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DISSERTATION

DEREGULATION OF CBP MEDIATED TRANSCRIPTION BY THE HTLV-I TAX PROTEIN

Submitted by

Karen L. Van Orden

Department of Biochemistry and Molecular Biology

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, CO

Summer 1999

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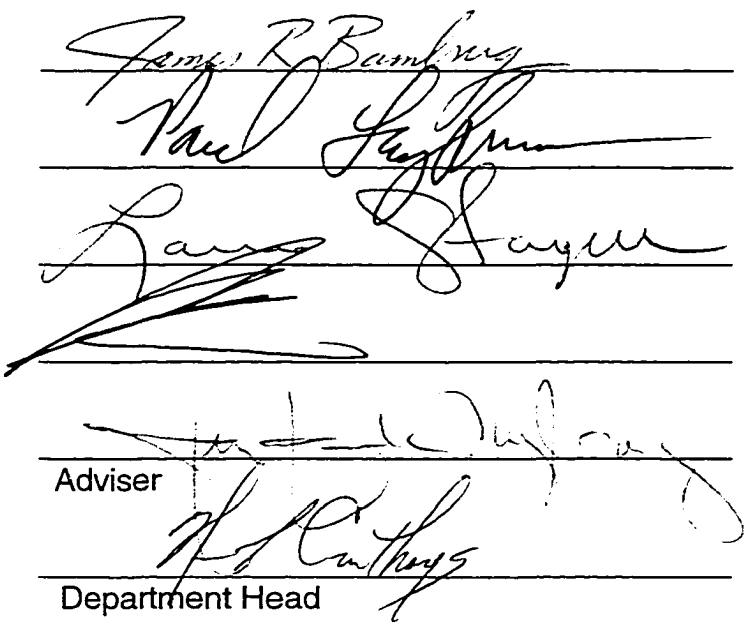
WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KAREN L. VAN ORDEN ENTITLED DEREGULATION OF CBP MEDIATED TRANSCRIPTION BY THE HTLV-I TAX PROTEIN BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work



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ABSTRACT OF DISSERTATION

DEREGULATION OF CBP MEDIATED TRANSCRIPTION
BY THE HTLV-I TAX PROTEIN

Human T-cell leukemia virus, type I (HTLV-I) is a retrovirus that causes an aggressive form of leukemia. A viral transcription factor called Tax is essential for efficient transcription from the viral promoter. At the viral promoter, Tax works through the cellular transcription factor CREB and three CREB response elements, called viral CREs, to form a stable nucleoprotein complex. Recently, Tax was shown to interact with the coactivator CBP in vitro. Here we utilize transient transfection assays to show that CBP plays a functional role in Tax transactivation through the viral CRE in vivo. Furthermore, Tax activity is dependent on interaction with a specific domain of CBP called KIX.

In addition to Tax, many cellular transcription factors bind to the KIX domain of CBP. We hypothesized that the high affinity binding of Tax to the KIX domain may occlude binding of these other factors, thus inhibiting transcription mediated through these factors in vivo. To test this hypothesis, we examined the interplay between Tax and c-jun, a second KIX binding factor. We observe reciprocal repression between Tax and c-jun in vivo and biochemical assays indicate that Tax competes with c-jun for binding to the KIX domain in vitro. Together, these data indicate that the Tax-KIX interaction has potential to disrupt other functional KIX interactions.

We next hypothesized that coactivator competition may explain the observed transcriptional inactivation of the tumor suppressor p53 in HTLV-I infected cells. p53 was recently shown to utilize CBP to activate transcription making

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coactivator competition a likely mechanism. We show reciprocal repression between Tax and p53 in vivo and in vitro binding assays demonstrate that p53 binds the KIX domain of CBP. This interaction appears to be functional as expression of the KIX domain of CBP in vivo has a dominant negative effect on p53 activated transcription. Finally, we observe that Tax and p53 compete for KIX in vitro. In summary, Tax competes with p53 for the KIX domain of CBP thus inactivating p53 transcription function. This mechanism may extend to other transcription factor pathways and play a key role cellular transformation.

Karen L. Van Orden
Department of Biochemistry
and Molecular Biology
Colorado State University
Fort Collins, CO 80523
Summer 1999

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CHAPTER 1
Introduction to HTLV-I, Tax and CBP

1.1 HUMAN T-CELL LEUKEMIA VIRUS, TYPE I

Human T-cell leukemia virus, type I (HTLV-I) was isolated from leukemic tissue in 1978 and became the first documented human retrovirus (161). Though animal retroviruses capable of inducing tumor formation had already been isolated from chickens and mice, the existence of such viruses in humans had been seriously doubted. The idea that an infectious agent, carrying its genetic material in the form of RNA, not DNA, could be maintained inside human cells and could further transmit disease seemed impossible at the time. Thus the discovery of HTLV-I played a large role in the realization that the flow of genetic information does not always start with DNA and initiated an expansion in our perception of viral pathogenic mechanisms (54).

Shortly after its discovery, HTLV-I was identified as the causative agent of Adult T-cell leukemia (ATL) (77, 93, 135, 161, 162, 168, 179, 216, 218, 227, 228). However, the mechanism of viral pathogenesis remained elusive. HTLV-I was not like the previously characterized transforming retroviruses. Sequence

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analysis revealed that it does not code for any cellular oncogenes as is seen in many of the acute leukemia viruses (179). Furthermore, its integration into the host genome was found to be completely random (178). This finding indicated that HTLV-I is different from the chronic leukemia viruses which must integrate at specific sites in the host genome where they can promote expression of growth related genes. Thus, HTLV-I was realized to transform cells by a novel, undefined mechanism and became a new focus for cancer research.

Future studies of HTLV-I revealed that the virus codes for a unique transcriptional activator named Tax. Tax is required for efficient transcription of the viral genome (30, 31, 41, 49, 180, 198). Perhaps more importantly, however, Tax deregulates host cell gene expression, and it is hypothesized that this function of Tax enables HTLV-I to transform infected cells (reviewed in 44, 20). As a result, Tax has become an area of intense research. To date, the exact molecular events by which Tax causes cells to undergo uncontrolled proliferation remain unclear. The studies summarized in this thesis suggest a new mechanism for Tax deregulation of gene expression through sequestration of a pleiotropic cellular coactivator called CREB Binding Protein (CBP).

1.1a HTLV-I ASSOCIATED DISEASE

HTLV-I is the etiological agent of ATL, an aggressive and fatal lymphoma of CD4+ T-cells. ATL patients are seropositive for HTLV-I antigens and HTLV-I particles can be observed budding from leukemic cells taken from these patients (77, 93, 135, 162, 168, 178, 216, 218, 227, 228). The disease is characterized by the appearance of leukemic cells with convoluted, flower-shaped nuclei and chromosomal abnormalities (52, 94, 207, 218). Clinical manifestations include enlarged lymph nodes, skin lesions from infiltrating leukemic T-cells, hypercalcemia, and spleenomegaly (53, 202, 207). Prognosis

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for ATL patients is very poor with a median survival time of only six months (188). Fortunately, ATL develops in less than 5% of HTLV-I-infected individuals and only after a long viral latency period of about 40 years (76, 143). Though the viral Tax protein is strongly linked to the development of ATL, the precise molecular pathway leading to disease remains to be elucidated.

HTLV-I has more recently been tied to a neurological degenerative disease Tropical Spastic Paraparesis (TSP), also called HTLV-I Associated Myelopathy (HAM) (56, 57, 86, 155, 166, 206). TSP is characterized by a progressive demyelination of the spinal cord. Clinical features include a chronic and gradual paralysis of the lower extremities and varying degrees of sensory loss (22, 85, 155). HTLV-I provirus is detectable in T-cells infiltrating the spinal cord, but not the neuronal or glial cells (105, 106, 141). The geographic occurrence of TSP coincides with that of ATL, and like ATL, only a very small percentage of HTLV-I infected individuals develop TSP (96, 121, 142). However the time for disease onset is much shorter, as TSP can develop just 6 months after infection (156). The factors which lead to this very different pathogenic course of HTLV-I remain to be defined.

HTLV-I has also been more loosely linked to several other unrelated diseases with diverse clinical symptoms (69, 113, 128, 138, 139, 148, 152, 211). However, the role HTLV-I plays in any of these diseases is unclear.

1.1b HTLV-I CLASSIFICATION

HTLV-I belongs to the *Retroviridae* family of viruses which also includes the Lentiviruses and the Spumaviruses (35). HTLV-I is grouped with HTLV-II, Bovine Leukemia Virus and the Simian T-cell leukemia viruses as all of these viruses are exogenous and are associated with lymphoma and neurological disease. Additionally, all of these viruses are classified as complex meaning

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they each encode genes that play a role in the regulation of viral expression (38).

1.1c HTLV-I GENOME

The viral genome is carried as two identical copies of single stranded RNA. Each strand of RNA is approximately 9,000 nucleotides in length and follows the general organization of retroviral genomes (figure 1.1) (36, 179). The genome is terminally redundant meaning that the sequences at the 3' and 5' ends are identical. These sequences play a functional role in the conversion of the RNA genome into DNA by the viral RNA-dependent DNA polymerase (reverse transcriptase). The U5 and U3 regions are untranslated regions and play important roles in both reverse transcription and replication. U3 contains the cis elements needed for activation of transcription from the viral promoter (210). Together, U3, R and U5 make up the viral Long Terminal Repeat (LTR). Adjacent to the U5 region is a primer binding site which is complementary to a cellular tRNA. tRNA can bind this sequence in the RNA and serves as a primer for the initiation of reverse transcription.

The internal genomic sequences encode the *gag*, *pol*, and *env* genes, which are common to all retroviruses (36). These are a series of overlapping reading frames that are differentially spliced to give rise to all of the viral structural proteins. For instance, *gag* codes for at least three proteins, which form the viral capsid. Additionally, a protease that serves to cleave polyprotein products is encoded within this region. The *pol* gene encodes reverse transcriptase and Integrase. Reverse transcriptase is responsible for the conversion of the RNA genome into DNA once inside the infected cell and Integrase serves to catalyze incorporation of the DNA into the host genome. Finally, *env* codes for the viral envelope glycoproteins.

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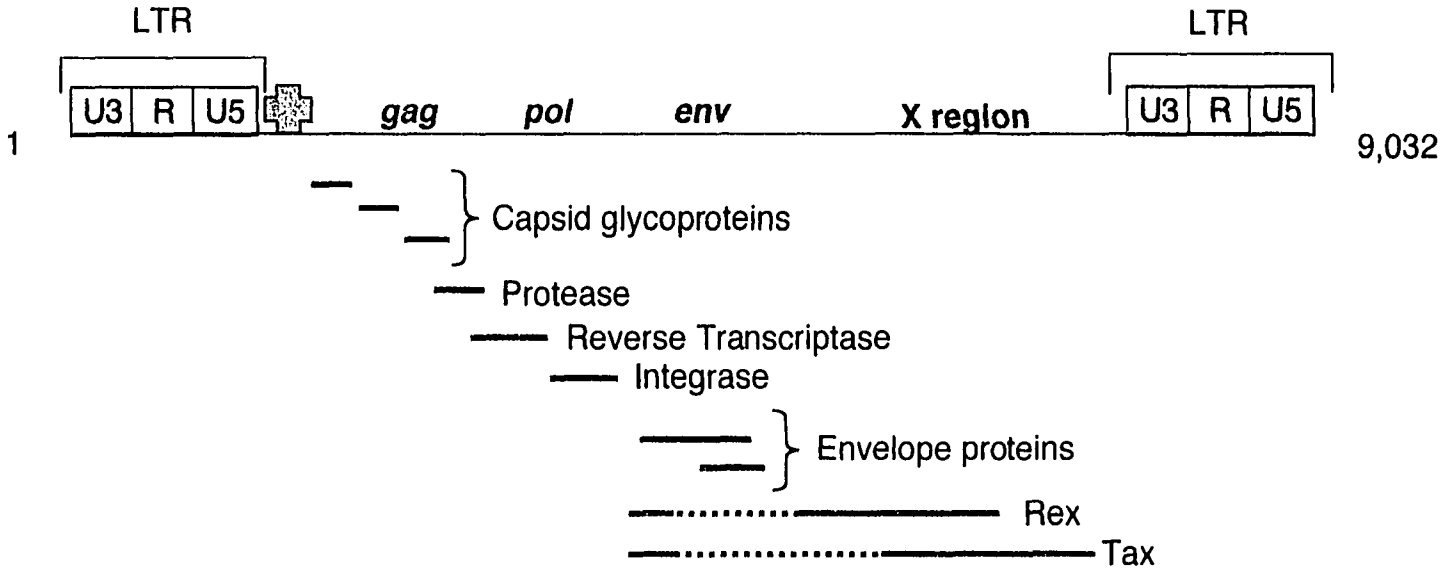

Figure 1.1 Organization of the viral genome. Long terminal repeat (LTR) sequences flank the coding region of the genome. A tRNA binding site is shown adjacent to the 3' U5 region. The arrangement of the gag, pol, env and X region gene sequences are also illustrated. Protein products of each gene are shown below.

In addition to the common retroviral genetic sequences, the HTLV-I genome contains two additional overlapping open reading frames located between the *env* and U3 regions (179, 187). These encode two regulatory proteins, Tax and Rex, which function in viral gene expression (31, 33, 108). The Rex protein is a suppressor of RNA splicing and allows for the expression and production of the viral structural proteins (72, 82, 83, 102). The Tax protein is a transcription factor and is a potent transactivator of viral gene expression (30, 31, 41, 49, 180, 197). Both Tax and Rex protein activities are critical to the viral life cycle (31) and will be discussed further in the next section.

1.1d HTLV-I INFECTIVITY AND LIFE CYCLE

Epidemiological studies have revealed that HTLV-I is endemic to localized regions of the world including southern Japan, the Caribbean basin and western Africa (23, 24, 75, 76, 78, 133, 175, 201). It is currently believed to infect 10-20 million people worldwide. The virus is transmitted by direct cell-cell contact usually through sexual intercourse, contaminated blood products, breast milk or in utero (34, 73, 99, 100, 101, 115, 134, 153, 154, 163, 215). The virus infects mature helper T-cells of the CD4+, CD8+ phenotype (71, 161, 218). However, the membrane receptor used by the virus to enter the cell is unknown.

The life cycle of the virus consists of two phases (figure 1.2) (36). The first phase is dependent primarily on viral proteins. In this phase of the life cycle, HTLV-I envelope proteins bind the T-cell receptor and the virus is taken into the cell. Once inside the cell, the viral capsid proteins are dislodged, allowing release of the viral genetic material. The viral RNA genome is subsequently copied to double stranded DNA by reverse transcriptase. To initiate this

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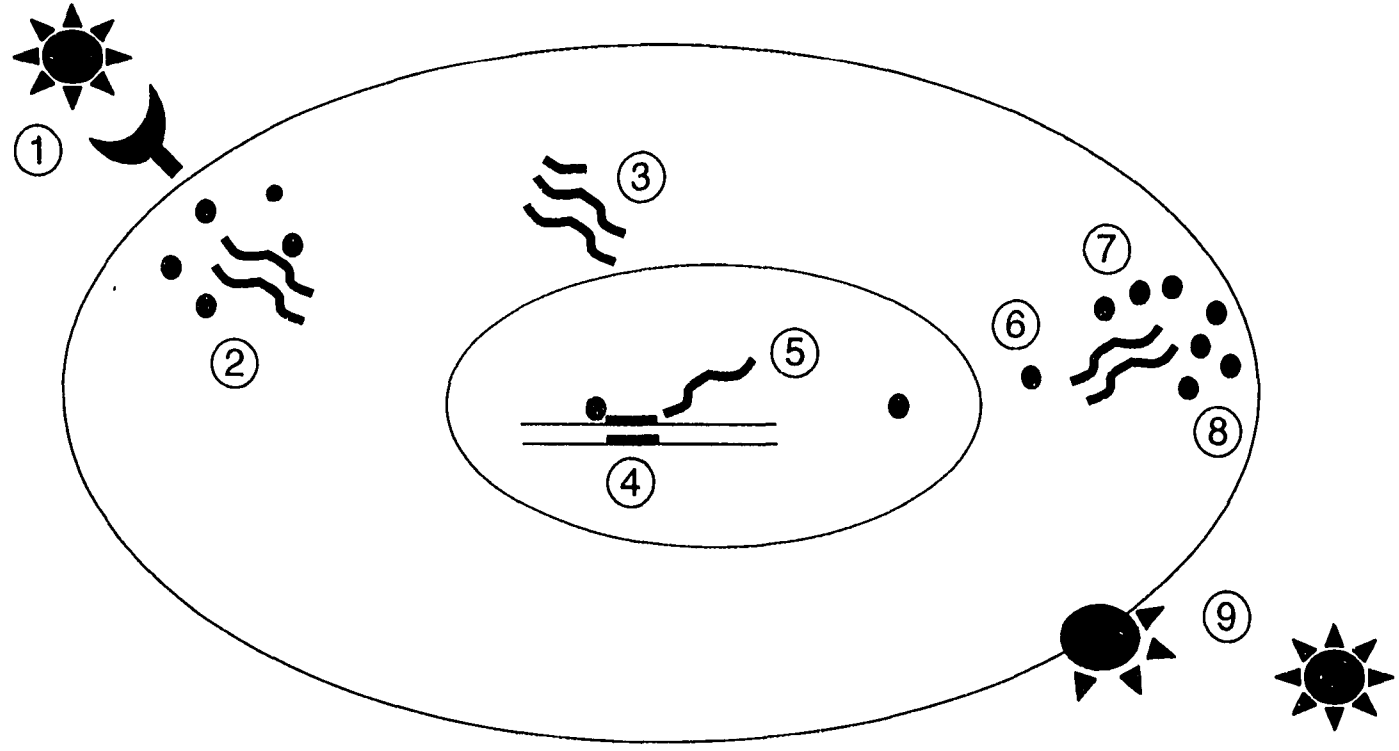

Figure 1.2 The viral life cycle. Step 1: The virus binds a receptor on the host cell membrane and is absorbed into the host cell. Step 2: Capsid proteins are dislodged and viral genetic material is released. Step 3: RNA genome is reverse transcribed into double stranded DNA during transport to the nucleus. Step 4: Viral genome is randomly incorporated into the host genome. Step 5: Transcription of viral regulatory genes. Step 6: Regulatory proteins are synthesized. These proteins feed back into the nucleus and promote transcription of the viral structural genes. Step 7: Synthesis of viral structural proteins. Step 8: Capsid proteins assemble at the cell membrane. Step 9: Newly assembled virions bud from the host cell membrane.

process, a cellular tRNA primes the 3' end of the genome, allowing reverse transcriptase to begin polymerization of the DNA copy. Reverse transcriptase also has intrinsic RNase H activity and uses this function to destroy the viral RNA after it has been copied to DNA. As this conversion from RNA to DNA takes place, the viral genome is transported to the host cell nucleus. Once synthesis of the double stranded DNA is complete, the viral genome is randomly incorporated into a host chromosome by the Integrase enzyme. The incorporated virus is called a provirus and is a permanent part of the host cell's genetic material.

The second phase of the viral life cycle is more highly dependent on host cell proteins. This phase involves transcription of the viral genome by the cellular RNA polymerase II machinery. New single stranded RNA copies of the viral genome as well as mRNA for viral proteins are made. In the early phase, mRNAs are doubly spliced by default giving rise to the regulatory proteins Tax and Rex. Tax feeds forward and activates the viral promoter stimulating expression of more viral RNA and protein. At the same time Rex functions as suppressor of splicing. Therefore, once Rex has been produced, unspliced and singly spliced RNA is produced (177, 212). These RNA products give rise to the structural proteins. Once structural proteins are made, new virions begin to assemble and associate with the host cell membrane. Finally, new viral particles bud from the host cell and are ready for infection of another host T-cell.

1.2 THE TAX PROTEIN

The viral Tax protein is a 40 kilodalton oncoprotein with no apparent homology to any cellular proteins (179). Tax was originally identified as a potent transactivator of the viral LTR and appears to play a key role in the viral life cycle, catalyzing the switch from latency to high level viral replication (44,

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review). This observation has sparked intense research on the mechanism of Tax transactivation of the viral promoter. The ensuing studies have generated a model for Tax transactivation of the viral LTR, which involves multiple complex interactions between the viral DNA, Tax and cellular proteins. Studies on Tax also revealed that Tax is pleiotropic in nature, aberrantly activating and repressing expression of a variety of cellular genes through a number of different cellular pathways. This information, together with the finding that Tax alone can transform cells, implicated Tax as the viral agent responsible for HTLV-I-associated leukemogenesis. Thus the Tax protein has become a tool for studying the molecular pathway leading to leukemia.

1.2a FUNCTIONAL DOMAINS OF TAX

Tax is a 353 amino acid nuclear phosphoprotein which functions as a transactivator of viral gene expression and a deregulator of cellular gene expression (42, 61, 62, 150, 191, 192, 193). However, precise definition of the functional domains of Tax has been enigmatic. Structural predictions based on amino acid sequence have been helpful, since high resolution structural information has yet to emerge. These analyses predict a Zinc finger motif within the amino terminus of Tax (amino acids 18-60) and an alpha helical domain at the carboxyl terminus (figure 1.3) (181, 194). This carboxyl terminal region is highly acidic and was initially hypothesized to serve as the transactivation domain. Mutations made within this region of Tax however have no effect on Tax transcription function, and in fact, this region has been shown to be completely dispensable for all known Tax functions (3, 182, 194, 224).

The functional domains of Tax have further been defined by the production of Tax mutants. Deletion mutants made from either the amino and carboxyl terminus of Tax, together with an array of point mutations that span the entire

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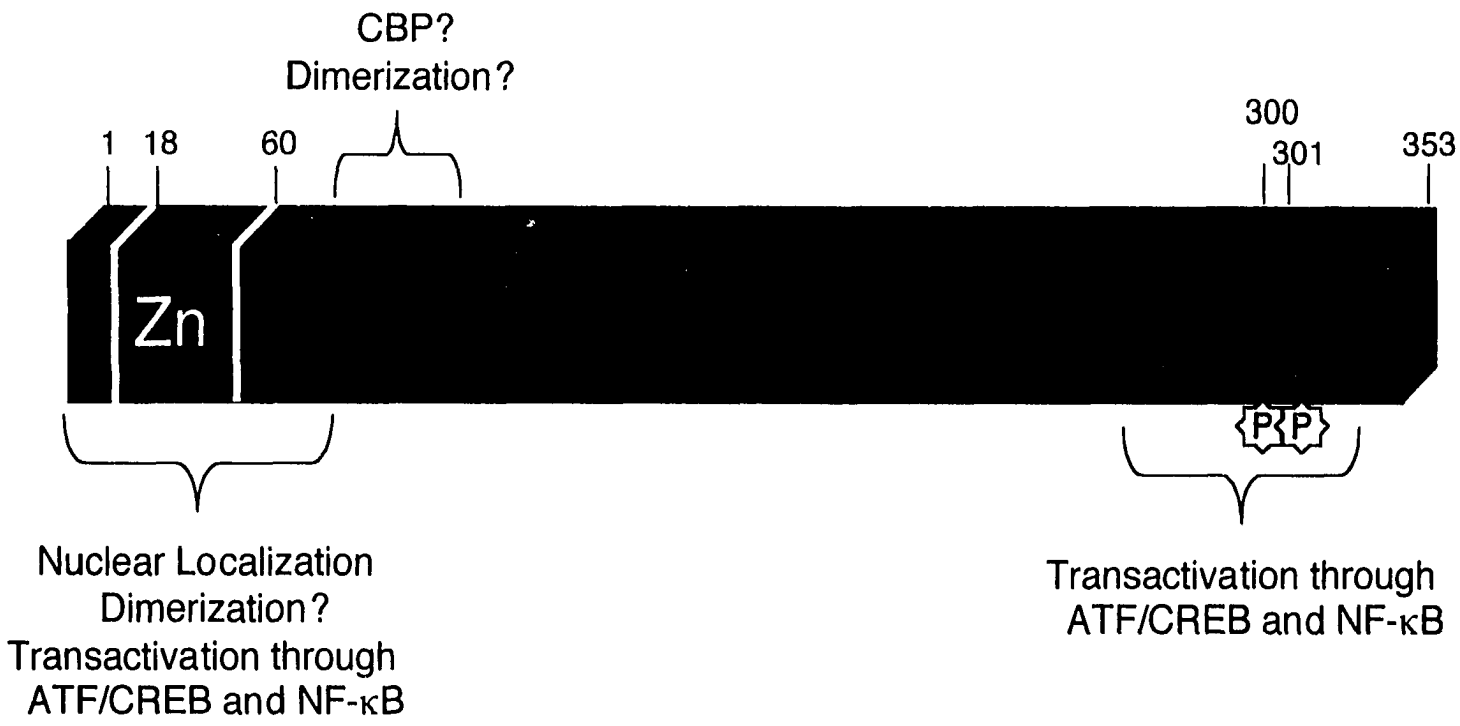

Figure 1.3 Functional domains of Tax. Location of the putative Zinc finger and phosphorylation sites are shown. Proposed functional domains are indicated above and below.

coding sequence, suggest that two distinct regions of Tax are critical for function (3, 45, 182, 194). The first is the extreme amino terminus of Tax that encompasses the putative Zinc finger. This region has been implicated in the nuclear localization of Tax as well as in Tax transactivation through both the HTLV-I LTR and the Human Immunodeficiency Virus LTR (61, 63, 182, 193, 194, 225). Transactivation through these two promoters occurs via two different cellular pathways, the CREB/ATF pathway and the NF-κB pathway respectively. Recent reports have suggested that Tax functions as a dimer (90, 91, 189, 205) and one group has localized the region of Tax necessary for dimerization to this area (90). However, another group demonstrated that Tax amino acids 123-204 are utilized for dimerization (205) and assignment of this function of Tax to a specific domain remains controversial.

