We also found that Tax and Bcl-3 form a stable complex *in vivo* and *in vitro*, and Bcl-3 expression potently reduces Tax activation of viral transcription. We hypothesized that Bcl-3 inhibition of Tax-dependent HTLV-1 transcription may result from stable complex formation between the two proteins. To test this possibility, purified Tax and Bcl-3 binding should be examined in DNA pull-down assays with the HTLV-1 promoter immobilized on magnetic beads. Using this system, we can test whether purified Bcl-3 binds to the HTLV-1 promoter and whether it displaces Tax. If Bcl-3 associates with Tax at the viral promoter, the Bcl-3 may block coactivator recruitment or may recruit transcriptional repressors, such as HDACs.

We observed elevated cyclin D1 RNA levels in HTLV-1 infected cells by RT-PCR and Tax stimulation of cyclin D1 expression in transient co-transfection assays. In addition, we found that Tax physically interacts with the cyclin D1 promoter *in vivo* and *in vitro*. Cyclin D1 is also activated by a complex containing p52 and Bcl-3. Because Tax and Bcl-3 form a stable complex *in vivo* and *in vitro*, we predicted that Tax and Bcl-3/p52 may synergize to stimulate transcription of cyclin D1. However, we did not observe this effect in transient co-transfection assays. These observations lead to several questions. Does Tax bind the ankyrin repeats of Bcl-3? If so, can both Tax and p52 interact with Bcl-3? To answer these questions, GST pull-down assays can be applied. GST tagged Bcl-3

constructs containing deletion and or mutation of the seven repeats in the ankyrin domain will be used to further characterize the Tax/Bcl-3 complex. Whether purified p50 or p52 form a stable ternary complex with Tax and Bcl-3 can be examined by addition of purified p50 or p52 and Tax to the reaction.

We determined that Tax requires the CRE element within cyclin D1 promoter, which contains a non-conserved G/C rich 5′ sequence. Further analysis of this interaction will contribute to understanding the mechanism and significance of this binding. For example, the importance of CREB phosphorylation for Tax binding to the cyclinD1 promoter can be examined by performing DNA immobilization assays in the presence of purified CREB or pCREB. Whether Tax binding to the cyclin D1 promoter increases recruitment of the CBP/p300 coactivator can also be tested using the assay.

We did not observe Tax transactivation of cyclin D1 in the *in vitro* transcription assay. The CEM nuclear extract does not contain NF-κB, which is also involved in Tax-dependent cyclin D1 transcription. To test the effect of exogenous NF-κB on Tax mediated activation of cyclin D1 *in vitro*, purified p50 and p65 from *E.coli* can be added to the *in vitro* transcription assays. These purified proteins can be also used for DNA pull-down assays to examine the effect of exogenous NF-κB on Tax association with the cyclin D1 promoter.

These studies merely begin to characterize Tax deregulation of key cellular regulatory proteins such as CREB, Bcl-3 and cyclin D1. It is likely that these events create an environment within the HTLV-1 infected cells that which promotes malignant transformation. Further studies of specific interactions

between Tax and Bcl-3 or CaM kinase pathway are needed to provide a more comprehensive understanding of the molecular basis of Tax function as a transcriptional activator and deregulator of cell function. The precise points of Tax deregulation within each of these key pathways may ultimately provide unique targets for pharmacological intervention to reduce proviral burden and the resultant development of HTLV-1-associated diseases.

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