The second critical region of Tax is located around amino acids 275 through 322. Point mutations and carboxyl deletions within this region abolish Tax transactivation properties (182, 194). Recently, Bex et al. identified two adjacent serine residues within this region (serines 300 and 301) as sites for Tax phosphorylation in vivo (21). These sites were shown to be critical for Tax transactivation function; however, the kinase responsible for mediating Tax phosphorylation remains to be identified. Finally, one recent report has implicated Tax amino acids 81 through 95 as being necessary for interaction with the cellular coactivator CBP (70). Supporting data for this observation has not yet emerged.

1.2b TAX TRANSACTIVATION THROUGH THE VIRAL LTR

It is well established that Tax is necessary for efficient transcription from the viral promoter and is believed to be critical in the switch from latency to high level viral replication. Tax works through three 21 base pair repeats in the viral

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promoter (figure 1.4) (27, 51, 157, 169, 186). Each of these repeats contains an off-consensus cAMP Response element (CRE) flanked by G and C rich sequences which are necessary for recognition by Tax (28, 50, 149). Together, these sequences are termed viral CREs and have been shown to serve as weak binding sites for the basic leucine zipper (bZIP) family of cellular transcription factors (88, 136). Tax appears to specifically work through CRE binding protein (CREB). Tax interacts with the bZIP region of CREB and serves to enhance and stabilize CREB binding to the viral CRE sites (2, 3, 16, 18, 28, 43, 119, 158, 222, 223, 224, 225, 231, 232). Additionally, Tax makes contacts with the minor groove of the DNA specifically within the GC rich flanking sequences (104, 118, 127). These GC rich flanks are absolutely required for Tax responsiveness and entry into the complex. Together, Tax, CREB and the viral CRE DNA create a stable ternary complex (figure 1.5). Within this complex, CREB serves primarily as a scaffolding molecule while Tax serves to recruit the cellular coactivator CBP to activate transcription (58, 111). Tax interacts with a specific region of CBP called the KIX domain with high affinity. The KIX domain therefore serves as an attachment site between the ternary complex and CBP. Once tethered to the ternary complex, CBP promotes strong transcriptional activation. Furthermore, Tax enables the virus to bypass the need for CREB phosphorylation in the recruitment of CBP, strongly activating viral gene expression without the proper cellular signals. Thus it is believed that Tax transactivation is mediated through the coactivator properties of CBP.

1.2c TAX TRANSFORMATION OF CELLS AND TRANSGENIC MICE

Both cell culture studies and transgenic mouse models support the notion that Tax is the transforming agent of HTLV-I. Initial cell culture studies made

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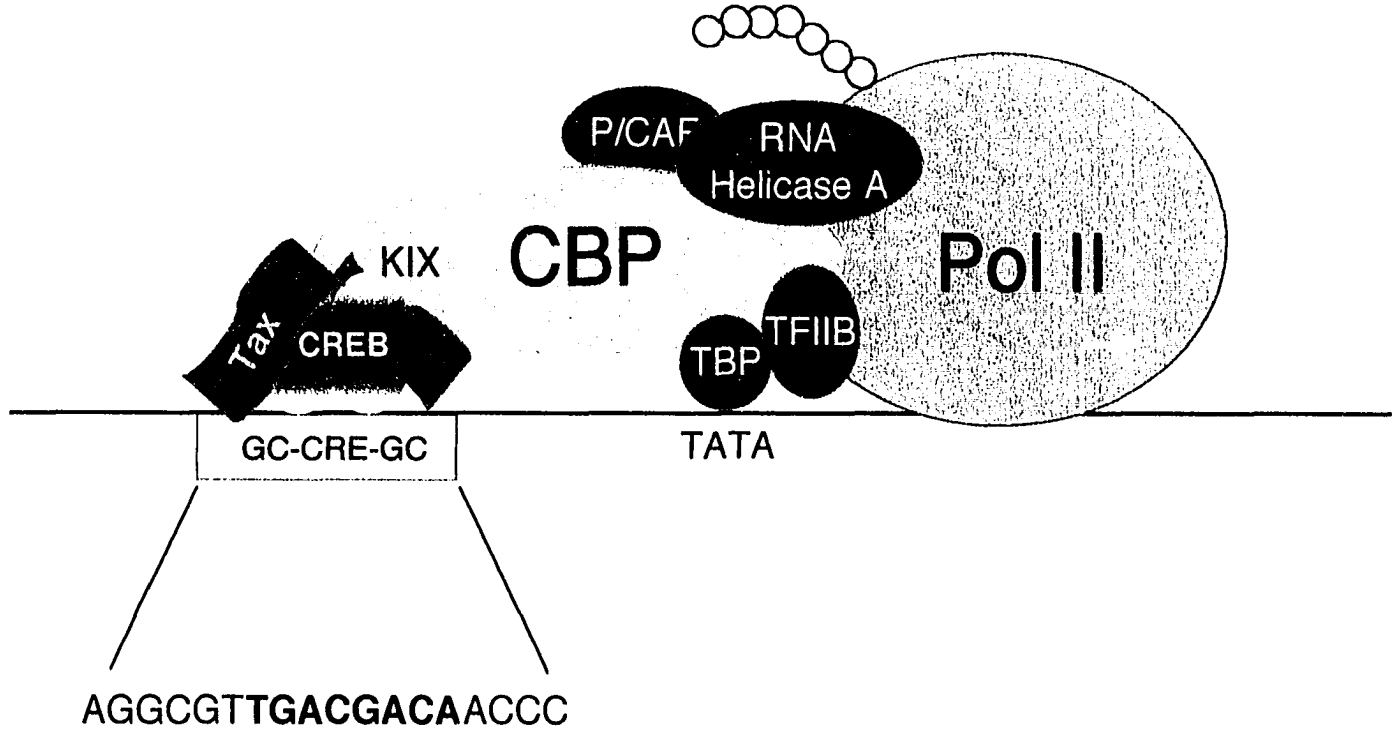

Figure 1.4 Model for Tax transactivation of the viral LTR. Tax interacts with the cellular transcription factor CREB and the viral CRE DNA elements at the viral promoter. This nucleoprotein complex serves to recruit the cellular coactivator CBP. Activation of transcription is mediated through the coactivator properties of CBP.

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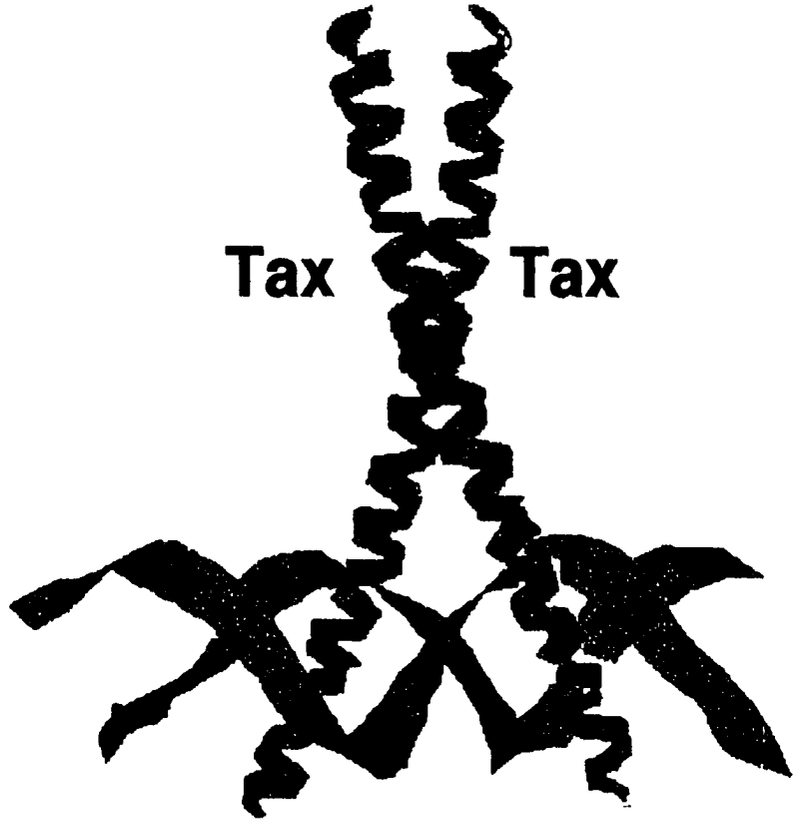

Figure 1.5 Schematic diagram of the ternary complex. Tax interacts with the bZip region of CREB, shown in blue, as well as with the minor groove of the DNA. This complex serves as a high affinity attachment site for the cellular coactivator CBP.

use of viral vectors to introduce the Tax gene into primary lymphocytes and showed that Tax specifically immortalizes the helper T-cell (CD3+, CD4+) population (4, 64). These cells show enhanced levels of CD25 and the IL-2 receptor α subunit (IL-2Rα), and are hyper-responsive to IL-2. Through the use of Tax mutants, several subsequent studies propose that Tax immortalization of cells in culture is heavily dependent on its ability to deregulate the cellular NF-κB pathway (84, 219). However, this remains controversial, and it has been suggested that other functions of Tax are important for full transformation to occur (170, 130).

Transgenic mice have also been made to study the role of Tax in cellular transformation. In early studies, fertilized eggs were microinjected with the Tax gene under the control of the viral LTR (74, 146). These mice express Tax mainly in muscle and thymus tissue and develop soft tissue tumors called neurofibromas. In a more recent attempt at making Tax transgenic mice, Tax expression was targeted to mature T-lymphocytes using the T-cell specific human gramzyme B promoter (66). These mice develop leukemic tumors in their ears, tails and legs as well as spleenomegaly. Together these studies support an independent role for Tax in the leukemogenesis associated with HTLV-I.

1.2d TAX DEREGULATION OF CELLULAR GENE EXPRESSION

Tax is a pleiotropic transcription factor which, in addition to activating the HTLV-I promoter, can deregulate the expression of many cellular genes. Tax has been shown to have both activation and repression activities. These effects of Tax are mediated through a variety of cellular transcription factors. Deregulation of cellular gene expression is believed to be the mechanism by which Tax transforms cells. Thus the deregulatory function of Tax is the focus of

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much of the research in the field as it may serve as a general model for cellular transformation.

1.2e ACTIVATION OF CELLULAR GENE EXPRESSION

Tax has been shown to activate many genes involved in cell growth and proliferation and it is believed that the aberrant activation of cellular genes initiates the pathway towards T-cell immortalization. Specifically, Tax has been shown to activate transcription of Interleukin-2 (IL-2), IL-2Rα, transforming growth factor β and several other growth control genes (12, 37, 81, 103, 120, 129, 190, 214). Additionally, there is some evidence that Tax activates the expression of the immediate early response genes, c-fos, fra-1, c-jun, junD, egr-1 and egr-2 (46, 98, 144). Tax stimulation of these genes has been shown to be mediated primarily through the NF-κB pathway and, to a lesser degree, other transcription factors such serum response factor (SRF) (5, 6, 12, 47, 48, 120, 190).

1.2f TAX ACTIVATION THROUGH THE NF-κB PATHWAY

Several studies have shown that the NF-κB pathway is constitutively active in HTLV-I transformed cells and recently a large effort has gone into understanding this phenomenon (12, 112, 114, 122, 132, 140, 219). These studies have revealed that activation of this pathway by Tax occurs in a unique manner. In a normal resting T-cell, NF-κB is bound by an inhibitor, IκB, and is sequestered in the cytoplasm. Upon T-cell activation, the inhibitor is phosphorylated and subsequently degraded by the proteosome complex. NF-κB is then free to travel to the nucleus and activate target genes (reviewed in 10, 11, 17). Tax serves a dual role in constitutively activating this pathway. First, Tax binds and stimulates the activity of a kinase, MEKK1, a signaling molecule

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in the pathway leading to phosphorylation of IκB (55, 112, 132, 226). As a result, IκB is constitutively phosphorylated and NF-κb is free to activate transcription in HTLV-I transformed and Tax-expressing cells. At the same time, Tax targets the inhibitor to the proteosome complex and strengthens the interaction between IκB and the proteosome (171). It is believed that these two activities of Tax operating in concert results in constitutive activation of the NF-κB pathway in HTLV-I transformed or Tax-expressing cells. Tax upregulation of this pathway has been implicated in the observed activation of many growth related genes, including IL-2 and IL-2Rα (12, 37, 65, 120, 129, 190, 214).

1.2g TAX ACTIVATION THROUGH OTHER TRANSCRIPTION FACTOR PATHWAYS

Early reports demonstrated that Tax stimulates DNA binding of many different transcription factors including ATF1, ATF2, CREB, SRF, and AP-I (8, 43, 131, 199, 231, 232). Thus it was hypothesized that Tax could upregulate cellular gene targets by increasing the DNA binding activity of transcription factors which activate expression of these genes. For example, Tax activates expression of c-fos, egr-1 and egr-2 through SRF and its DNA response element, the CArG Box (5, 46, 144). Tax directly interacts with SRF and stimulates SRF binding to DNA (8, 47). However, it remains unclear whether Tax activation of these genes occurs simply through increasing transcription factor binding to DNA or whether additional factors are involved.

1.2h REPRESSION OF CELLULAR GENE EXPRESSION

In addition to its ability to aberrantly activate cellular gene expression, the Tax protein can also inappropriately repress some promoters. To date only a handful of genes have been shown to be repressed by Tax. Interestingly,

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however, these genes tend to be linked to DNA repair and cell growth control. For instance, Tax represses expression of β-polymerase (89). β-polymerase is a base-excision DNA repair enzyme (195). Down regulation of β-polymerase by Tax is believed to be significant in the accumulation of genetic abnormalities observed in ATL cells. Additionally, Tax represses the promoters for the tumor suppressor p53 and lck, a non-receptor tyrosine kinase involved in T-cell activation (117, 209). Tax mediated repression of these select genes suggests that repression may be critical in the HTLV-I transformation pathway.

Tax repression of cellular gene expression is mediated through the basic Helix-Loop-Helix (bHLH) family of transcription factors and their DNA response element, the E box (208). Tax does not interfere with bHLH DNA binding, and a direct interaction between Tax and any of the bHLH proteins has never been observed (183, 208). It is hypothesized that Tax repression of cellular gene expression through the bHLH proteins may be mediated through additional factors that remain to be identified.

1.2i TAX DEREGULATION OF CELL CYCLE

Several recent reports have demonstrated that Tax directly deregulates several checkpoints within the cell cycle (figure 1.6). Tax disrupts the G1/S checkpoint by two mechanisms. First, Tax interferes with the function of the tumor suppressor $p16^{INK4A}$ (125, 200). $p16^{INK4A}$ is an inhibitor of the CDK4/Cyclin D complex, a kinase that normally serves to phosphorylate pRb and hence activate E2F-mediated transcription and progression into S phase (137, 159, 184, review). $p16^{INK4A}$ is therefore an important regulator of cell cycle progression. Tax binds to $p16^{INK4A}$ and disrupts $p16^{INK4A}$ inhibition of CDK4/cyclin D, causing a loss of proper CDK4/cyclin D regulation. Second, it was recently shown that Tax activates E2F-mediated transcription

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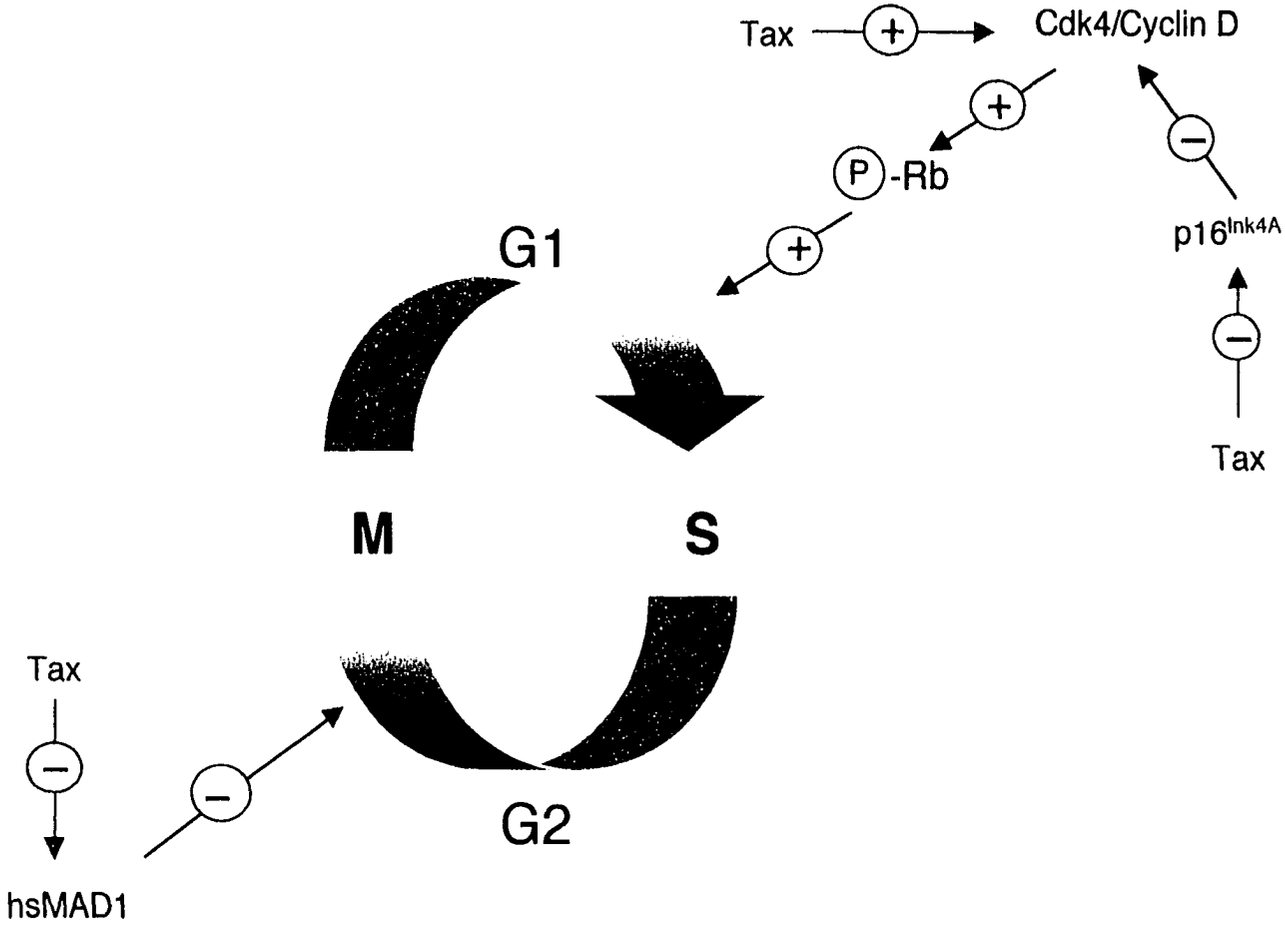

Figure 1.6 Tax deregulates the cell cycle. Tax interferes with both the G1/S and G2/M checkpoints. Tax simultaneously represses $p16^{Ink4A}$ and activates Cdk4 to inappropriately drive the cell into S phase. Additionally, Tax represses hsMAD1 function, disrupting the mitotic spindle checkpoint, a cellular mechanism to ensure the proper segregation of chromosomes before cell division.

independently of $p16^{INK4A}$ (116, 147). Tax was shown to directly increase CDK4 kinase activity resulting in hyperphosphorylation of pRb. Thus, Tax has two mechanisms for disrupting the G1/S checkpoint; interference with the $p16^{INK4A}$ inhibitor and activation of Cdk4 kinase activity. Both activities lead to hyperphosphorylation of Rb with consequential progression into S phase.

Tax also disrupts the mitotic spindle checkpoint. In a two-hybrid screen, Tax was found to bind hsMAD1, a mitotic spindle checkpoint protein that controls for proper alignment of chromosomes on the spindle before cell division (92, 174). HsMAD1 functions as a heterodimer with hsMAD2. Tax binds hsMAD1 within the same region needed for dimerization with hsMAD2, thereby inactivating hsMAD1 by preventing dimerization. As a result, the mitotic spindle checkpoint is disrupted and it is hypothesized that this function of Tax may lead to the karyotypic abnormalities and multinuclei seen in Tax-expressing cells.

1.3a CBP

CBP is a multifunctional coactivator which plays a regulatory role in many cellular processes including growth, differentiation and apoptosis (87, review). As a coactivator, CBP does not bind DNA but serves as a bridge between upstream activators and the general transcription machinery. Hence, CBP is thought to recruit the RNA polymerase II holoenzyme to activated promoters. In light of recent studies however, CBP function as a transcriptional coactivator appears to be much more complex and seems to extend well beyond its ability to serve as a bridging molecule. For example, CBP has been implicated in modulating chromatin structure as it carries both intrinsic and extrinsic histone acetyltransferase activities (14, 151, 220). Additionally, CBP appears to be a central integrator of cellular signaling as many pathways seem to converge upon CBP (185, review). Thus, CBP regulation may serve as an additional

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level of control for cellular gene expression. This point is enunciated in the recent emergence of evidence implicating CBP as a tumor suppressor as loss of proper CBP function has been linked to malignancy (60, review).

1.3a CBP AND p300

Shortly after its discovery, CBP was realized to be highly homologous to a previously identified protein, p300 (7). Sequence comparison indicates that CBP and p300 are 75% homologous and 63% identical across their entire length (figure 1.7) (60, review). Some conserved regions show higher degrees of homology, such as within the cysteine/histidine rich domains C/H1, C/H2 and C/H3. The strong similarity between CBP and p300 led to the initial hypothesis that CBP and p300 are functionally redundant. Several studies however, have demonstrated that while there are some overlapping functions between CBP and p300, there are also specific functions which are unique to one or the other.

1.3b INTERACTIONS WITH TRANSCRIPTION FACTORS

CBP was initially identified and named for its ability to bind to phosphorylated CREB (32). Since that time, CBP has been shown to be quite pleiotropic in nature as it interacts with a broad range of unrelated cellular transcription factors (figure 1.8) (87, review). These factors include the nuclear hormone receptors, c-Jun, c-Fos, c-Myb, p53 and many more (9, 13, 15, 39, 68, 95, 123). Each of these factors binds to a distinct domain of CBP and utilizes CBP to potentiate transcription from their respective target promoters. Some regions of CBP appear to be particularly important for transcription factor binding. For instance, the KIX domain of CBP binds to approximately ten different transcription factors, including Tax. The solution structure of this particular domain of CBP has recently been solved bound to phosphorylated CREB (165).

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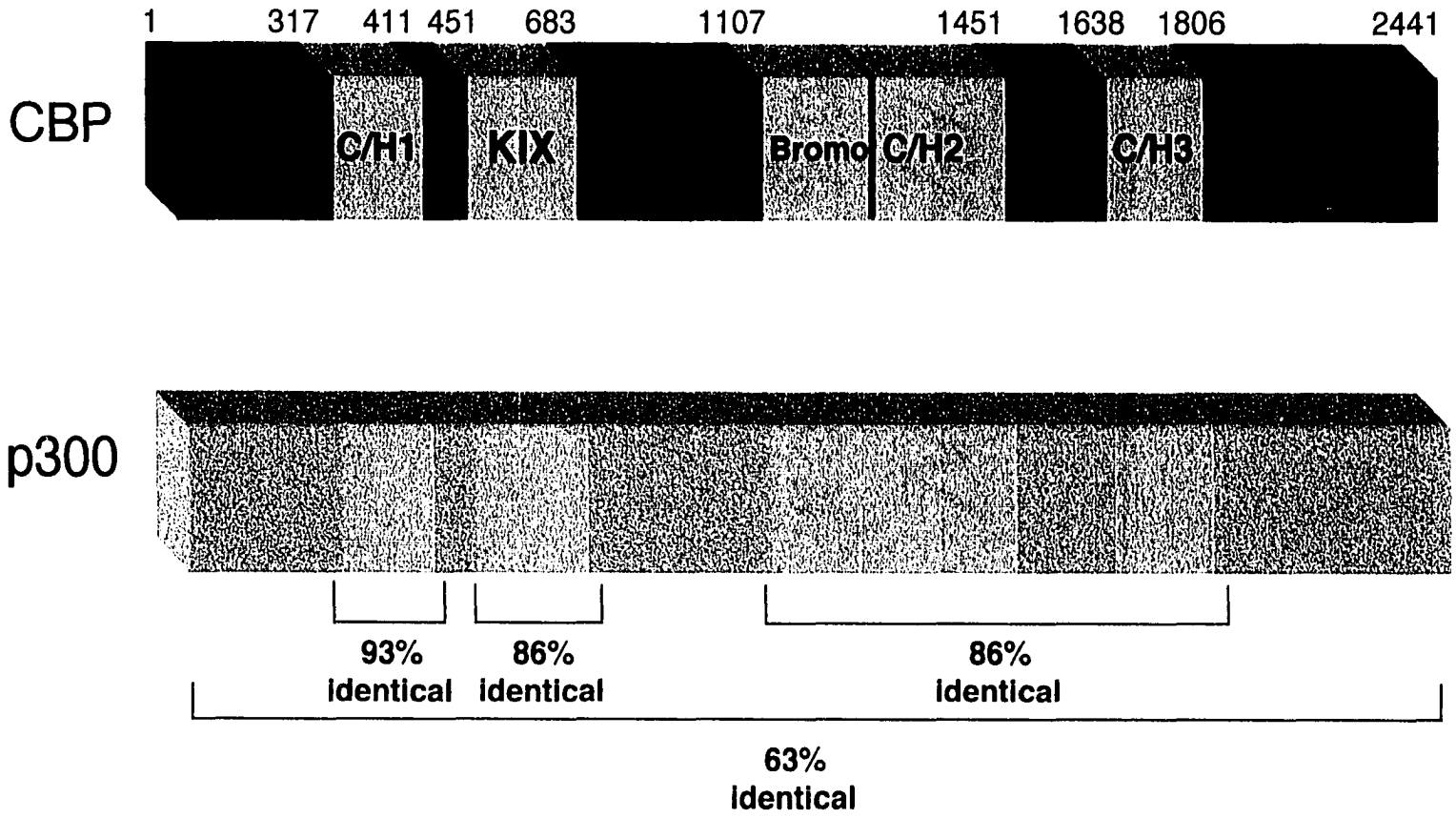

Figure 1.7 Sequence comparison of CBP and p300. Percent identity between CBP and p300 is indicated.

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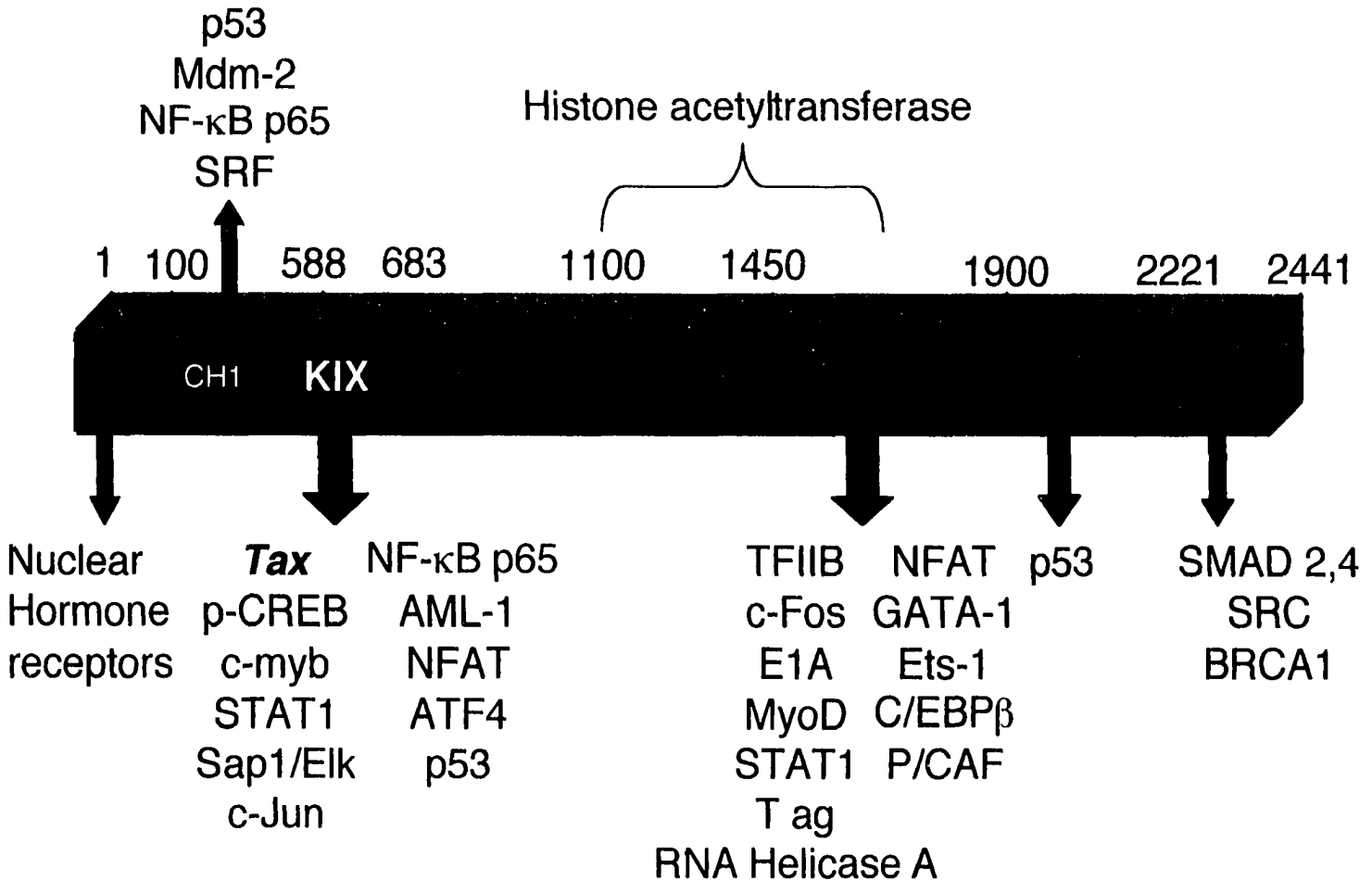

Figure 1.8 Schematic diagram of CBP. Relevant protein domains are illustrated. Transcription factors which bind CBP are also indicated.

The structure indicates that the KIX domain of CBP consists of three alpha helices that together form a hydrophobic groove. Phosphoryated CREB fits down in the groove suggesting that though many transcription factors recognize this domain of CBP, KIX can only accommodate binding of one factor at a time. This finding suggests that transcription factors may compete for access to this site creating an additional layer of transcription regulation and coordination mediated through CBP. It will be interesting to see if a similar structural motif exists in other transcription factor binding sites on CBP.

In addition to binding upstream activators, CBP also interacts with the general transcription machinery. In vitro and in vivo studies have shown CBP complex formation with both TBP and TFIIB (1, 40, 110, 229). Additionally, CBP co-purifies with RNA Polymerase II (Pol II) from HeLa nuclear extracts (97). CBP does not appear to directly interact with any of the RNA Pol II subunits, however biochemical studies have identified RNA Helicase A as a bridging molecule between CBP and RNA Pol II (145). RNA Helicase A interacts with both CBP and RNA Pol II in vitro and the three proteins form a complex in vivo. RNA Helicase A is a double stranded DNA/RNA helicase. Thus the participation of RNA Helicase A in the CBP-RNA Pol II complex suggests that CBP recruitment to a given promoter may permit localized DNA unwinding. Thus, CBP coactivation properties may involve both recruitment of the general transcription machinery as well as directed changes in the DNA through chromatin remodeling and DNA unwinding which facilitate transcription initiation.

1.3c CBP HISTONE ACETYLTRANSFERASE ACTIVITY

Both CBP and p300 possess intrinsic histone acetyltransferase (HAT) activity (14, 151). Previous biochemical studies have correlated histone

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hyperacetylation with transcriptionally active chromatin regions (29, review). This hyperacetylation occurs at lysine residues within the amino terminal tail region of histones. This modification neutralizes the positive charge associated with the lysine residues and presumably destabilizes inter-nucleosome interactions and disrupts higher order chromatin structure, making the DNA more accessible to transcription machinery (126). Thus the finding that CBP has HAT activity supports the notion that CBP may acetylate histones at targeted promoters and that this activity may play a crucial role in CBP coactivation. However, the exact mechanism of how CBP HAT activity and histone acetylation may facilitate transcription remains to be determined.

In addition to intrinsic HAT activity, CBP also has an associated HAT, p300/CBP associated factor (p/CAF) (220). p/CAF binds CBP at a region that is adjacent to CBPs defined intrinsic HAT domain (14, 151, 220). The finding that CBP has both intrinsic and associated HAT activity raises the question of why there is an association between two proteins with overlapping function. One possibility is that both are needed for efficient histone acetylation and chromatin rearrangement in vivo. Another possibility is that CBP and p/CAF have distinct acetylation substrates and complementary function. Recent studies have suggested that the latter may be true. Schiltz et al. have determined the acetylation specificities of p300 and p/CAF on core histones in vitro and have found that p/CAF has a very narrow preference for lysines on histones H3 and H4 (176). p300, on the other hand, was found to acetylate all four core histones and had a broader range of preferred lysine residues. Furthermore, biochemical studies have demonstrated transcription factor-specific requirements for CBP and/or p/CAF HAT activity for transcription activation (109, 164, 217). For instance, MyoD-mediated transcription requires the presence of both CBP and p/CAF in the activation complex (164). However, only p/CAF

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appears to contribute HAT activity while CBP apparently serves as a complex scaffold and to recruit RNA Pol II. These findings further support the notion that CBP and p/CAF have overlapping yet distinct functions.

The CBP HAT story took an interesting turn with appearance of several recent reports demonstrating that CBP can also acetylate both regulatory and general transcription factors. The first of these reports showed that CBP acetylates the tumor suppressor p53. CBP specifically acetylates p53 within the carboxyl terminal 30 amino acids, a region previously implicated in regulating p53 DNA binding activity (67, 107). CBP acetylation of p53 occurs at two conserved lysine residues and increases p53 sequence-specific DNA binding activity in a dose dependent manner. Acetylation of these sites has been detected in vivo and appears to increase in response to DNA damage (124). Thus p53 acetylation by CBP has been proposed to serve a regulatory role in activating p53 function. Subsequently, CBP has been shown to acetylate other transcription activators as well as the general transcription factors TFIIEβ and TFIIF (26, 80, 230). The functional relevance of CBP acetylation of these other protein substrates remains to be elucidated.

1.3d CBP TRANSLOCATIONS AND LEUKEMIA

A direct role for CBP in leukemogenesis has recently emerged (60, review). The CBP gene has been identified in somatic chromosomal translocations that lead to a type of acute myeloid leukemia (AML) (figure 1.9) (25, 59). As a result of this genetic rearrangement, most of the CBP coding region is fused in frame to MOZ, a gene which encodes a monocytic zinc finger protein. The cellular function of MOZ is unknown but is suspected to be an acetyltransferase based on sequence homologies (167). This translocation appears to keep both the MOZ and CBP proteins mostly intact and it is hypothesized that the resulting

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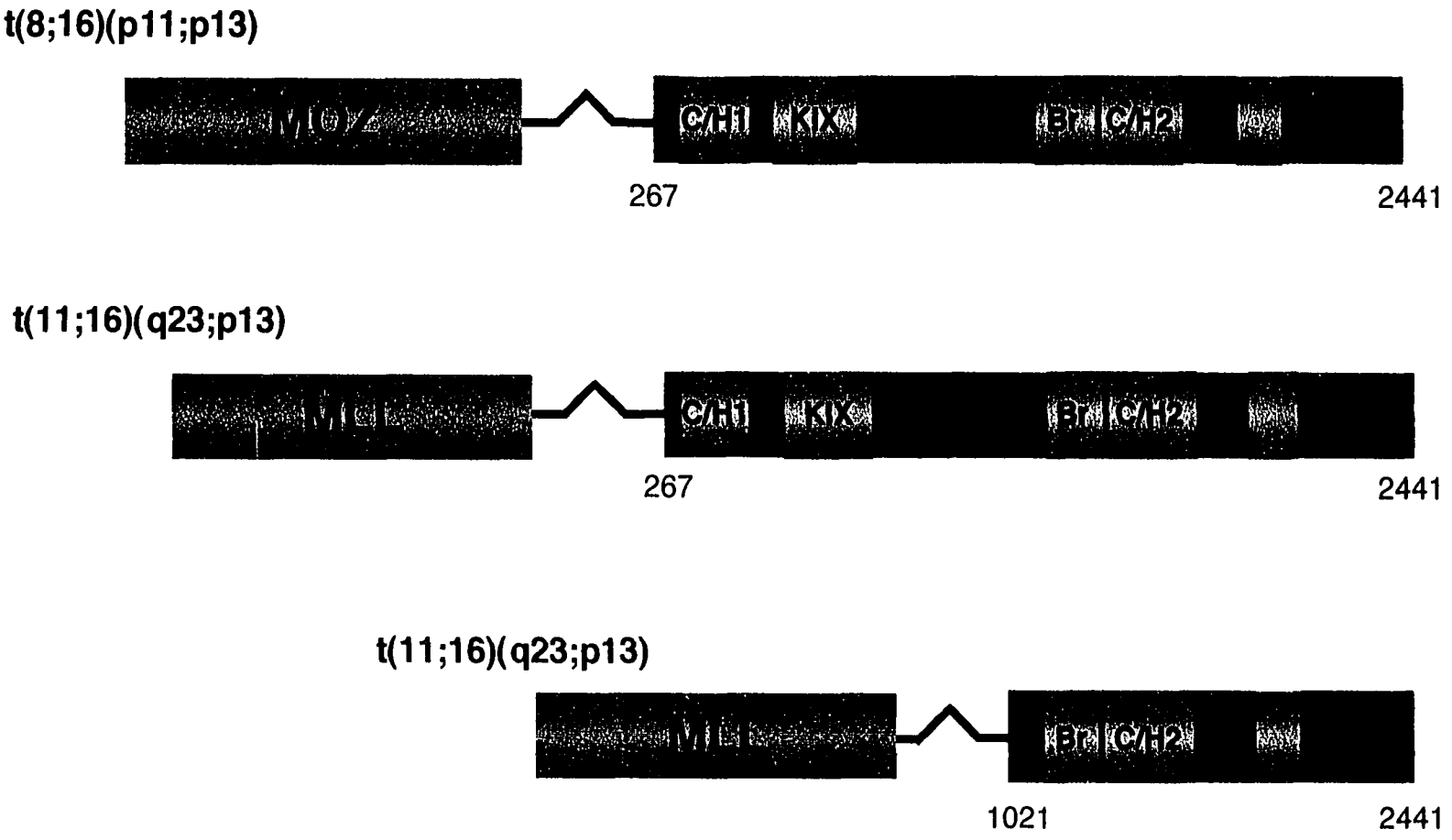

Figure 1.9 Fusion proteins generated through CBP translocations. The t(8;16)(p11;p13) translocation fuses a large portion CBP to MOZ, a putative acetyltransferase. The t(11;16)(q23;p13) translocation fuses CBP to another putative acetyltransferase, MLL. Both identified CBP breakpoints are shown. Note that both of these retain the CBP histone acetyltransferase domain.

fusion protein may be a hyperactive acetyltransferase. How aberrant acetyltransferase activity might lead to leukemogenesis is currently unknown.

CBP has also been linked to a second translocation associated with treatment-related malignancies which arise as a result of previous cancer therapies (172, 196, 203). In this case, CBP becomes fused to MLL, another protein implicated in chromatin remodeling based on sequence homologies (figure 1.9) (19). In these translocations, CBP appears to have two possible breakpoints (196). One of these is exactly the same as that identified in the AML translocation. The second occurs further downstream in CBP but still maintains a large portion of the CBP protein including the HAT domain. A p300-MLL translocation has also recently been identified in a patient with treatment-related leukemia (79). Once again these are in-frame, and most likely gain-of-function, fusions. Though the exact cellular consequences of these translocations is unknown, it is interesting that in both the AML and treatment-related translocations, CBP and p300, histone acetyltransferases, are fused to other proteins also implicated in chromatin rearrangement. These findings suggest a critical role for chromatin structure in the regulation of cellular gene expression and cellular homeostasis.

1.3e RUBINSTEIN-TAYBI SYNDROME

Rubinstein-Taybi syndrome (RTS) is a developmental disorder characterized by mental and growth retardation, broad thumbs and toes, and facial abnormalities (173). Additionally, RTS patients have a propensity for malignancy. Recently, RTS was linked to chromosomal mutations within one CBP allele (160). These mutations include both chromosomal rearrangements as well as point mutations within the CBP coding region and result in loss of CBP protein function. As a result, RTS patients have only a 50% normal dose of

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functional CBP protein. These findings suggest that CBP is critical for normal cell function as well as normal developmental processes. Furthermore, they indicate that p300 is incapable of substituting for the loss of CBP function. Thus, CBP appears to be limiting in the cell and both copies of the CBP gene are required to produce sufficient amounts of CBP protein to fulfill cellular needs.

1.3f CBP TRANSGENIC MICE

Both CBP and p300 transgenic mice have been generated and characterized (204, 221). Homozygous mutants of either CBP or p300 die in utero, having defects in both morphogenesis and cell differentiation. These findings demonstrate that CBP and p300 are both essential for development and suggest that they can not functionally compensate for one another. Compound heterozygous mutant mice (p300+/-; CBP+/-) also exhibit full embryonic lethality. Heterozygous knockouts of either CBP or p300 are only partially lethal where 45% survive. Characterization of the viable CBP and p300 heterozygous mice from this study has not yet been published. However, another group has generated CBP heterozygous mice and has reported skeletal abnormalities reminiscent of those seen in RTS patients (204).

Together these findings indicate that at least 75% of the normal combined dose of CBP and p300 is required for viability. This dosage sensitivity may be due to the fact that CBP and p300 are utilized by many transcription factor pathways, where even a 25% decrease in the combined concentration of the proteins leaves the cell unable to perform critical processes. Another possibility is that CBP and p300 each have distinct, essential roles in the cell and that a 50% decrease in either one impairs these activities. This second possibility is supported by the finding that in p300-/- mouse embryo fibroblasts, the retinoic acid receptor is unable to activate transcription in response to retinoic acid

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(221). On the other hand, CREB is fully responsive to PKA in these cells and activates transcription to a level similar to that seen in wild type cells. Thus it appears that a decrease in either CBP or p300 protein concentration can disable distinct cellular pathways and that CBP and p300 can not entirely substitute for one another.

1.3g COMPETITION FOR LIMITING CBP

The finding that CBP haplo-insufficiency leads to severe developmental disorders, both in humans and mice, implies that CBP is limiting in some cell types and that p300 is unable to overcome CBP deficiencies. This notion is further supported by biochemical studies suggesting coactivator competition between transcription factors. For example, it has been proposed that the nuclear hormone receptors and AP-I compete for CBP and that this results in the observed nuclear hormone receptor inhibition of AP-I mediated transcription activation (95). A similar mechanism of coactivator competition has been proposed for the observed reciprocal repression between p53 and NF-κB (213). These studies suggest that CBP is a central integrator of many transcription factor pathways and that competition for limiting amounts of CBP generates transcription factor cross talk, a novel mechanism for regulation of gene expression.

Based on these findings, its seems likely that the HTLV-I Tax protein may deregulate a large number of cellular genes through its interaction with the KIX domain of CBP. Tax has been shown to interact with the KIX domain with high affinity in the context of the viral CRE nucleoprotein complex at the HTLV-I promoter. Additionally, the Tax protein is intermittently produced at very high levels in an infected T-cell (191). Consequentially, Tax may sequester the available, limiting amount of CBP, leaving other factors which interact with the

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KIX domain of CBP unable to activate transcription. Thus, the Tax-KIX interaction may derail CBP mediated transcription regulation and result in widespread repression of host cell gene expression. Tax sequestration of CBP may represent a novel mechanism for Tax deregulation of host cell gene expression and contribute to HTLV-I associated leukemogenesis.

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CHAPTER 2
BINDING OF THE HUMAN T-CELL LEUKEMIA VIRUS TAX PROTEIN TO THE COACTIVATOR CBP INTERFERES WITH CBP-MEDIATED TRANSCRIPTIONAL CONTROL

Chapter two describes a study of competition between Tax and the oncoprotein c-Jun for the KIX domain of CBP. This work will be published in the journal *Oncogene*. Jian-ping Yan and Aida Ulloa are co-authors on the paper as they cloned and expressed the KIX deletion mutants and point mutants used in figure 2.5. The work appears here exactly as in the publication. However, data cited as "data not shown" in the manuscript are shown here as supplemental figures. The citation for the publication appears below.

Van Orden, K., J.-P. Yan, A. Ulloa and J. K. Nyborg. 1999. *Oncogene* **18** (in press)

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2.1 ABSTRACT

The HTLV-I oncoprotein Tax is required for high level viral transcription and is strongly linked to HTLV-I-associated malignant transformation. Tax stimulates HTLV-I transcription through high affinity binding to the KIX domain of CBP, a pleiotropic coactivator. Several cellular proteins, including c-jun, also bind to KIX and utilize CBP as a coactivator. To test whether Tax binding to KIX may disable cellular CBP function, we examined the potential interplay between Tax and c-jun for binding to KIX. We show that Tax represses the transcription function of c-jun in vivo and demonstrate that both transcription factors bind to an overlapping minimal region of KIX in vitro. c-jun binding to KIX is displaced by Tax, indicating that their binding is mutually exclusive and providing a molecular basis for the observed repression. The competition between Tax and cellular transcription factors for CBP represents a novel pathway for HTLV-I dependent deregulation of gene expression, and may have significant implications for cellular homeostasis and transformation in the HTLV-I infected T-cell.

2.2 INTRODUCTION

It is estimated that between 11 and 20 million people worldwide are infected with the human T-cell leukemia virus type I (HTLV-I). While most infected individuals remain asymptomatic, a small percentage develop an aggressive and fatal lymphoproliferative disease called adult T-cell leukemia (ATL) several decades following retroviral infection (18, review). A single HTLV-I-encoded protein, called Tax, has been strongly implicated in the oncogenic transformation associated with HTLV-I infection (23, 24). Tax is a regulatory protein that is required for high level expression of the HTLV-I genome. Tax stimulates transcription through a series of complex protein-DNA

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and protein-protein interactions. The transcriptional control region of HTLV-I carries three 21 bp repeats, called viral CREs, that serve as binding sites for members of the basic-leucine zipper (bZIP) family of cellular transcription factors (2, 11, 12, 15, 19, 31, 48, 49). Tax specifically interacts with the bZIP domain of the transcription factor CREB, as well as with nucleotides which immediately flank the CREB binding site in the viral CRE (3, 4, 11, 15, 19, 28, 31, 34, 49). These interactions by Tax lead to the formation of a stable ternary complex on the HTLV-I promoter that serves as a high affinity binding site for the recruitment of the multifunctional cellular coactivator CREB binding protein (CBP) (20, 29). Tax anchoring of CBP to the HTLV-I promoter is believed to result in strong transcriptional activation mediated through the coactivator functions of CBP (8, 33, 47).

CBP is a very large protein, 2441 amino acids in length, with several discrete domains that bind a variety of structurally unrelated transcription factors (40, review). One of these CBP domains, called KIX (aa 451-719) has been shown to interact with phosphorylated CREB, c-myb, c-jun, and Tax (7, 10, 16, 17, 20, 29, 30, 32). Because Tax shares the same CBP binding site as several important cellular transcription factors, we hypothesized that Tax binding to KIX might inhibit access of other transcription factors to CBP, thus altering patterns of cellular gene expression. This hypothesis is based in part on observations that CBP may be limiting in the cell (35), thus creating a competition between transcription factors for available CBP. Competition for limiting CBP has been proposed as a mechanism for the observed repression of AP-1 transcription activity by nuclear hormone receptors (27). Furthermore, there is evidence that the adenovirus E1A protein antagonizes cellular gene expression through disruption of transcription factor/CBP interactions (9, 43, 47).

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To test the hypothesis that Tax binding to the KIX domain of CBP may at least partially disable cellular CBP function, we examined the potential interplay between Tax and c-jun for binding at the KIX domain of CBP. We selected the cellular protooncoprotein c-jun for this analysis, as c-jun regulates a large number of target genes, and has a role in cell proliferation, differentiation and transformation (5). We demonstrate that Tax represses the transcription activity of c-jun, and reciprocally, c-jun represses the transcription activity of Tax. Specific Tax point mutants, defective for KIX binding in vitro (25), are defective for c-jun repression in vivo. Binding assays show that, like Tax (46), c-jun interacts with a structural domain of KIX (~aa 588-665) that is composed of an extensive hydrophobic core (36). Consistent with these observations, we also demonstrate that Tax displaces c-jun binding to KIX in vitro.

These data have potentially important implications for cellular homeostasis, and possibly cellular transformation. Intermittent high level Tax expression in an HTLV-I-infected T-cell may lead to occupation of the KIX domain by Tax, with the subsequent partial inactivation of CBP. This inactivation would likely disable specific cellular transcription factor pathways, with resulting global deregulation of cellular genes that are regulated by these factors.

2.3 RESULTS

Previous research has demonstrated that the HTLV-I Tax protein and the cellular protooncoprotein c-jun both interact with the KIX domain of CBP, and utilize CBP as a transcription coactivator (Fig. 2.1) (7, 10, 20, 29). This interaction within a common domain of CBP raises the possibility that Tax and c-jun may compete for the available CBP in the cell, thus mutually repressing the function of the transcription factors. To test this hypothesis, we measured

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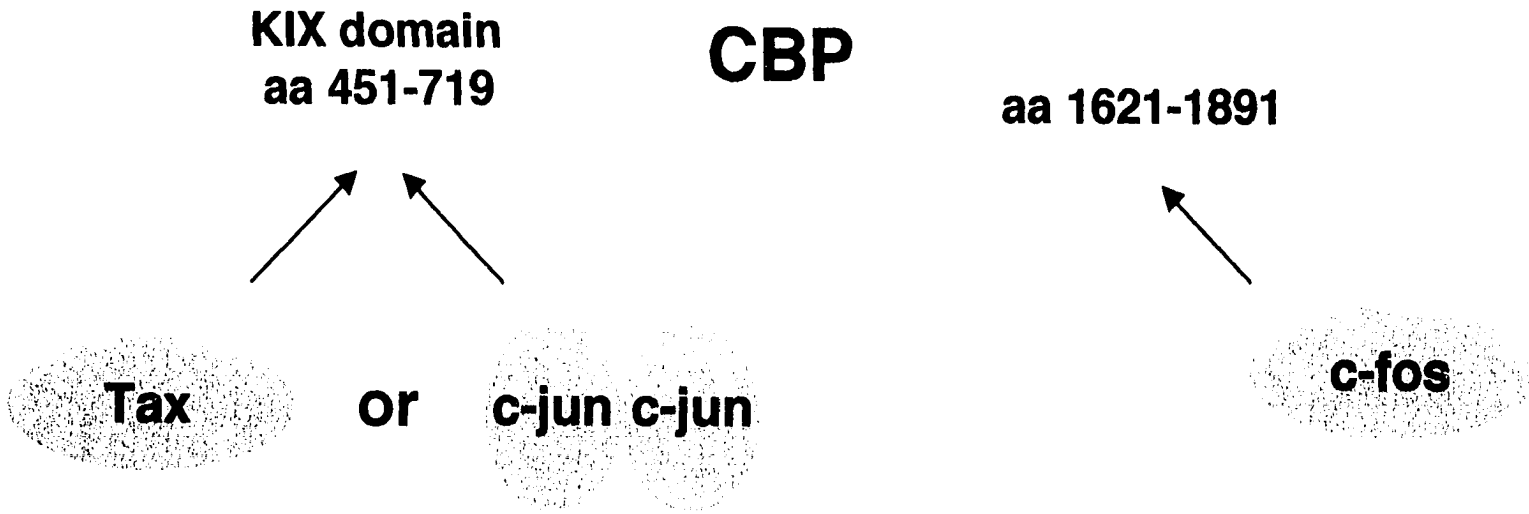

Figure 2.1 Schematic illustration of CBP showing the amino acid position of the KIX domain, which interacts with Tax and c-jun. The region of CBP that interacts with c-fos is also shown.

the transcription function of Tax and/or c-jun using transient cotransfection assays in HTLV-I-negative Jurkat T-cells. The transcription function of Tax and c-jun was measured by luciferase reporter plasmids carrying three copies of either the Tax-responsive viral CREs (viral CRE-Luc), or c-jun-responsive AP-1 sites (AP-1-Luc), respectively, cloned upstream of the thymidine kinase minimal promoter (Fig. 2.2) (also see 20).

2.3a Tax represses the transcription function of c-jun.

We first tested the transcription activity of the c-jun-responsive AP-1-Luc reporter plasmid in the presence of expression plasmids for c-jun and Tax. As expected, cotransfection of the RSV-c-jun expression plasmid (6) strongly activated transcription from the AP-1-containing promoter (14-fold) (Fig. 2.3A, lanes 1, 2). Although c-jun normally functions as a heterodimer with c-fos, under transfection conditions the high level expression of c-jun likely drives homodimer formation, enabling c-jun to directly activate transcription through the AP-1 enhancer elements (42). To test whether Tax can repress the transcription function of c-jun, we cotransfected the Tax expression plasmid (14). Figure 2.3A shows that Tax repressed c-jun-mediated transcription from the AP-1-Luc reporter plasmid (3-fold repression) (compare lanes 2 and 3). This same amount of the Tax expression plasmid strongly activated transcription from the viral CRE-Luc reporter plasmid (75-fold), indicating that the Tax expression plasmid was functioning properly, and Tax protein was not toxic to the cells (Fig. 2.3A, lanes 4, 5). Since Tax is a highly pleiotropic transcriptional deregulator, it is possible that the observed repression of c-jun occurred through Tax repression of transcription of the RSV-driven c-jun expression plasmid. To test this possibility, we measured the effect of cotransfected Tax on an RSV-driven luciferase reporter plasmid. We did not

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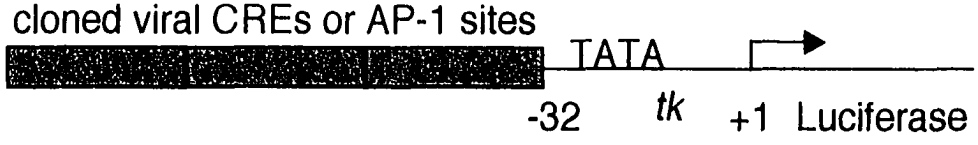

Figure 2.2 Schematic illustration of the AP-1 and viral CRE luciferase reporter constructs used in transient cotransfection assays. Note that the two constructs are identical except for the transcription factor binding sites cloned upstream of the thymidine kinase (*tk*) minimal promoter (see 20).

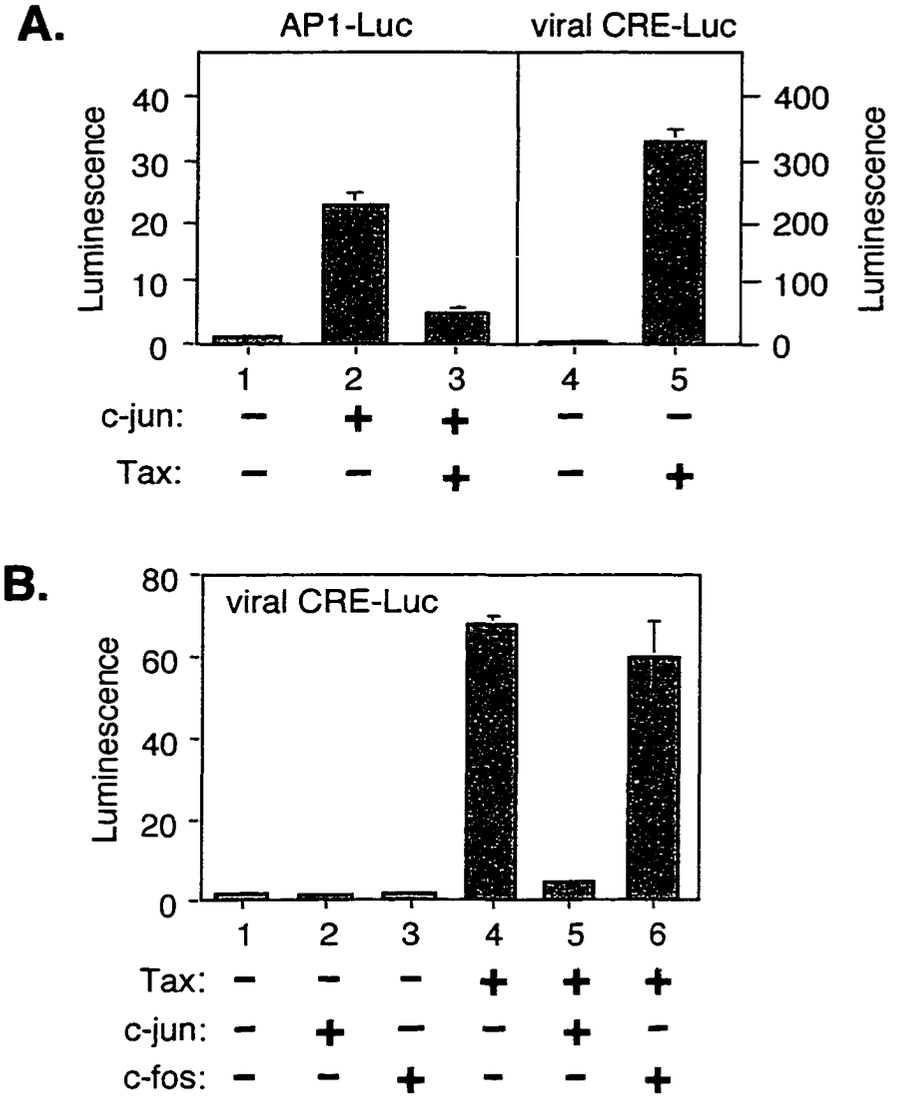

Figure 2.3 Reciprocal repression between Tax and c-jun. Transient cotransfection assays were carried out in the HTLV-I negative human Jurkat T-cell line. (A) Either the AP-1-Luc reporter (400 ng) (lanes 1-3) or the viral CRE-Luc reporter (400 ng) (lanes 4, 5) was cotransfected with the c-jun expression plasmid (RSV-c-jun [6]; 200 ng) and/or the Tax expression plasmid (HTLV-I-Tax [14]; 200 ng) as indicated. Although different luminescence scales are shown, the two reporter constructs were assayed within the same experiment. The values shown are the luminescence +/- the standard error from two independent experiments performed in triplicate. (B) Cells were cotransfected with the viral CRE-Luc reporter plasmid (400 ng) (lanes 1-6), the Tax expression plasmid (50 ng) (lanes 4-6), and either the c-jun (lanes 2, 5) or c-fos (lanes 3, 6) expression plasmids (400 ng). The values shown are the luminescence +/- the standard error from one experiment performed in triplicate.

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observe changes in RSV-directed transcription at any of the Tax expression plasmid concentrations tested (Fig. 2.7). Furthermore, Western blot analysis using an anti-Tax antibody indicated that c-jun had no effect on Tax synthesis from the cotransfected HTLV-I-driven expression plasmid (Fig. 2.8).

2.3b c-jun represses the transcription activity of Tax.

The above observation supports the hypothesis that both Tax and c-jun utilize CBP to mediate transcription, and that Tax can compete with c-jun for available CBP. If this hypothesis is correct, then titration of c-jun into transfection reactions should similarly repress Tax-activated transcription from the viral CRE. To test this hypothesis, we performed the reciprocal experiment shown in figure 2.3B. As expected, Tax strongly stimulated expression from the viral CRE-luciferase plasmid (32-fold activation) (compare lanes 1 and 4). Figure 2.3B shows that transfection of c-jun into the assay strongly repressed Tax transactivation (13-fold repression, lanes 4, 5), while having no effect on the viral CRE promoter in the absence of Tax (lane 2). As a control, we also tested the cotransfection of an expression plasmid for c-fos, since c-fos binds to a distinct region of CBP, and therefore would be predicted to not interfere with binding to KIX. Transfection of the c-fos expression plasmid had no effect on Tax transactivation through the viral CRE-Luc reporter plasmid (Fig. 2.3B, lane 6). Under these same conditions, c-fos potentiated c-jun transcription from the AP-1-Luc reporter plasmid indicating that the c-fos expression construct was functioning properly (Fig. 2.9). Transfection of c-fos had no effect on the transcription activity of viral CRE-Luc in the absence of Tax (Fig. 2.3B, lane 3). These studies suggest that Tax and c-jun compete for binding to the KIX domain of CBP in vivo, and that their binding is mutually exclusive.

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2.3cTax point mutants defective for c-jun transcriptional repression.

To investigate the specificity of Tax repression of c-jun mediated gene expression, we tested two Tax point mutants with amino acid substitutions at positions 88 and 89 (K88→A and V89→A). These point mutations have previously been shown to disrupt Tax interaction with the KIX domain of CBP (25). Figure 2.4A shows that, as expected, both Tax point mutants were defective for transactivation through the viral CRE reporter plasmid (compare lane 2 to 3 and 4). Figure 2.4B shows the effect of these mutations in Tax on repression of c-jun transcriptional activity. As compared to wild type Tax, K88→A was fully defective for c-jun repression, whereas V89→A was partially defective for c-jun repression. Western blot analysis indicated that both Tax mutant proteins were expressed at comparable levels in the transfection assay (Fig. 2.10). Comparison of the data presented in figures 2.4A and 2.4B indicates that the point mutation in Tax at position 88 abolished both the activation and repression functions of Tax, whereas the point mutation at position 89 only partially affected both functions. The observation that these point mutations similarly affected both the activation and repression functions of Tax is fully consistent with a role for CBP in both of these pathways. Furthermore, since these Tax mutants were originally identified as defective for interaction with the KIX domain of CBP, these data specifically implicate the KIX domain in the competition for CBP in vivo.

2.3d Tax and c-jun interact with similar amino acids within KIX.

c-jun has previously been shown to bind to a region of the KIX domain (aa 461-662) (10), however, the minimal region of KIX competent for c-jun binding has not been defined. Since we were interested in determining whether both Tax and c-jun bind to an overlapping region of KIX, possibly explaining the in

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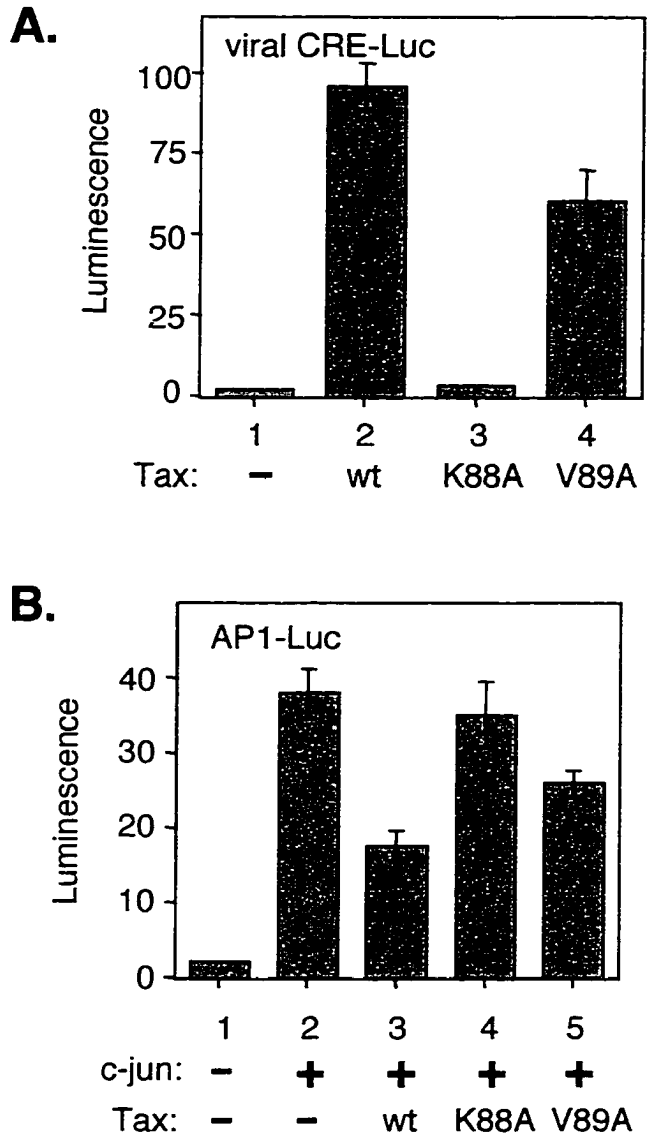

Figure 2.4 Tax mutants are defective for both activation and repression activities. (A) Transient cotransfection assays were carried out in Jurkat T-cells with 400 ng of the viral CRE reporter plasmid and 200 ng of the expression plasmids for CMV promoter-driven wild type Tax (39) (lane 2), Tax K88A (lane 3) or Tax V89A (25) (lane 4). The values shown are the luminescence +/- the standard error from two experiments performed in triplicate. (B) The AP-1-Luc reporter plasmid (400 ng) was cotransfected with 200 ng of the expression plasmids for c-jun (lanes 2-5) and wild type Tax (lane 3), Tax K88A (lane 4) or Tax V89A (lane 5). The values shown are the luminescence +/- the standard error from three experiments performed in triplicate.

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vivo competition, we first needed to determine the minimal region of KIX to which c-jun binds. We have previously shown that aa 588-683 represents the minimal amino acids of KIX to which Tax binds with apparent wild type affinity (46). Figure 2.5 shows that purified, recombinant c-jun binds to full length GST-KIX (aa 451-719) in the GST pull-down assay (lane 2). Progressive amino terminal deletions of KIX revealed that, like Tax, KIX amino acid 588 represents the amino terminal border competent for wild type interaction with c-jun (GST-KIX Δ588-719) (Fig. 2.5, lanes 2-5). Carboxy terminal deletion of KIX to amino acid 665 still retained the ability to bind c-jun, but deletion to amino acid 655 diminished the interaction, indicating that c-jun recognizes a slightly smaller core domain of CBP than does Tax (Fig. 2.5, lanes 6-8).

The significant overlap between the minimal region of KIX required for interaction with c-jun and Tax suggests that both transcription factors recognize the hydrophobic core of KIX (36). To further test this hypothesis, we were interested in determining whether specific residues within the core of KIX interact with both transcription factors. We have previously identified a double point mutation in KIX (R600→G; E633→D) that abolished interaction with both Tax and phosphorylated CREB, and a second double point mutation (L603→P; K659→I) that abolished interaction with Tax, but had no effect on interaction with phosphorylated CREB (46). To test whether these KIX double point mutants were competent for interaction with c-jun, we analyzed the purified polypeptides using the GST pull-down assay. Figure 2.5 shows that the point mutations in both KIX proteins abolished interaction with c-jun; identical to the results obtained with Tax (lanes 9 and 10; see 46). These data suggest that Tax and c-jun make similar amino acid contacts within the hydrophobic core structure of KIX.

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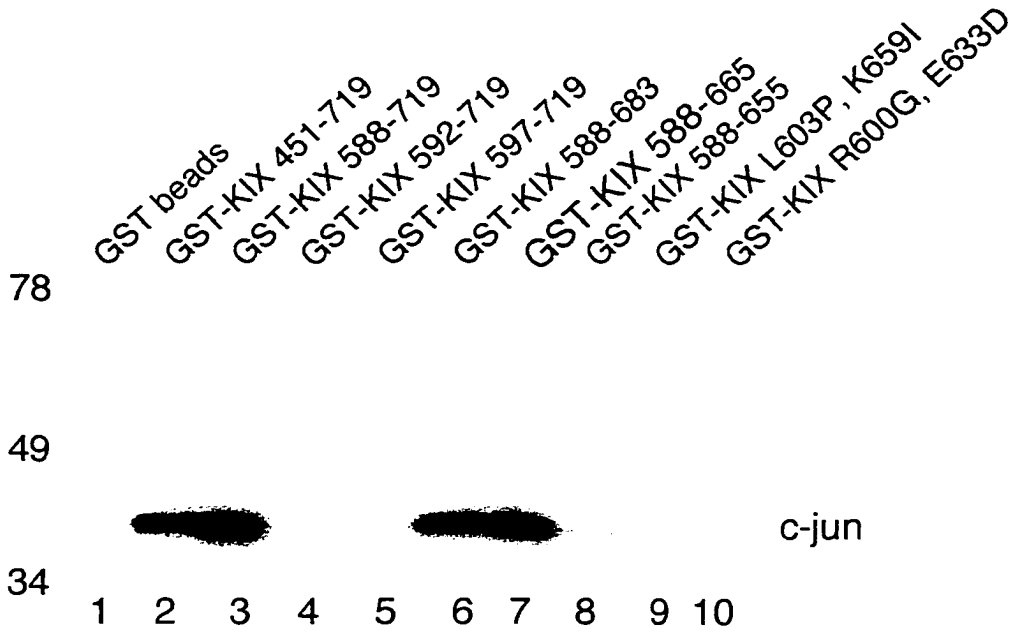

Figure 2.5 Identification of the minimal KIX domain required for c-jun binding. Ten pmol purified, recombinant c-jun were incubated with 100 pmol of either GST, GST-KIX, the indicated GST-KIX deletion mutant or the indicated double point mutant bound to glutathione agarose. The bound proteins were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with an anti-c-jun antibody. Protein standards are indicated at the left.

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2.3eTax competes with c-jun for KIX binding.

The extensive similarity in the KIX amino acids required for interaction with c-jun and Tax provided strong support for the hypothesis that their binding is mutually exclusive. To directly test this hypothesis, we measured whether Tax can compete with c-jun for binding to the KIX domain of CBP in vitro. Glutathione beads bound with GST-KIX aa 451-719 were incubated with an equimolar amount of c-jun and a 5-fold molar excess of Tax, and the relative binding of c-jun was measured. We hypothesized that if c-jun and Tax binding to KIX is mutually exclusive, then the presence of Tax in the c-jun binding reaction should reduce the amount of c-jun bound to KIX. Figure 2.6A shows that the co-incubation of purified Tax in the c-jun-GST-KIX binding reaction dramatically displaced c-jun from KIX (compare lanes 3, 4). The presence of a 5-fold molar excess of either the CBP-binding region of c-fos, or a protein unrelated to CBP (actin depolymerizing factor, ADF), had no effect on c-jun binding to KIX (Fig. 2.6A, compare lanes 3 with 5 and 6). Although this data strongly suggests that the presence of Tax prevents c-jun binding to KIX, it does not directly demonstrate that c-jun displacement is due to a Tax-KIX interaction. To test this idea, we titrated Tax into the c-jun-KIX binding reaction, and monitored both Tax and c-jun binding to KIX. Figure 2.6B shows that increasing amounts of Tax in the c-jun-KIX binding reactions resulted in a progressive increase in Tax binding to KIX, with a concomitant reduction in c-jun binding to KIX. These data suggest that elevated Tax protein concentrations drive interaction with KIX, and result in the physical displacement of c-jun.

2.4 DISCUSSION

The recent observation that the HTLV-I Tax protein binds to the KIX domain of CBP, and utilizes CBP as a transcriptional coactivator, reveals a

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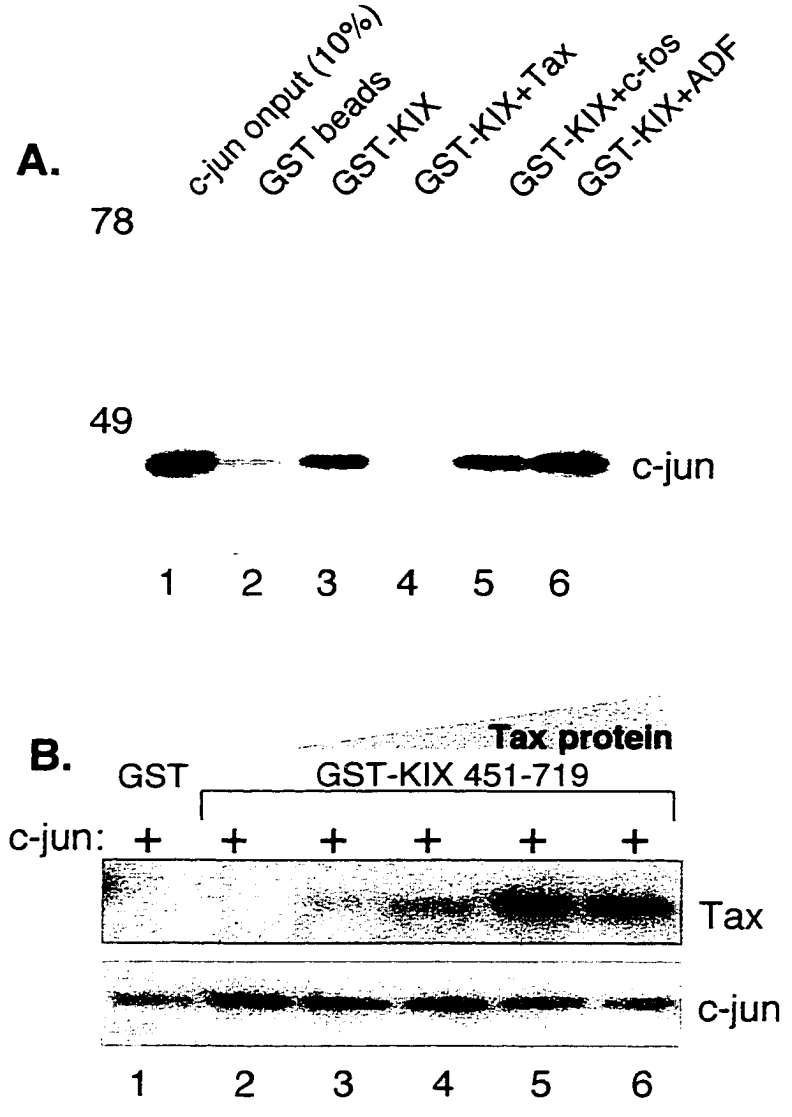

Figure 2.6 Tax and c-jun binding to KIX is mutually exclusive. (A) Tax inhibits c-jun binding to KIX. Purified c-jun (10 pmol) was incubated with GST or GST-KIX 451-719 in the presence of purified recombinant Tax, c-fos or ADF (50 pmol). The bound proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with an anti-c-jun antibody. Protein standards are indicated at the left. (B) Tax binding to KIX displaces c-jun. GST or GST-KIX 451-719 (10 pmol) was bound to glutathione agarose in the presence of 10 pmol c-jun and 10, 20, 30 or 40 pmol purified, recombinant Tax. The reactions were divided in half, and bound proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with either an anti-c-jun antibody or anti-Tax antibody.

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major new pathway by which Tax may deregulate cellular homeostasis (20, 29). In addition to Tax binding to KIX, several cellular transcription factors also bind to this, and other regions of CBP (40). In this report, we directly tested the hypothesis that Tax binding to the KIX domain represses the transcription activity of cellular transcription factors that utilize CBP. We selected the cellular protooncoprotein c-jun for this study, as c-jun binds to KIX to recruit the coactivator to c-jun-responsive promoters (10). We show that in the presence of Tax, the transcription activity of c-jun is repressed, and that reciprocally, c-jun represses the transcription activity of Tax. Furthermore, two Tax mutants, previously shown to be defective for interaction with CBP (25), were unable to repress c-jun transcriptional activity to the level observed with wild type Tax. We investigated the molecular basis for the apparent in vivo competition using in vitro binding and competition assays. We demonstrate that c-jun binds to a region of KIX that significantly overlaps with the minimal region of KIX required for Tax binding. Furthermore, Tax directly competes with c-jun for binding to KIX, indicating that their binding is mutually exclusive.

The KIX domain of CBP, loosely defined as amino acids 450 to 700, is the principal region of CBP that interacts with Tax, c-jun , and several additional transcription factors. Within the KIX domain, we have previously shown KIX aa 588-683 as the minimal region necessary for in vitro interaction with Tax (46). In this study, we defined a similar region of KIX, amino acids 588-665, as competent for interaction with c-jun. These observations are of interest, as Radhakrishnan et al. (36) have recently determined the solution structure of KIX amino acids 586-666 in complex with phosphorylated CREB. This domain of KIX was shown to be composed of three interacting α helices that form a compact hydrophobic core. In the solution structure of the protein-protein complex, two perpendicular α helices of phosphorylated CREB bind to a

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hydrophobic groove on the surface of KIX. Since Tax and c-jun both bind this region of KIX, it seems likely that these transcription factors participate in similar molecular interactions within this surface groove of KIX. This hypothesis is supported by our evidence showing that specific KIX point mutants are defective for both Tax (46) and c-jun interaction, indicating that both transcription factors make similar molecular contacts within CBP. These data suggest that the hydrophobic core of KIX acts a singular docking site for competing transcription factors.

The observations reported here indicate that Tax and KIX-binding transcription factors compete for CBP utilization in the HTLV-I infected T-cell. The extent, and thus the consequences, of the competition would depend upon several factors, including the relative abundance of each transcription factor, their relative KIX binding affinities, and the concentration of available CBP in the cell. Tax expression in an HTLV-I-infected cell is believed to be intermittent, but that during burst periods of Tax expression, Tax protein levels are high (0.15% of total cell protein; [41]). It seems likely that during these burst periods, Tax levels would exceed that needed for optimal proviral expression, and by mass action, the high concentrations of free Tax would bind to KIX, sequestering the limiting concentrations of intracellular CBP and at least partially altering CBP-mediated cellular gene expression.

These Tax-dependent effects on CBP function may also be linked with cellular transformation and adult T-cell leukemia, as a prominant role for CBP in hematopoetic malignancies is emerging (22, review). Chromosomal translocations involving CBP (and p300) are being identified with increasing frequency in patients with treatment-related acute and chronic myeloid leukemias and myelodysplastic syndrome (13, 21, 26, 37, 38, 44, 45). The molecular basis of CBP translocation-associated leukemogenesis is not known,

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however the available evidence strongly suggests that chromosomal translocations involving CBP result in reduced and/or defective coactivator function. Because of the pleiotropic role for CBP in cellular gene expression, it is likely that alterations in CBP function promote inappropriate regulation of cell cycle and differentiation genes, possibly through aberrant histone acetyltransferase activity. We propose that Tax binding to the KIX domain may mimic the deregulation that is achieved following chromosomal translocations involving CBP, with both scenarios promoting malignant transformation.

In summary, the studies presented herein demonstrate that Tax binding to CBP results in derailment of CBP coactivator function. Although we have focused on Tax trans-repression of c-jun transcription activity, it is likely that it is through disruption of one or more of the many other CBP-binding transcription factors that Tax may alter cellular homeostasis. These studies provide the biochemical foundation for future work on the mechanism of Tax deregulation of cellular gene expression through alterations in CBP-mediated transcriptional control.

2.5 MATERIALS AND METHODS

2.5a Cell culture and transfections. HTLV-I negative Jurkat T-lymphocytes were maintained in Iscove's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics. Transient cotransfection assays were performed at a cell density of 10^6 cells/ml using lipofectamine (Life Technologies). Cells were transfected for 5 hours, allowed to recover for 19 hours, and luciferase reporter gene expression was measured using a Turner TD 20-e luminometer.

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2.5b Recombinant plasmids. The viral CRE-Luc reporter plasmid carries 3 tandem copies of the third 21 bp repeat from the HTLV-I promoter cloned immediately upstream of the minimal HSV thymidine kinase promoter driving expression of the luciferase gene (20). The AP-1-Luc reporter plasmid is similar to the viral CRE-Luc reporter plasmid, except that three consensus AP-1 recognition sites were cloned immediately upstream of the thymidine kinase minimal promoter. These reporter plasmids were tested in the presence of the HTLV-I-Tax (14), IEX-Tax (39), Tax CMV-K88→A, Tax CMV-V89→A (25), RSV-c-jun (6), or RSV-c-fos expression plasmids.

2.5c Expression and Purification of Recombinant Proteins. pTax-His_6 (50), GST-KIX 451-719 (20), the GST-ΔKIX deletion mutants and double point mutants (46) were expressed in *E. coli* and purified to apparent homogeneity as described in the above references. Purified c-jun protein was purchased from Promega. Purified c-fos, amino acids 216-379 (which contain the CBP binding region, [9]) was expressed in *E. coli* and purified to apparent homogeneity (unpublished construct; purified c-fos protein was a gift from Katie Campbell and the Kevin Lumb laboratory, Department of Biochemistry and Molecular Biology, Colorado State University). Actin depolymerizing factor (ADF) was prepared as described (1), and provided as a gift from the James Bamburg laboratory, Department of Biochemistry and Molecular Biology, Colorado State University.

2.5d In Vitro Binding Assays. GST pull-down assays with c-jun were performed by incubating 100 pmol of GST, GST-KIX, GST-ΔKIX derivatives, or the GST double point mutants with 12.5 μl of swollen glutathione-agarose (Sigma) in 400 μl 0.5X superdex buffer (12.5 mM Hepes [pH 7.9], 6.25 mM

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$MgCl_2$, 75 mM KCl, 5 μM $ZnSO_4$, 0.5 mM EDTA, 10% Glycerol, 0.05% NP-40) at 4°C for 1 hour. The beads were then washed twice with 0.5X superdex buffer and incubated with 10 pmol c-jun at 4°C for 1 hour in 400 μl of the same buffer. The beads were washed twice with 0.5X superdex buffer, resuspended in SDS-PAGE sample buffer, and electrophoresed on a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with rabbit IgG against mouse c-jun (amino acids 91-105) (Santa Cruz Biotechnology Inc.) and subsequently with ^{125}I labeled protein A. The blots were imaged, analyzed and quantitated using PhosphorImager analysis and ImageQuant.

GST pull-down competition assays were carried out by incubating 10 pmol GST or GST-KIX with 12.5 μl swollen glutathione agarose in 400 μl 0.5X superdex buffer at 4°C for 1 hour. The beads were washed twice in 0.5X superdex buffer and then incubated with 10 pmol c-jun and 50 pmol Tax, c-fos or ADF at 4°C for 1 hour. In the Tax titration experiment, 10, 20, 30, or 40 pmol of Tax were incubated with 10 pmol c-jun. The remainder of the assays were carried out as described above.

2.6 ACKNOWLEDGMENTS We thank Katie Campbell, Kevin Lumb and James Bamburg for gifts of proteins, and Robert Harrod and Chou-Zen Giam for the Tax mutant plasmids. We also thank Brian Lenzmeier and Holli Giebler for critical reading and discussions of the manuscript. This work was supported by a grant from the American Cancer Society (VM-170).

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Supplemental Figures

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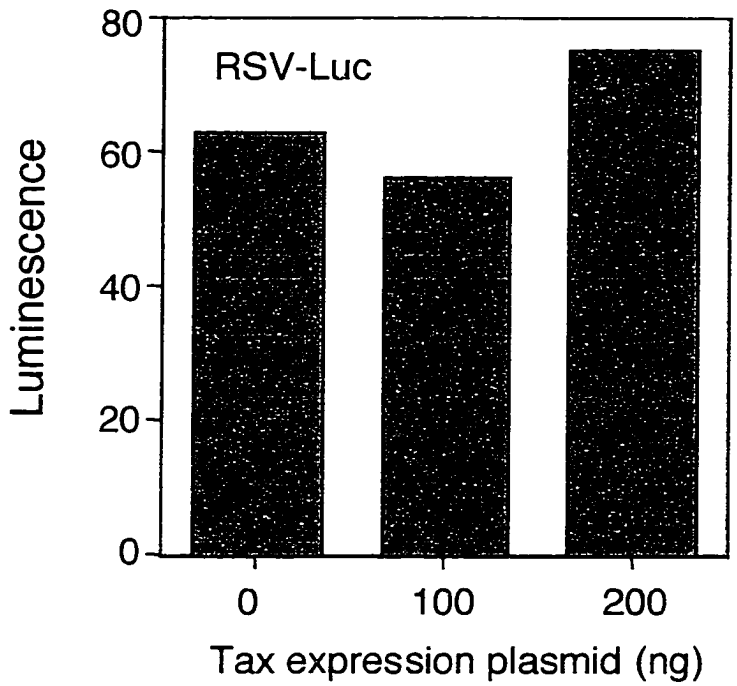

Figure 2.7 Tax has no effect on the RSV promoter. Transient cotransfection assays were carried out in the HTLV-I negative human Jurkat T-cell line with 400 ng of the RSV-Luc reporter. The Tax expression plasmid was cotransfected as indicated. The luminescence values shown are the average of duplicate samples.

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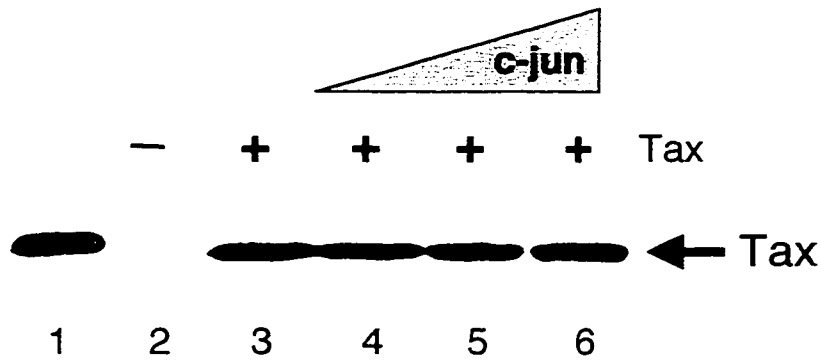

Figure 2.8 c-jun has no effect on synthesis of Tax protein from the HTLV-I-Tax expression plasmid. Jurkat T-cells were transfected in duplicate with 500 ng of the Tax expression plasmid (lanes 3-6) and 250, 500 and 1000 ng of the c-jun expression plasmid (lanes 4-6). Cells were harvested after 24 hours. Duplicates were combined, resuspended in SDS sample buffer, and run on a 12% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with an anti-Tax antibody. Purified recombinant Tax (20 ng) was loaded in lane 1 of the gel as a control.

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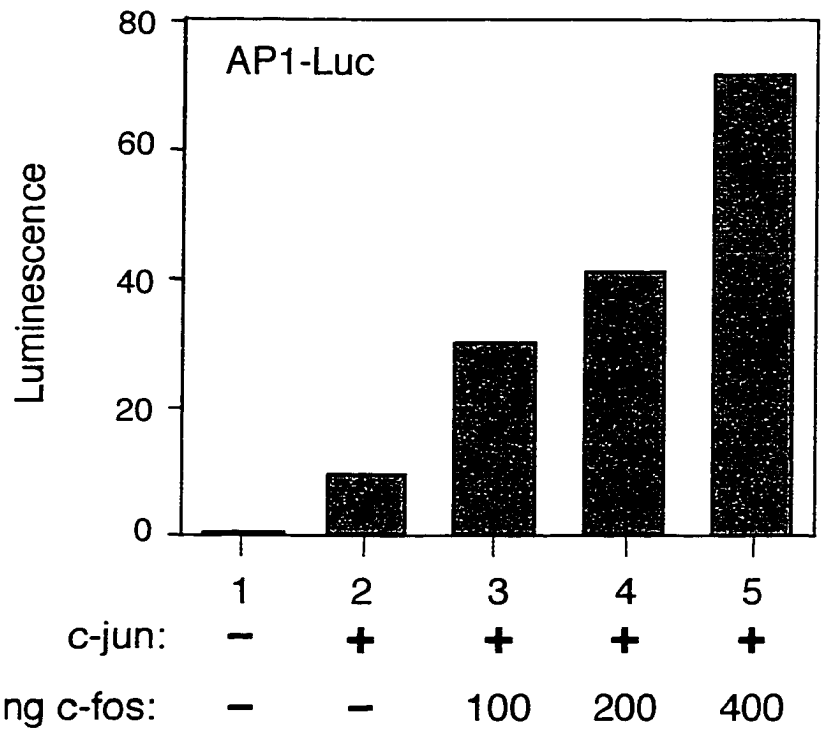

Figure 2.9 c-fos potentiates c-jun activated transcription. Jurkat T-cells were transfected with 400 ng of the AP-1-Luc reporter, 200 ng of the c-jun expression plasmid (lanes 2-5) and the indicated amounts of the c-fos expression plasmid (lanes 3-5). The luminescence values shown are the average of duplicate samples.

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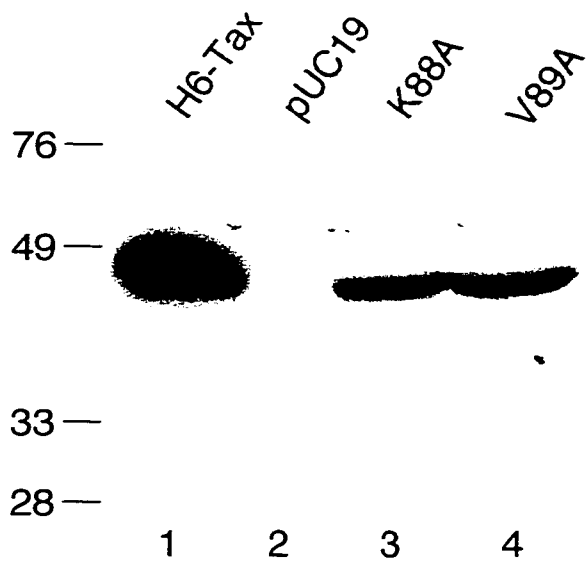

Figure 2.10 Tax mutants K88A and V89A are properly expressed in transient transfection assays. Jurkat T-cells were transfected in duplicate with 500 ng of pUC19 (lane 2), the expression plasmid for Tax K88A (lanes 3) or the expression plasmid for Tax V89A (lane 4). Cells were harvested after 24 hours. Duplicates were combined, resuspended in SDS sample buffer, and run on a 12% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with an anti-Tax antibody. Purified recombinant Tax (20 ng) was loaded in lane 1 of the gel as a control.

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CHAPTER 3
THE ONCOPROTEIN TAX INHIBITS p53 FUNCTION THROUGH COMPETITION FOR THE KIX DOMAIN OF CBP

Chapter three describes a study showing that Tax functionally inactivates the tumor suppressor p53 through competition for the KIX domain of CBP. This work has been submitted for publication. Holli A. Giebler and I are co-first authors on the paper. Isabelle Lemasson and Melissa Gonzales are also authors. My experiments are shown as figures 3.1B, 3.3A, 3.7, 3.8A, 3.8B, 3.13 and 3.14. Data cited as "data not shown" in the manuscript are shown here as supplemental figures. The citation for the manuscription appears below.

Van Orden, K., H. A. Giebler, I. Lemasson, M. Gonzales and J. K. Nyborg. 1999. The oncoprotein Tax inhibits p53 function through competition for the KIX domain of CBP. submitted.

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3.1 ABSTRACT

The HTLV-I oncoprotein Tax is required for high level viral transcription, and is strongly linked to HTLV-I-associated malignant transformation. Tax stimulates gene expression through high affinity binding to the KIX domain of CBP, a pleiotropic cellular coactivator protein. Here we report that the activation domain of the tumor suppressor p53 also binds to KIX to functionally utilize the coactivator. We demonstrate reciprocal transcriptional repression between Tax and p53 in vivo, suggesting intracellular coactivator competition. We biochemically confirm coactivator competition by directly showing that both transcription factors bind to KIX in a mutually exclusive fashion. These data provide molecular evidence for the observed intracellular competition, and suggest that Tax attenuates p53 function by abrogating a novel p53-KIX interaction. Specific disruption of the p53-KIX complex by Tax may represent a pivotal event in the HTLV-I transformation pathway.

3.2 INTRODUCTION

Adult T-cell leukemia (ATL) is an aggressive and fatal hematological malignancy that is etiologically linked to infection with the human T-cell leukemia virus, type I (HTLV-I). Although there is a clear link between HTLV-I infection and ATL, only a small percentage of infected individuals develop leukemia, and this generally follows a prolonged, asymptomatic latency period (reviewed in 14). The molecular mechanism of malignant transformation by the virus is poorly understood; however, expression of the virally-encoded Tax protein appears to be a necessary event in the leukemogenic pathway (20, 19). Tax is a regulatory protein that is critical for high level HTLV-I transcription, and thus is required for propagation of the virus.

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To activate HTLV-I transcription, Tax binds to specific DNA sequences (termed viral CREs) in the transcriptional control region of the virus. Through a series of elaborate protein-protein and protein-DNA interactions, Tax binds to the cellular protein CREB and the viral CRE DNA, to form a stable ternary complex (1, 3, 8, 9, 15, 29, 35, 44, 61). Within this nucleoprotein complex, CREB primarily serves as a scaffold that stabilizes Tax on the promoter DNA, whereas Tax serves as a high affinity binding site for the recruitment of the multifunctional cellular coactivator CREB binding protein (CBP) (18, 32). The tethering of CBP to the ternary complex promotes the strong transcriptional activation associated with Tax (27). Therefore, in the presence of Tax, the virus is able to bypass the need for CREB phosphorylation in the recruitment of CBP, strongly activating viral gene expression in the absence of cellular signalling pathways (18).

CBP is a pleiotropic coactivator that mediates the transcription function of a wide variety of transcription factors (53). CBP, and the related protein p300, appear to stimulate transcription by facilitating communication between upstream activators and the basal transcription machinery (59). Furthermore, CBP and p300 carry intrinsic and associated acetyltransferase activities, and are believed to play a role in chromatin remodeling and possibly transcription factor acetylation (6, 42). Tax has been shown to specifically interact with the KIX domain of CBP (amino acids 588-683), and this molecular interaction results in the efficient recruitment of the coactivator to the HTLV-I promoter (18, 32, 58). Solution structure determination revealed that these amino acids of KIX compose three α-helices that interact to form a hydrophobic core (48). A groove on the surface of KIX is believed to serve as a binding platform for the many transcription factors that interact with this domain.

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In addition to KIX, CBP carries multiple transcription factor interaction domains that enable physical and functional recruitment of the coactivator to many classes of genes. It is noteworthy that in spite of its central role in mediating gene expression, CBP appears to be present at limiting concentrations in the cell (45). Limiting coactivator abundance may create competition between transcription factors, thus providing an additional layer of regulated gene expression. Consistent with this idea, overexpression of Tax has been shown to repress the transcription function of the cellular proteins c-myb and c-jun, a functional antagonism that appears to result from direct transcription factor competition for the KIX domain (12, 56). Several other reports demonstrate transcription factor cross-talk, apparently through competition for KIX and/or other regions of CBP (7, 16, 26, 52, 54, 59). Consistent with these and other results, it is tempting to speculate that the strong physical interaction between Tax and KIX may partially sequester CBP, and that this inactivation may be linked to the widespread cellular gene deregulation associated with HTLV-I infection.

In an effort to understand HTLV-I-associated leukemogenesis, several studies have examined the activity of p53 in HTLV-I transformed T-cells. p53 is a tumor suppressor protein that induces cell cycle arrest or apoptosis in response to DNA damage, thus preventing the transmission of genetic mutations to the daughter cells (reviewed in 31). Loss of p53 activity has been identified in 60% of the human malignancies examined (24, 36), consistent with a role for p53 in genome surveillance and the suppression of oncogenic transformation. In HTLV-I-infected and Tax-expressing cells, p53 is present, generally at elevated levels, with a relatively low frequency of mutation (2, 49, 57). However, p53 is functionally inactive, as these cells no longer respond appropriately to a variety of p53 stimuli (10, 17, 40, 46). For example, gamma

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irradiation, which normally induces p53 transcriptional activity, has little or no effect on the expression of a panel of known p53-responsive genes in HTLV-I transformed cell lines (46). Consistent with these observations, expression of the Tax protein increased the sensitivity of a mouse cell line to ionizing radiation-induced DNA damage (50). Tax expression also abrogated both p53-induced cell cycle arrest and apoptosis. Finally, Tax expression specifically inhibits p53 transcriptional activity, as measured by a p53-responsive reporter construct (2, 41). These data provide strong evidence that Tax inactivates p53, inhibiting the physiologically relevant functions of this tumor suppressor protein (41). Thus, inactivation of p53 by Tax may be a pivotal molecular event in the transformation pathway.

Unfortunately, very little is known about the molecular basis for Tax inactivation of p53 transcription function. However, several studies have shown that CBP and p53 physically interact, and that this interaction participates in p53 transcriptional activation (4, 23, 37). Since both Tax and p53 utilize CBP, coactivator competition is a plausible explanation for the observed repression. One mechanism of coactivator competition involves both transcription factors binding to a common region of CBP. As described above, Tax interacts strongly with the KIX domain of CBP, whereas p53 has been shown to bind to the C/H1 domain, and an ill-defined carboxy-terminal region (4, 22, 23, 37). The molecular basis of this paradox is investigated in this report.

Here, we demonstrate that Tax represses p53 transcription function, and as would be predicted from coactivator competition, p53 reciprocally represses Tax transcription function. The interference appears to arise from intracellular competition for the KIX domain of CBP, suggesting that p53 binds both physically and functionally to KIX. We demonstrate biochemically that both

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recombinant and endogenous p53 bind specifically to KIX, and that this interaction directly involves the activation domain of p53. Finally, we show that Tax and p53 bind to KIX in a mutually exclusive fashion, providing molecular evidence for the intracellular competition. Together, these data indicate that Tax inactivates p53 by abrogating a novel p53-CBP interaction, providing key insight into the events that lead to HTLV-I associated leukemogenesis.

3.3 RESULTS

3.3a Reciprocal repression between Tax and p53

Recently, several reports have demonstrated that Tax inactivates p53 transcription function in vivo (2, 10, 17, 40, 41, 46). Additionally, both Tax and p53 have been shown to utilize the transcriptional coactivator CBP, consistent with the possibility that both proteins may compete for CBP in HTLV-I-infected cells. However, several studies have shown that p53 and Tax bind to distinct regions of CBP, making direct competition for the coactivator unlikely. We were interested in investigating the molecular basis for Tax inactivation of p53 transcription function. To begin, we first confirmed Tax repression of p53 in our transient transfection assay system. The transcription function of p53 was measured using a reporter plasmid carrying 13 copies of the p53-response element driving expression of the luciferase gene (pG13-luc). In HTLV-I-negative, p53-negative (11, 60) Jurkat T-cells, we show that, as expected, transfection of a p53 expression plasmid strongly stimulated expression from the p53-responsive reporter plasmid (Fig. 3.1A, lanes 1, 2). Under these conditions of p53 activation, cotransfection of a Tax expression plasmid repressed p53-mediated activation in a dose-dependent fashion (Fig. 3.1A,

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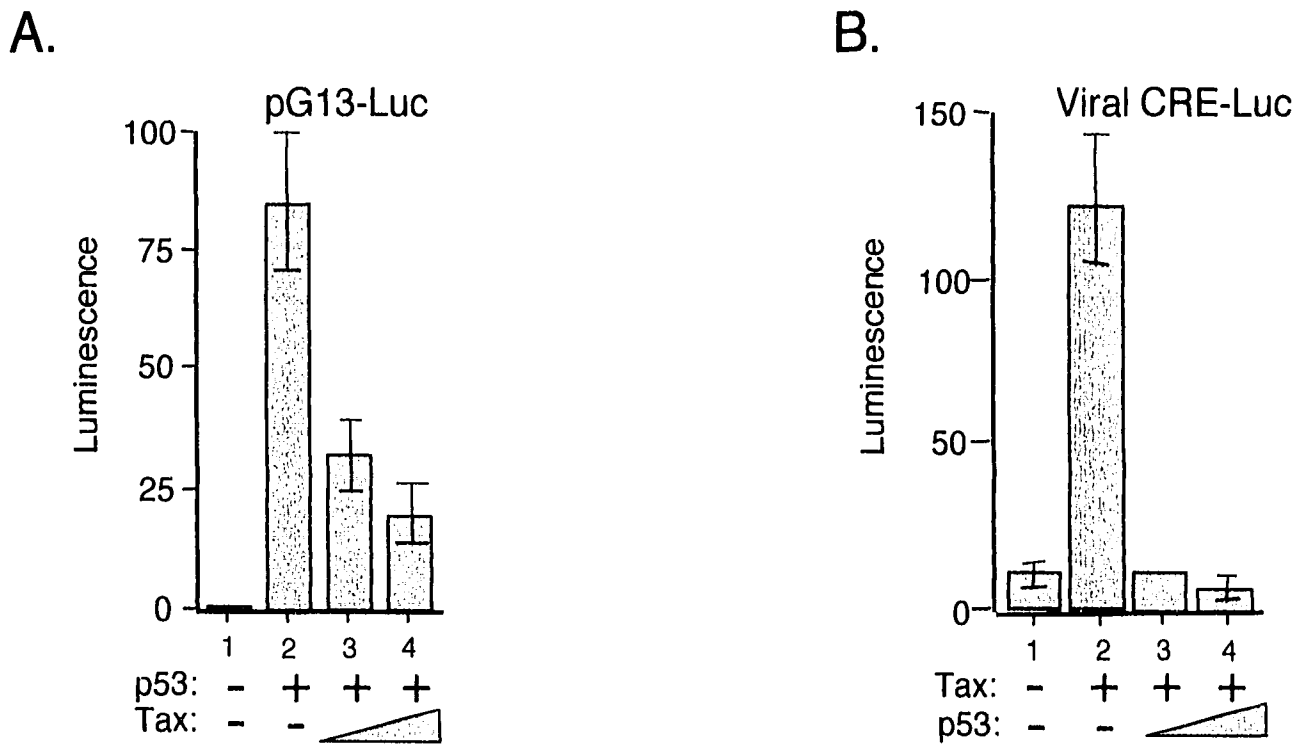

Figure 3.1 Reciprocal repression between Tax and p53. Transient cotransfection assays were carried out in HTLV-I negative Jurkat T-cells. (A) The pG13-Luc reporter plasmid (400ng) was cotransfected with a constant amount of the p53 expression plasmid (pC53-SN3; 400ng) and an increasing amount of the Tax expression plasmid (IEX-Tax; 200 and 400 ng), as indicated. Values shown are the average luminescence +/- the standard error from one experiment performed in triplicate. (B) The Tax-responsive viral CRE-Luc reporter plasmid (400 ng) was cotransfected with a constant amount of the Tax expression plasmid (IEX-Tax; 200 ng) and an increasing amount of the p53 expression plasmid (pC53-SN3; 50 and 100 ng), as indicated. The values shown are the average luminescence +/- the standard error from two experiments performed in triplicate.

lanes 3, 4). The repression by Tax was dependent upon the p53 expression plasmid, as Tax had no effect in the absence of cotransfected p53 (Fig. 3.11). Tax also repressed p53-activated expression from the human *bax* promoter (Bax-luc), indicating that Tax repression of p53-activated transcription also occurs on a naturally occurring p53-responsive promoter (Fig. 3.12).

If repression of p53 by Tax occurs as a consequence of competition for CBP, then overexpression of p53 should similarly repress Tax function. To test this possibility, we performed the reciprocal experiment using a reporter plasmid carrying three copies of the Tax-responsive viral CREs (viral CRE-Luc) driving expression of the luciferase gene (18). Transfection of the Tax expression plasmid strongly activated transcription from the Tax-responsive promoter (Fig. 3.1B, lanes 1, 2). Cotransfection of increasing amounts of the p53 expression plasmid repressed Tax transactivation in a dose-dependent fashion (Fig. 3.1B, lanes 3, 4). As shown in figure 3.1A (lanes 1, 2), cotransfection of the p53 expression plasmid was not toxic to the cells. The reciprocal repression observed with these two transcription factors led us to further investigate whether the interference may be a result of competition for limiting amounts of CBP in the cell.

3.3b Dominant negative repression of p53 activity by KIX

The observation of reciprocal transcriptional repression between p53 and Tax strongly suggests that CBP may mediate cross-talk between these two transcription factors. This hypothesis, however, is inconsistent with previous reports showing that Tax binds to the KIX domain of CBP, whereas p53 binds to a region near the carboxy-terminus of CBP (4, 18, 23, 32, 37, 58). These two regions of CBP are separated by up to 1400 amino acids, making direct

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competition, and/or induced conformational changes in CBP unlikely. Although an interaction between p53 and KIX has not been previously reported, we were interested in testing whether p53 binds the KIX domain, and if so, whether this interaction promotes the transcriptional interference observed with Tax. We examined p53 transcription function in transient transfection assays in the presence of increasing amounts of an expression plasmid for KIX (RSV-$KIX_{aa\ 459\text{-}679}$). We reasoned that if p53 bound to the KIX domain of CBP in vivo, then overexpression of KIX should sequester p53 away from the endogenous CBP/p300. KIX should therefore have a dominant negative effect on p53 transcriptional activity, as KIX does not have intrinsic transcriptional coactivation properties. We have previously shown that this KIX expression plasmid inhibits Tax transactivation in vivo (18). Figure 3.2 shows that titration of an expression plasmid for KIX produced a dose-dependent repression of p53 transcription function (lanes 3, 4). These data suggest that p53 transcription function requires interaction with the KIX domain of CBP, resulting in the reciprocal repression observed with p53 and Tax.

3.3c Tax activation and repression functions utilize a similar pathway

To more closely examine whether Tax repression of p53 transcriptional activity was mediated through CBP, we tested a Tax mutant that has been shown to be defective for interaction with KIX (25). This Tax mutant, carrying a single amino acid substitution at position 88 (K88→A), is fully defective for Tax transactivation in vivo (25, 56). Since Tax K88→A is reported to be specifically defective for interaction with KIX, we hypothesized that if Tax repression of p53 occurred through competition for the KIX domain of CBP, it would also be

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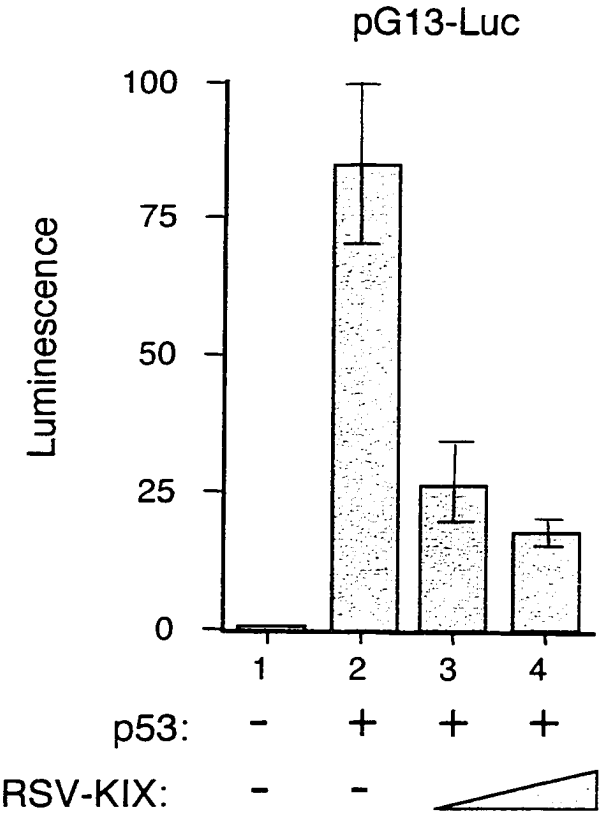

Figure 3.2 Dominant negative repression of p53 activity by the KIX domain of CBP. The p53 responsive pG13-Luc reporter plasmid (400 ng) was cotransfected with a constant amount of the p53 expression plasmid (400 ng), and an increasing amount of the KIX expression plasmid (RSV-KIX; 200 and 400 ng), as indicated. The values shown are the average luminescence +/- the standard error of one experiment performed in triplicate.

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unable to repress p53 function. Figure 3.3A shows that Tax K88→A is completely defective for p53 repression, as compared with wild type Tax (compare lanes 3 and 4). Both the mutant and wild type Tax proteins were expressed from the same promoter (CMV), and Western blot analysis indicated that the mutant was properly expressed in the transfection assay (Fig. 3.13). To confirm that Tax K88→A was defective for interaction with KIX, we tested the mutant protein in an in vitro binding assay. A purified GST fusion protein carrying the KIX domain (GST-C/H1-$KIX_{aa302-683}$, see figure 3.4) was bound to glutathione-agarose beads and used in a GST pull-down assay with Tax. Equal amounts of purified wild type Tax and Tax K88→A were tested for binding to KIX. Figure 3.3B shows that, as compared to wild type Tax, K88→A demonstrated significantly reduced interaction with KIX (compare lanes 3 and 4). The data obtained with Tax K88→A is consistent with the hypothesis that Tax interaction with the KIX domain of CBP results in repression of p53 transcriptional activity. It is noteworthy that Tax K88→A is fully defective for both activation and repression, strongly supporting the idea that a common mechanism is utilized in both pathways.

3.3d p53 binds KIX in vitro

Previous studies have shown that p53 physically interacts with a poorly defined carboxy terminal region of CBP to activate transcription (4, 23, 37). However, the in vivo results presented above strongly support the idea that p53 also interacts with KIX. To directly test this idea, a purified GST-KIX fusion protein (GST-$KIX_{aa588-683}$; Fig. 3.4) was bound to glutathione-agarose beads, and used in a GST pull-down assay with full-length purified, recombinant p53. This KIX construct has previously been shown to represent the minimal region

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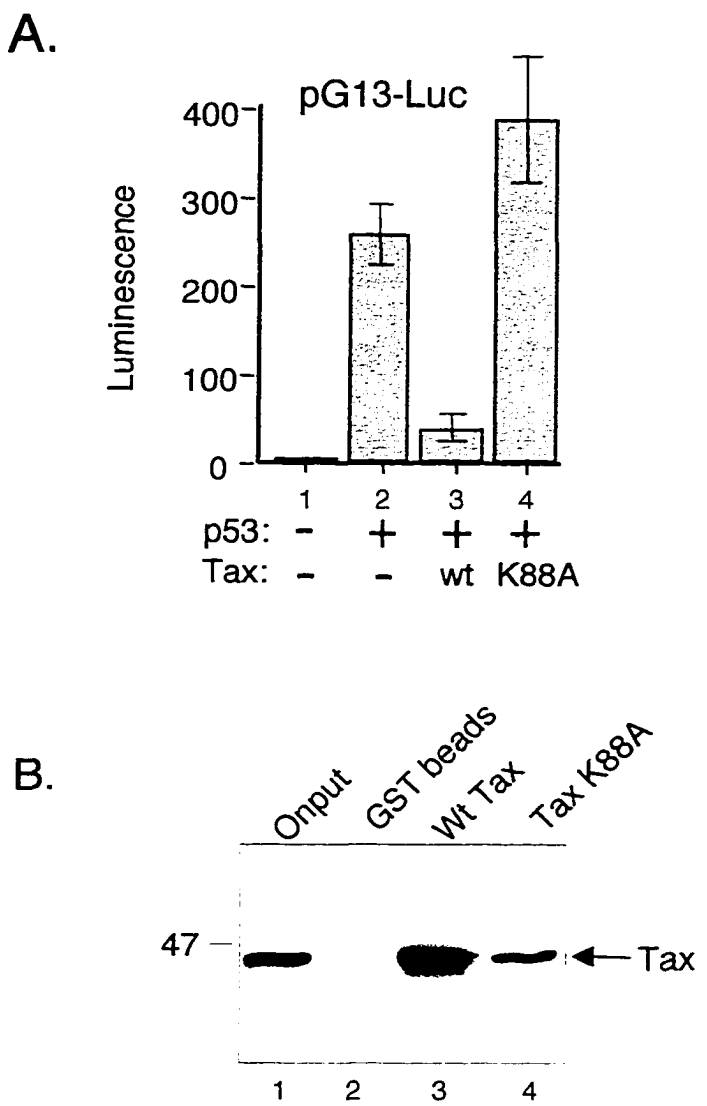

Figure 3.3 Tax mutant is defective for KIX binding and repression of p53 activity. (A) The pG13-Luc reporter plasmid (400 ng) was cotransfected with a constant amount of the p53 expression plasmid (400 ng), and 200 ng of the expression plasmid for either wild type Tax (lane 3), or Tax K88→A (lane 4). Values shown are the average luminescence +/- the standard error of two experiments performed in triplicate. (B) GST pull-down assay comparing wild type Tax and K88→A Tax binding to KIX. Purified, recombinant wild-type or mutant Tax (20 pmol) was incubated with either GST alone or GST-KIX (200 pmol) bound to glutathione agarose beads. Bound Tax protein is indicated. Five percent of onput wild type Tax is shown (lane 1).

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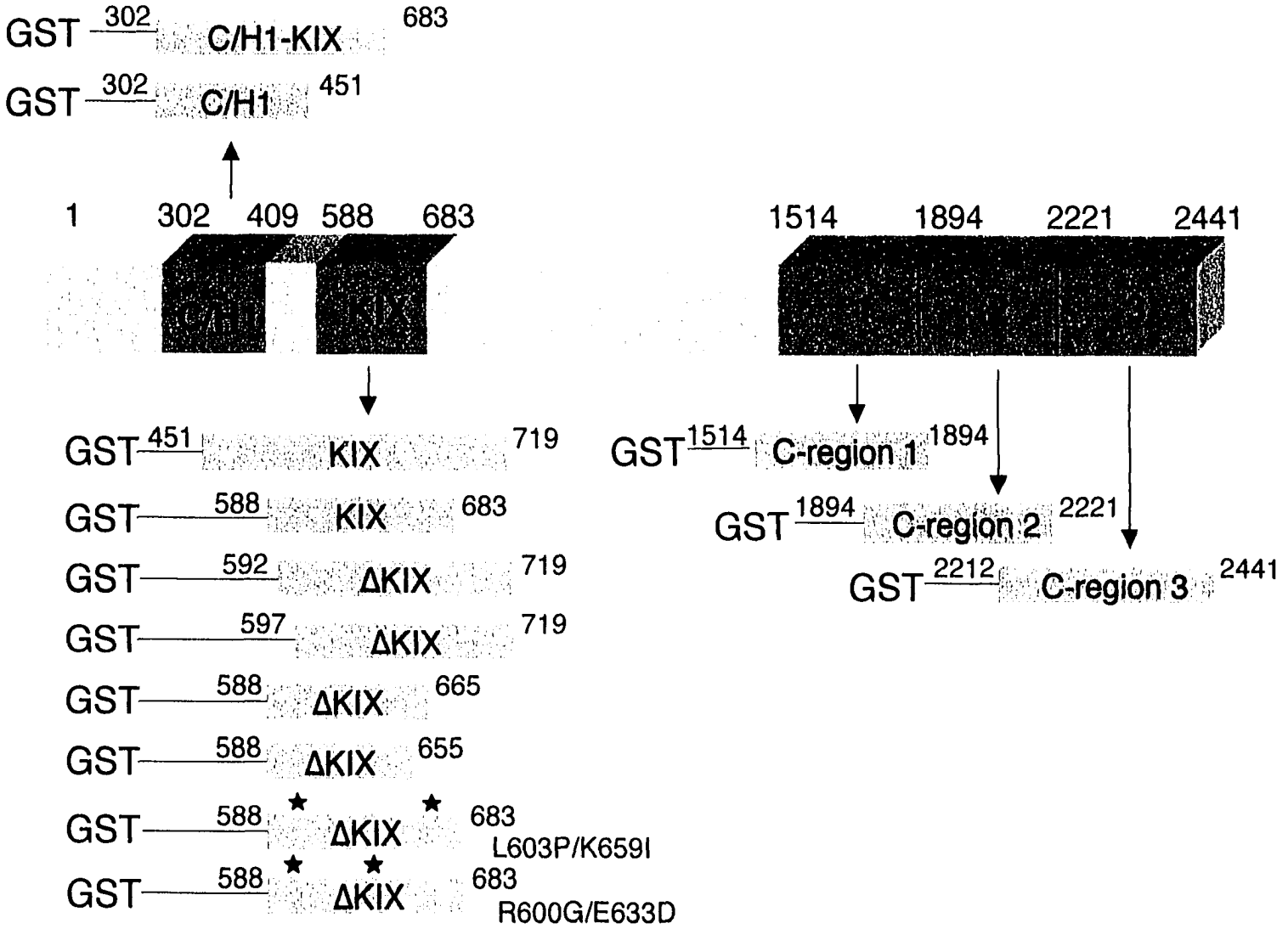

Figure 3.4 Schematic illustration of the GST-CBP fusion proteins used in this study.

of KIX required for wild type interaction with Tax (58). Figure 3.5A shows that p53 bound strongly to the KIX domain of CBP (lane 6). For comparison, we also tested the binding of p53 to the other regions of CBP reported to interact with p53. These include C/H1 (aa302-451), a CBP domain involved in p53 degradation (22) (lane 9), and three carboxy terminal regions previously implicated in p53 transcriptional activation (aa1514-1894; 1894-2221; 2212-2441) (4, 23, 37) (lane 3-5). All of these GST-CBP fusion constructs are illustrated in figure 3.4. Recombinant p53 bound strongly and specifically to C/H1, C/H1-KIX, KIX, and to carboxy terminal region 2 (aa1894-2221) (Fig. 3.5A, compare lanes 4, 6, 9, 10).

We next determined the minimal region of KIX competent for p53 binding. Progressive amino terminal deletions of GST-KIX revealed that amino acid 588 represents the amino terminal border competent for wild type interaction with p53 (GST-$KIX_{aa588-683}$) (Fig. 3.5B, lanes 4-6). p53 interacted weakly, yet similarly, with GST-$KIX_{aa592-719}$ and GST-$KIX_{aa597-719}$, possibly due to the deletion of a tryptophan residue at position 591 (Fig. 3.5B, compare lanes 5, 6). This tryptophan residue participates in a side chain hydrogen bond interaction that contributes to stabilization of the primary hydrophobic core (48). Progressive carboxy terminal deletions of KIX revealed that amino acid 683 represents the carboxy terminal border competent for wild type interaction with p53. KIX deletions to amino acid 665 (GST-$KIX_{aa588-665}$) and 655 (GST-$KIX_{aa588-655}$) abolished detectable interaction with p53 (Fig. 3.5B, lanes 7, 8). Structural analysis and secondary structure predictions suggest that amino acids between 683 and 665 participate in extension of the third α helix, thus contributing to stabilization of the folded KIX structure (48). Therefore, $KIX_{aa588-665}$, and $KIX_{aa588-655}$ are likely unable to form the stable core KIX structure. Finally, we

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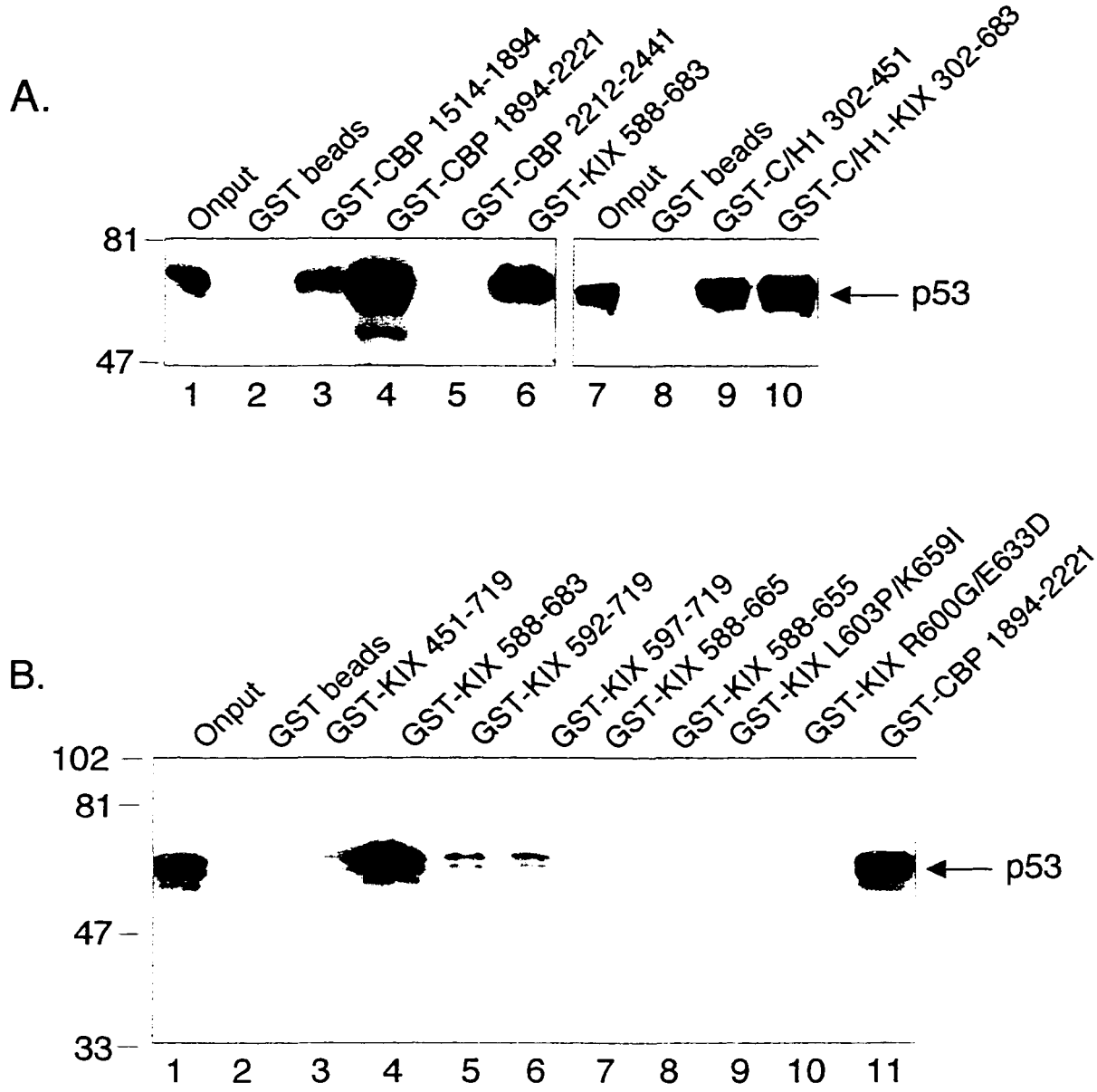

Figure 3.5 p53 binds to the KIX domain of CBP in vitro. (A) Purified, recombinant p53 (5 pmol) was incubated with GST alone, or the indicated GST fusion proteins (50 pmol). Bound p53, and protein standards, are indicated. Five percent of onput p53 is shown (lanes 1 and 7). (B) Purified p53 (5 pmol) was incubated with GST alone, or the indicated GST-ΔKIX fusion proteins (50 pmol). As a positive control, p53 binding to GST-$CBP_{aa1894-2221}$ was also tested. Bound p53 and protein standards are indicated. Five percent of onput p53 is shown (lane 1).

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were interested in identifying specific amino acids in KIX that abolished interaction with p53. Two GST-$KIX_{aa588-683}$ proteins, containing double point mutations at positions 603 and 659, or positions 600 and 633, abolished interaction with p53 (Fig. 3.5B, lanes 9, 10). It is interesting to note that the pattern of p53 binding to the KIX deletion mutants and point mutants precisely parallels the pattern of Tax binding to KIX (58), suggesting that both transcription factors recognize similar amino acids within the hydrophobic core structure of KIX. Both serine-133 phosphorylated CREB and c-jun recognize a slightly smaller region of KIX (43, 56, 58). In carrying out these studies, we also tested the binding of p53 to a larger region of KIX, GST-$KIX_{aa451-719}$, and observed only a weak interaction (Fig. 3.5B, lane 3). We have previously observed that GST-$KIX_{aa451-719}$ interacts weakly with other KIX-binding transcription factors, including c-jun (56), suggesting that it forms an inappropriately folded structure that may prevent transcription factor access to the core structural domain of KIX. It is possible that the use of larger KIX molecules in previously published studies may have precluded detection of the p53-KIX interaction (4, 23).

3.3e The p53 activation domain interacts with KIX

To further establish the biological relevance of the p53-KIX interaction, we were interested in identifying the domain of p53 involved in KIX interaction. Since it seemed most plausible that the activation domain would play a role in coactivator recruitment, we first tested whether an antibody directed against the p53 activation domain (α-p53 aa11-25) might block p53 binding to KIX in a GST pull-down experiment. In the same experiment, we also tested the effect of an antibody directed against the carboxy terminus of p53 (α-p53 aa371-380), and

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an unrelated antibody (α-EGFR). Figure 3.6A shows that the anti-p53 activation domain antibody strongly blocked p53 binding to KIX (lanes 4, 5). Equal concentrations of the other two antibodies had no detectable effect on p53 binding to KIX (Fig. 3.6A, lanes 6-9). These results suggest that a region near or within the activation domain of p53 participates in KIX binding.

To determine whether specific activation domain amino acids are involved in the interaction, we introduced a double point mutation (L22→Q; W23→S) into the activation domain of p53, and tested the ability of the mutant protein to bind KIX. We selected these two amino acids in the p53 amino terminus, as the effect of these mutations on p53 transcription function has been well characterized (38). Figure 3.6B shows the results of a GST pull-down assay where we tested the binding of purified wild type and mutant p53 proteins to several regions of CBP. Surprisingly, the double point mutation in the p53 activation domain completely abolished interaction with KIX (Fig. 3.6B, lanes 7,8). The activation domain mutations exhibited reduced binding to the two carboxy terminal regions of CBP, consistent with previous studies (23) (Fig. 3.6B, lanes 9-12). Although the preparation of GST-$KIX_{aa588-683}$ used in this experiment was less active for p53 binding, the interaction with mutant p53 protein was still significantly decreased (we typically observe significant variation in the activity of our GST-$KIX_{aa588-683}$ preparations). As a negative control, we also tested mutant p53 binding to C/H1, as amino acids within the core domain of p53 (aa90-160) have been shown to interact with this region of CBP (22). No significant difference in the binding of wild type and mutant p53 protein to C/H1 was observed (Fig. 3.6B, lanes 5,6). Together, these data indicate that the activation domain of p53 interacts directly with KIX.

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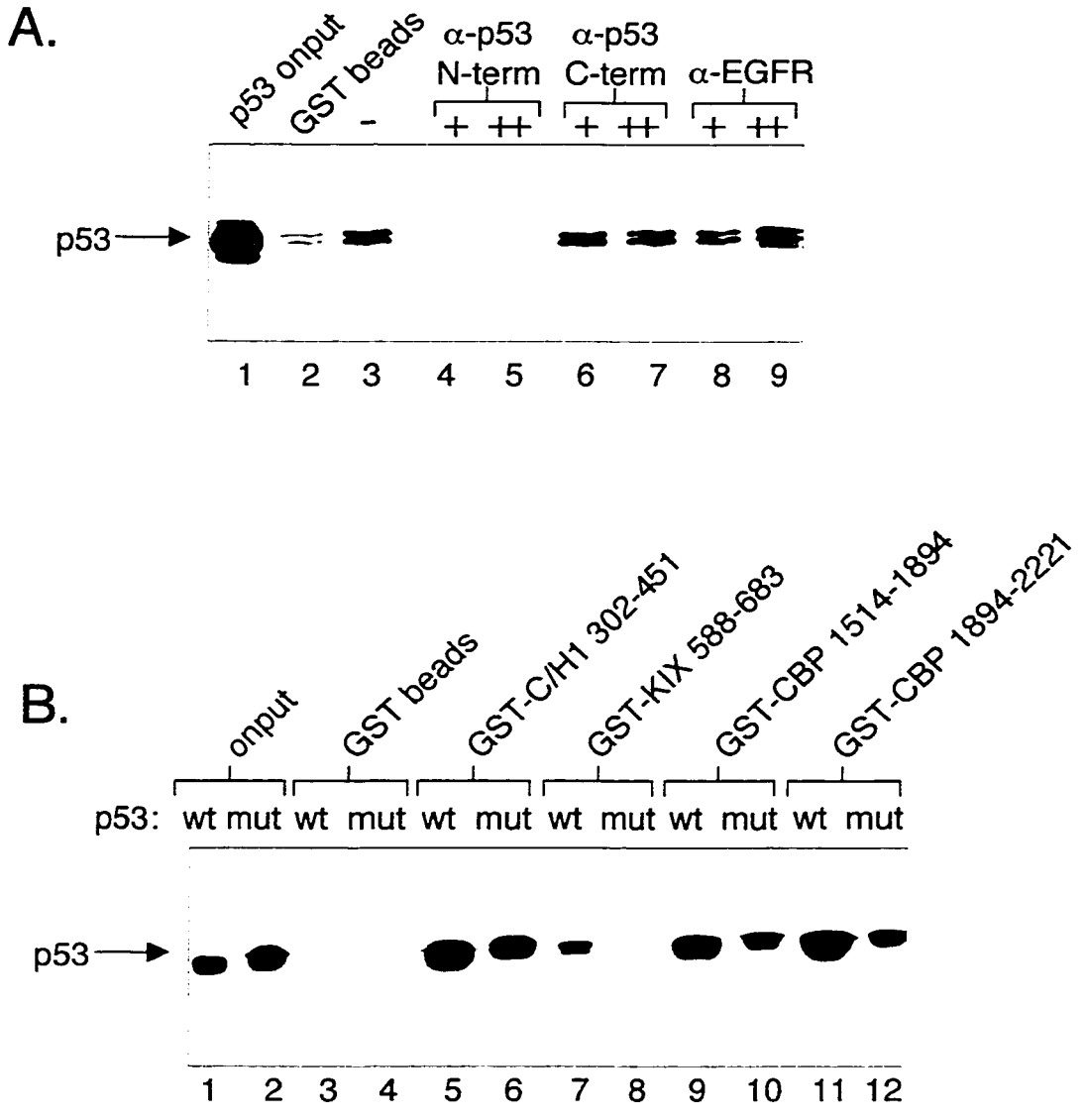

Figure 3.6 The amino terminus of p53 is critical for interaction with KIX. (A) Purified p53 (20 pmol) was incubated with GST alone, or GST-$KIX_{aa588-683}$ (20 pmol) in the presence of an increasing amount of antibody directed against either the p53 amino terminus, or the p53 carboxy terminus (7 or 20 pmol). An unrelated antibody was used as a negative control (anti-EGFR). Bound p53 is indicated. Two percent of onput p53 is shown (lane 1). (B) Purified wild type p53 or activation domain mutant p53 (L22→Q; W23→S) (15 pmol) was incubated with GST alone, or the indicated GST fusion proteins (30 pmol). Bound p53 protein is indicated. Seven percent of onput wild type or mutant p53 is shown (lanes 1 and 2).

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We next introduced this activation domain double point mutation into our mammalian p53 expression plasmid. We reasoned that if p53 repression of Tax transcription function occurs through competition for KIX, then this p53 mutant should also be defective for Tax repression. Figure 3.7 shows that, as compared with wild type p53, the p53 activation domain mutant only modestly repressed Tax function (lanes 3, 4). Both wild type and mutant p53 proteins were cloned in the same plasmid and expressed from the same promoter. Western blot analysis confirmed that both proteins were properly expressed (Fig. 3.14). These data support our hypothesis that Tax and p53 compete for the KIX domain of CBP in vivo.

3.3f Endogenous p53 binds KIX

We next tested whether naturally occurring p53 protein, present in the physiologically relevant T-cell milieu, interacts with KIX. For this experiment, we compared the binding of p53 to KIX, C/H1, and C/H1-KIX $_{aa302-683}$ (Fig. 3.4). To perform this experiment, we incubated the GST-CBP fusion proteins with nuclear extracts from the HTLV-I-infected human T-cell line SLB-1. Figure 3.8A shows the result of the GST "pull-out" assay. As expected, the endogenous p53 protein interacted strongly with the KIX domain (GST-KIX$_{aa588-683}$), but not with a carboxy terminal deletion mutant of KIX (GST-KIX$_{aa588-665}$) (Fig. 3.8A, lanes 6,7). p53 exhibited the highest apparent affinity for GST-C/H1-KIX $_{aa302-683}$, and also bound well to the C/H1 domain (Fig. 3.8A, lanes 4, 5). The strong binding of p53 to C/H1-KIX was not unexpected, as this larger CBP region encompasses two independent p53 recognition elements.

The use of HTLV-I-infected SLB-1 cells enabled direct comparison of the CBP-binding pattern of both p53 and Tax in the same experiment. To do this,

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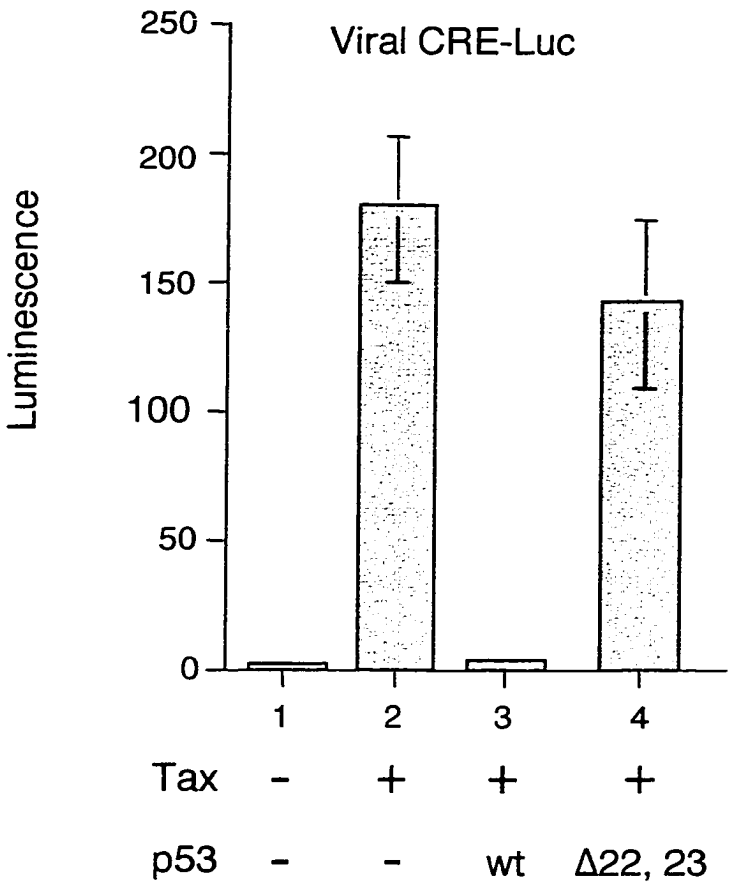

Figure 3.7 Amino terminal mutant of p53 is defective for repression of Tax activity. Transient cotransfection assays were carried out in HTLV-I negative Jurkat T-cells. The Tax-responsive viral CRE-Luc reporter plasmid (400 ng) was cotransfected with a constant amount of the Tax expression plasmid (IEX-Tax; 200 ng) and 50 ng of either the wild type or mutant p53 expression plasmid (pC53-SN3 wt or Δ22,23) as indicated. The values shown are the average luminescence +/- the standard error from two experiments performed in triplicate.

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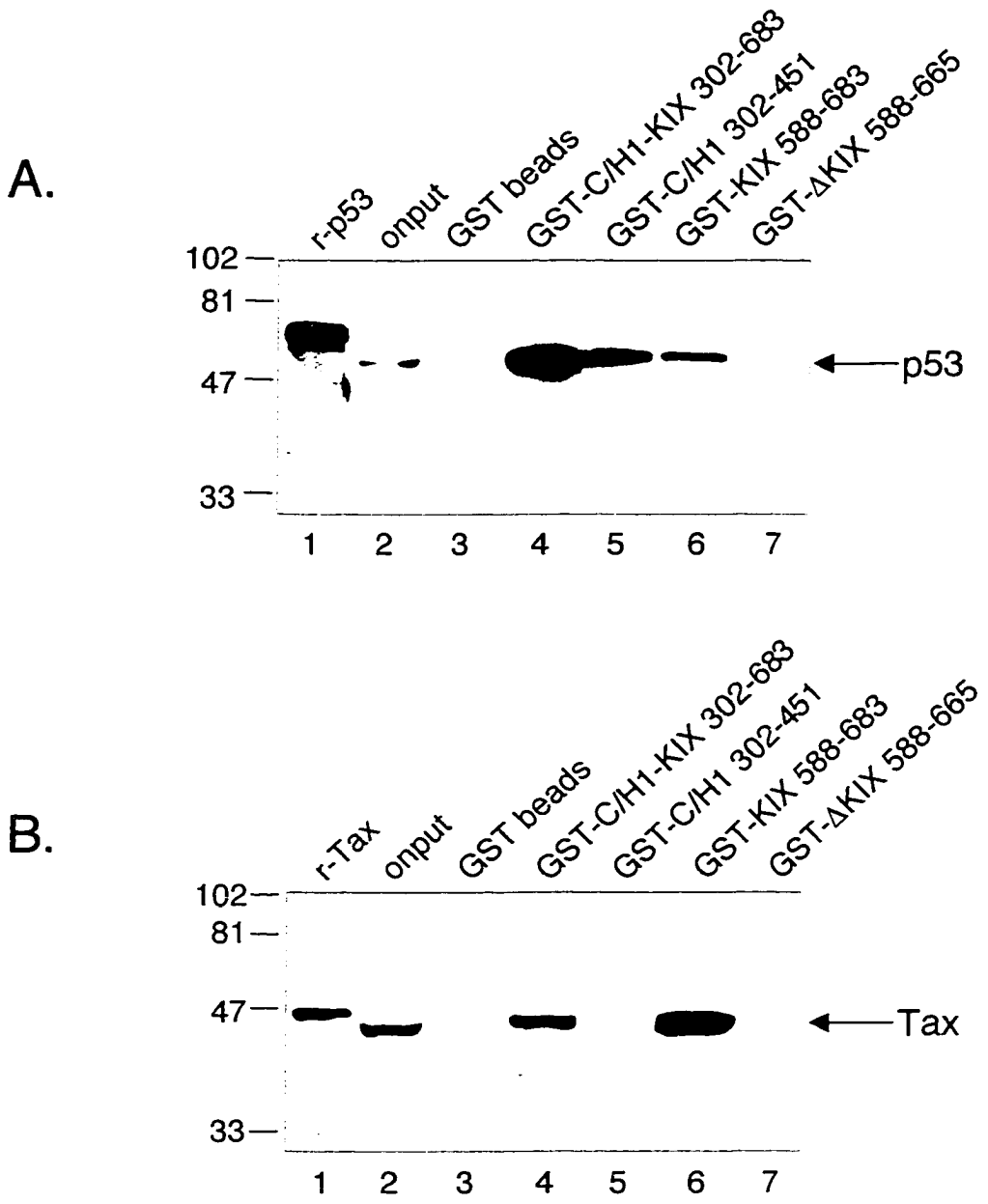

Figure 3.8 Endogenous p53 protein binds to KIX. (A) Nuclear extract prepared from the HTLV-I infected cell line SLB-1 (1.5 mg) was incubated with GST alone, or the indicated GST fusion proteins (50 μg). p53 was detected by Western blot analysis and bound protein is indicated. One percent of onput nuclear extract is shown (lane 2). (B) The Western blot shown in A was reprobed using an anti-Tax antibody. Bound Tax protein is indicated.

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we stripped the p53 blot, and re-probed using an anti-Tax antibody. Figure 3.8B shows that, as expected, Tax bound to GST-$KIX_{aa588-683}$, but not a deletion mutant of KIX (GST-$KIX_{aa588-665}$) (Fig. 3.8B, lanes 6, 7). Unlike p53, Tax did not interact with the C/H1 domain, and recognized the C/H1-KIX (GST-C/H1-$KIX_{aa302-683}$) fusion protein with affinity similar to that of KIX alone (Fig. 3.8B, lanes 4,5).

3.3g Tax and p53 compete for KIX in vitro

The observation that p53 and Tax physically and functionally interact with KIX provides support for the idea that their binding is mutually exclusive, thus providing a molecular explanation for the transcriptional interference observed in vivo. To directly test this hypothesis, we examined whether increasing concentrations of Tax can displace p53 from KIX in vitro. For this experiment, glutathione beads were bound with GST-C/H1-$KIX_{aa302-683}$, then incubated with purified, recombinant p53. We selected C/H1-$KIX_{aa302-683}$ for this experiment, as the presence of the two adjacent p53 binding sites represent the p53-CBP interaction in a more physiologically relevant context. Increasing amounts of Tax were included in the binding reactions containing p53, and the resulting protein-protein interactions were monitored by Western blot analysis. Figure 3.9A shows that increasing amounts of Tax resulted in a reduction in p53 binding to C/H1-$KIX_{aa302-683}$, with a concomitant increase in Tax binding (lanes 2-5). As shown above, Tax only binds KIX in this GST-C/H1-KIX fusion construct, whereas p53 binds to both domains. The observation that p53 binding is only partially inhibited by Tax likely reflects the unperturbed p53 interaction with C/H1. We next tested whether the Tax point mutant, Tax K88→A, could effectively compete with p53 for KIX binding (Fig. 3.9B). We

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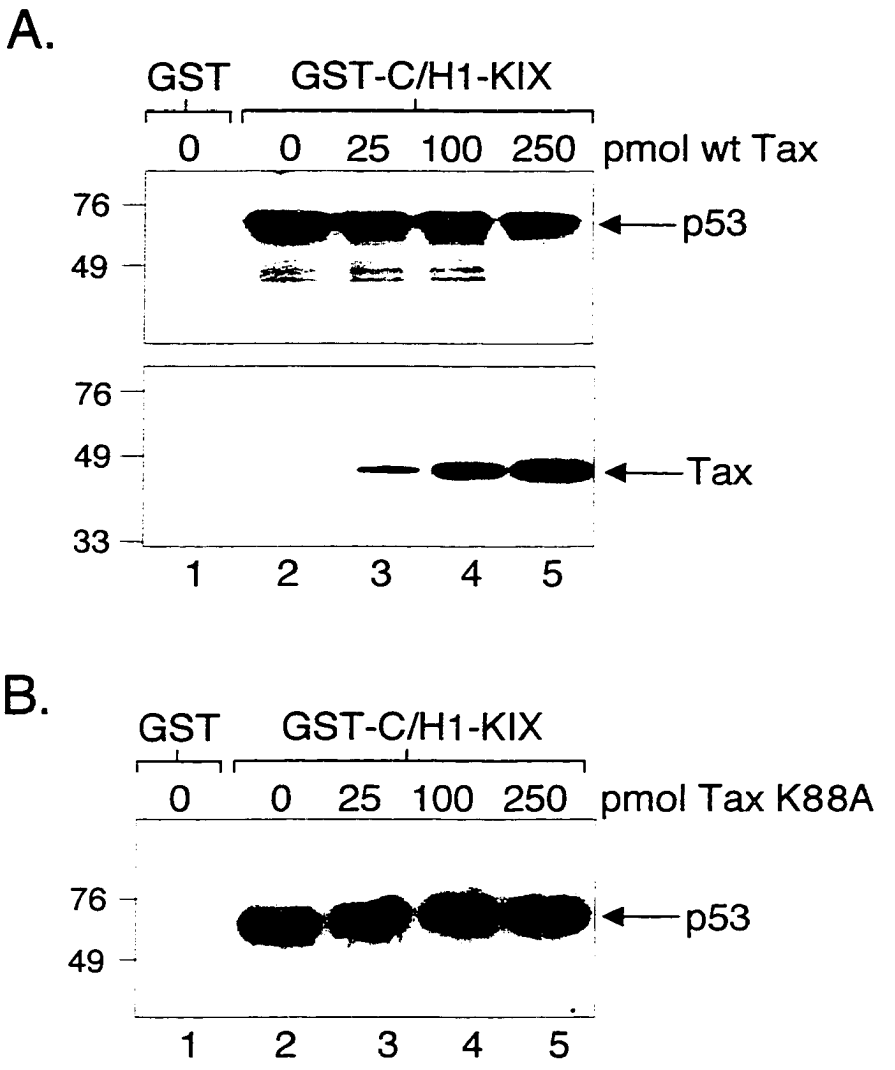

Figure 3.9 Tax inhibits p53 binding to KIX. (A) Purified p53 (25 pmol) was incubated with GST alone, or GST-C/H1-KIX$_{aa302-683}$ (25 pmol) in the presence of the indicated amounts of purified Tax protein. p53 was detected by western blot analysis and bound p53 protein is indicated in the upper panel. The western blot was re-probed using an anti-Tax antibody and bound Tax is indicated in the lower panel. (B) Tax K88→A can not inhibit p53 binding to KIX. Purified p53 (25pmol) was incubated with GST alone, or GST-C/H1-KIX $_{aa302-683}$ (25 pmol) in the presence of the indicated amounts of purified Tax K88→A protein. p53 was detected by western blot analysis and bound p53 protein is indicated.

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have previously shown that this Tax mutant is defective for binding to C/H1-KIX $_{aa302-683}$ (Fig. 3.3B, above). Figure 3.9B shows that, as expected, increasing amounts of Tax K88→A had no effect on p53 binding to C/H1-KIX $_{aa302-683}$ (lanes 2-5). We then performed a reciprocal competition experiment, binding Tax to GST-C/H1-KIX$_{aa302-683}$ fusion protein, and adding increasing concentrations of p53. Titration of p53 into the binding reaction dramatically reduced Tax interaction with p53, with a concomitant increase in p53 binding (Fig. 3.10, lanes 3-6). Together, these data indicate that the binding of p53 and Tax to KIX is mutually exclusive, and that elevated concentrations of either protein drives exclusive interaction with KIX.

3.4 DISCUSSION

In this report, we show that p53 interacts physically and functionally with the KIX domain of the cellular coactivator CBP. We provide both in vivo and in vitro data that strongly support the idea that the p53-KIX interaction is physiologically relevant for p53 coactivator recruitment. For example, expression of the KIX domain in transient transfection assays inhibits p53 transcriptional activity, suggesting that p53 interacts with KIX in vivo, and that this interaction is critical for p53-mediated transcription. We further show that purified recombinant p53 and, perhaps more significantly, p53 protein present in a T-cell nuclear extract, recognize a minimal region of KIX (aa588-683). This region corresponds to a defined structural domain of CBP that consists of a pleiotropic transcription factor binding site on the surface of a hydrophobic core (48).

The strength of the p53-KIX interaction is comparable to that observed with the previously identified, p53-interacting carboxy terminal regions of CBP.

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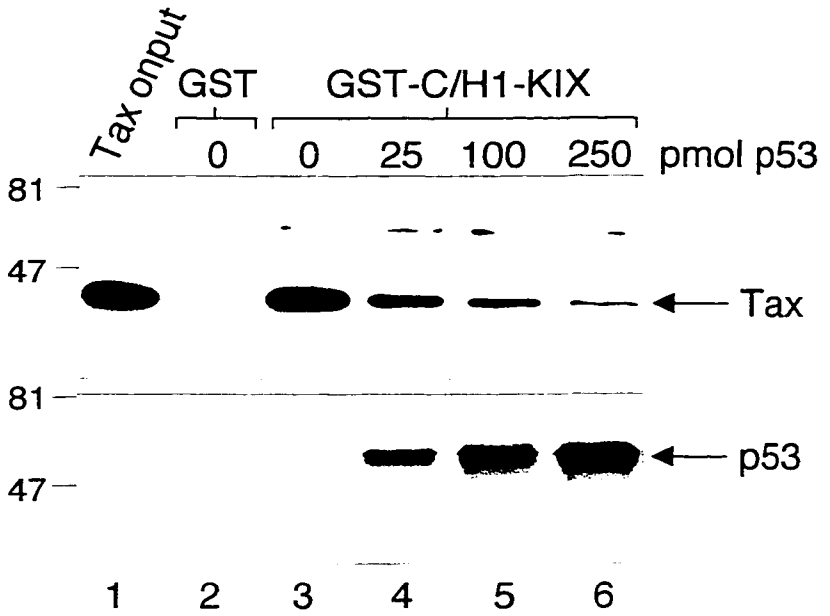

Figure 3.10 p53 inhibits Tax binding to KIX. Purified Tax (25 pmol) was incubated with GST alone, or GST-C/H1-$KIX_{aa302-683}$ (25pmol) in the presence of the indicated amount of purified p53 protein. Tax was detected by western blot analysis and bound Tax protein is indicated in the upper panel. The western blot was re-probed using an anti-p53 antibody and bound p53 is indicated in the lower panel. Protein standards are indicated at the left. Four percent of onput Tax protein is shown (lane 1, upper panel).

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Significantly, a double point mutation in the activation domain of p53 severely reduces Tax repression, and abolishes interaction with KIX. Furthermore, an antibody directed against the activation domain of p53 disrupts the interaction with KIX. These data indicate that the activation domain of p53 is directly involved in KIX binding, and provides strong evidence for the functional relevance of the p53-KIX interaction. Although it is clear that p53 interacts with multiple regions of CBP, our data suggests that the p53-KIX interaction is critical for p53 transcription function in vivo.

Several previous studies have shown that Tax inhibits many of the tumor suppressor functions of p53, however the molecular mechanism of this inhibition has remained elusive. The finding that p53 interacts with KIX suggests coactivator competition as a plausible mechanism for Tax inactivation of p53 transcription function. Consistent with coactivator competition, we also observed p53 repression of Tax transactivation. In support of this observation, we have previously shown that, like p53, Tax also recognizes KIX amino acids 588-683 (58). Thus, it appears that both proteins recognize the same surface structure of KIX, and suggests that their binding is mutually exclusive. This is directly demonstrated using a competition binding assay in vitro which suggests that Tax specifically disrupts the p53-KIX interaction. Since the p53-KIX interaction appears to be critical for p53 transcription function (Fig. 1C), these data provide a molecular explanation for Tax repression of p53 in vivo. Together, these observations are consistent with previous studies showing that in HTLV-I-infected and Tax-expressing cells, p53 is unable to activate target genes in response to various stimuli (10, 17, 40, 46). These data support a model for Tax repression of p53 transcription function that is mediated through competition for the KIX domain of CBP.

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A previous report examining Tax repression of p53 (47) suggests that a possible mechanism for p53 inactivation arises from a Tax-dependent increase in amino-terminal p53 phosphorylation. However, this observation is in contrast with a subsequent finding by the same group showing that phosphorylation within this region of p53 increases p53 function and CBP recruitment (34). The precise biological consequences of Tax-dependent phosphorylation of p53 remain elusive. Our direct demonstration that p53 utilizes the KIX domain for coactivator recruitment, together with the evidence that Tax and p53 binding to KIX is mutually exclusive, provides a sound framework for the mechanism of p53 inactivation.

HTLV-I associated ATL is well characterized by chromosomal instability and karyotypic abnormalities. However, studies of ATL patients reveal that p53 mutations are relatively rare, as compared with other human malignancies. The development of such severe genetic mutations seems unlikely in the presence of functional p53. Since it is well established that p53 plays a critical tumor suppressor role, it seems likely that Tax inactivation of p53 transcription function may be pivotal in the leukemogenic process. We propose that the high affinity binding of Tax to the KIX domain of CBP results in the inactivation of p53 transcriptional activity, obviating the need for p53 mutation. Although Tax levels are generally low in an HTLV-I-infected T-cell (30), intermittent burst periods of Tax expression may sequester CBP and derail p53 transcription function. This event would likely promote an environment in the infected T-cell that is tolerant of mutations and chromosomal instability. Thus, Tax disruption of the p53-KIX interaction may be paramount in the in HTLV-I transformation pathway.

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3.5 MATERIALS AND METHODS

3.5a Cell culture, transient cotransfection assays and mammalian expression plasmids. HTLV-I negative Jurkat T-cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin-streptomycin. For transient cotransfection assays, cells were grown to a density of 10^6 cells/ml and transfected with Lipofectamine (Life Technologies) and a constant amount of DNA for 5 hours. The cells were allowed to recover for 24 hours before harvest. Cells were lysed and luciferase activity was measured with a Turner Designs Model TD 20-e Luminometer.

Expression plasmids for p53, pC53-SN3 (5), wild type Tax, IEX-51), the CMV-K88A Tax point mutant (25), and RSV-KIX (CBP amino acids 459 to 679) (18) have been previously described. The luciferase reporter plasmids pG13-Luc (28) and viral CRE-Luc (18) have also been described.

The expression plasmid for the double point mutant of p53 (L22→Q, W23→S) was created by PCR amplification (*Pful* polymerase) of the wild-type pC53-SN3 plasmid using Stratagene's Quik-Change Site-Directed Mutagenesis Kit.

3.5b Cloning, expression and purification of recombinant proteins. GST-$KIX_{451-719}$, the GST-ΔKIX deletion mutants, and the GST-KIX point mutants have previously been described in detail (18, 58). GST-CH/1-KIX was made by PCR amplification of the CBP cDNA sequence corresponding to amino acids 302-683 (pRC/RSV-CBP; 39), and insertion of the fragment into the BamH1 site of pGex2T (Pharmacia). GST-CH/1 was made by digestion of the GST-CH/1-KIX PCR product with EcoR1, releasing a 471 bp fragment

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corresponding to CBP amino acids 302-451. This fragment was inserted into the BamH1/EcoR1 site of pGex2T. Both expression plasmids were transformed into SCS1 cells (Stratagene), and the proteins purified as previously described (58).

Three GST fusion proteins encompassing the carboxy terminal region of CBP were made by PCR amplification of the appropriate sequences from pRc/RSV-CBP (39). The PCR fragment from region 1, which carries sequences corresponding to amino acids 1514-1894, was inserted into the BamH1 site of pGex2T. PCR fragments from both region 2, which carries sequences corresponding to amino acids 1894-2221, and region 3, which carries sequences corresponding to amino acids 2212-2441, were inserted into the EcoR1/BamH1 site of pGex2T. The expression plasmids for regions 1 and 3 were transformed into SCS1 cells, and region 2 was transformed into BL21(DE3) pLysS (55). Cell cultures were expanded, induced, and the GST proteins purified by glutathione-agarose chromatography. Histidine-tagged p53 (H_6-p53) was cloned by PCR amplification of the full-length, wild type p53 cDNA (p53-H-19; 24). The PCR product was inserted into the EcoR1/BamH1 site of pRSETA, transformed into BL21(DE3) pLysS, expanded and induced as previously described (55). p53 was purified to greater than 90% homogeneity by Ni^{+2}-NTA agarose chromatography (Qiagen). The purified protein was dialyzed against p53 dialysis buffer (20 mM Tris, pH 8.0, 0.5 mM EDTA, 0.1 M KCl, 20% glycerol), aliquoted and stored at –70° C. The histidine-tagged double point mutant of p53 (L22->Q, W23->S) was created as described above using the wild-type H_6-p53 plasmid and Stratagene's Quik-Change Site-Directed Mutagenesis Kit. The DNA was transformed into BL21(DE3) pLysS, expanded, induced, and purified exactly as wild-type H_6p53. Tax protein was

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expressed from pTaxH_6 expression plasmid (62) and purified as previously described (18).

3.5c GST pull-out assays. HTLV-I-infected SLB1 human T-cells were grown to a density of $1x10^6$ cells/ml in IMDM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin-streptomycin. The nuclei were prepared using an adaptation of a previously published nuclear extract protocal (13). Briefly, cells were harvested and washed once with ice-cold phosphate buffered saline (PBS) with 1g/L $MgCl_2$. The cell pellet was resuspended in hypotonic lysis buffer (10 mM Tris, pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$), homogenized and the nuclei isolated by centrifugation. Aliquoted nuclear pellets were frozen until immediately prior use. To perform the GST pull-out experiments, thawed nuclei were resuspended in PBS with 0.1% NP40 and protease inhibitors and sonicated three times (30 s each) at 50% output with a Bronson Sonifier. Extracts were cleared by centrifugation, and total protein concentration was determined by Bradford assay. Glutathione agarose beads (25µl) were equilibrated in PBS containing 0.1% NP40, then incubated with 50 µg of the indicated GST fusion protein at 4°C for 1 hour. The beads were washed twice with equilibration buffer, then incubated with 1.5 mg of SLB1 nuclear extract at 4°C for 2 hours. The beads were washed twice in equilibration buffer, and resuspended in SDS sample dyes. The eluted proteins were separated by electrophoresis on a 10% SDS gel, transferred to nitrocellose and probed with an anti-p53 antibody (DO-1; Santa Cruz). The blots were developed using chemiluminescence (Pierce). Finally, the membranes were stripped with 0.1M Glycine (pH2.0) for 3-5 hours at room temperature and reprobed with an anti-Tax antibody, prepared against the C-terminal 13 amino acids of Tax.

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3.5d GST pull-down assays. All GST pull-down experiments were performed using 12.5µl of glutathione agarose beads equilibrated in 0.5X Superdex buffer (1X Superdex contains 25mM Hepes, pH7.9, 12.5 mM $MgCl_2$, 10 µM $ZnSO_4$, 150 mM KCl, 20% Glycerol, 0.1% NP40, 1mM EDTA). The indicated amount of purified GST-CBP fusion protein was incubated with the beads for 1-2 hours at 4°C, then washed 2 times with 0.5X Superdex buffer. The indicated amount of the second protein was then added to the washed beads and incubated for 1-2 hours at 4°C. The beads were washed twice as before, and bound proteins were eluted with SDS sample dyes. Bound proteins were separated by electrophoresis on a 10% SDS gel, transferred to nitrocellulose and probed with the appropriate antibody. The following antibodies were used in this report: anti-Tax antibody (epitope corresponding to the carboxy-terminal 13 amino acids), anti-p53 antibody (DO-1; Santa Cruz Biotech, epitope corresponding to amino acids 11-25), anti-p53 antibody (Ab-1; Calbiochem, epitope corresponding to amino acids 371-380), and anti-His antibody (H-15; Santa Cruz Biotech). The antibody inhibition pull-down experiments were performed as above, except that the indicated amount of p53 was preincubated with the indicated amount of either anti-p53 (DO-1), anti-p53 (Ab-1), or anti-EGFR (Santa Cruz Biotech; cell surface epitope) for 15 minutes at 4°C prior to addition to either GST alone or GST-$KIX_{588-683}$.

3.6 ACKNOWLEDGEMENTS. We thank R. Harrod and C.-Z. Giam for the Tax point mutant (K88A). This work was supported by Public Health Service grant CA-55035 from the National Institutes of Health, and VM-170 from the American Cancer Society.

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Supplemental Figures

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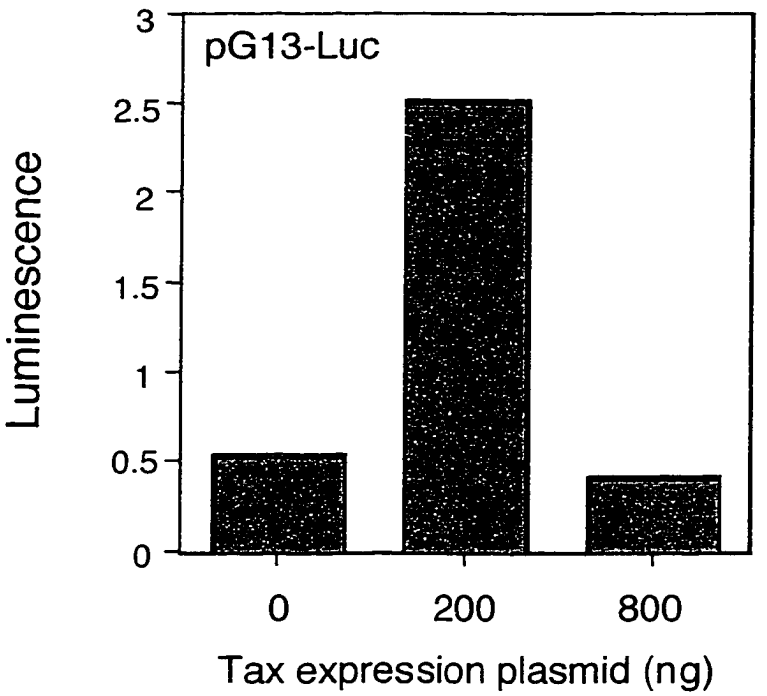

Figure 3.11 Tax has no effect on expression from the pG13-Luc reporter in the absence of p53. Transient cotransfection assays were carried out in Jurkat T-cells using 400 ng of the pG13-Luc reporter plasmid and the indicated amounts of the Tax expression plasmid (IEX-Tax). The values shown are the average luminescence from duplicate samples.

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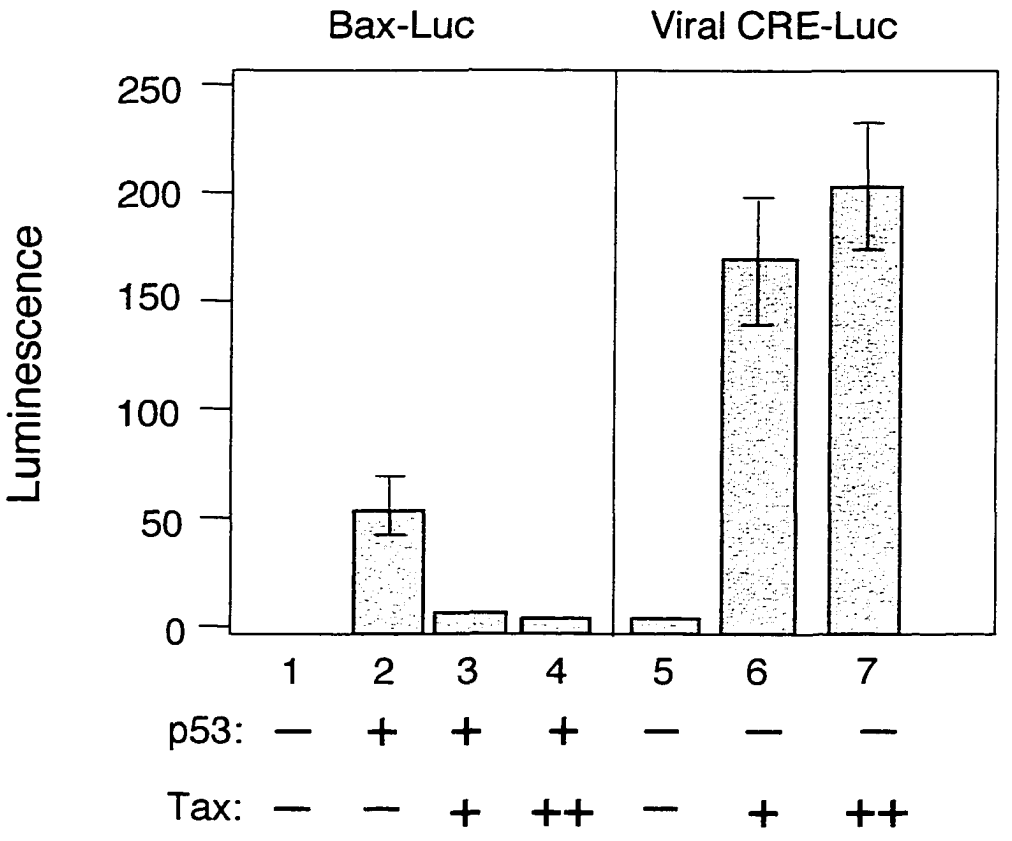

Figure 3.12 Tax represses p53 mediated activation of the human bax promoter. Transient cotransfection assays were carried out in HTLV-I-negative Jurkat T-cells. The p53 responsive Bax-Luc reporter plasmid (400 ng; lanes 1-4) or the Tax responsive viral CRE-Luc reporter plasmid (400 ng; lanes 5-7) was cotransfected with a constant amount of the p53 expression plasmid (pC53-SN3; 400 ng) and/or increasing amounts of the Tax expression plasmid (HTLV-I-Tax; 100 or 200 ng) as indicated. The luminescence values shown are the average luminescence +/- the standard error from one experiment performed in triplicate.

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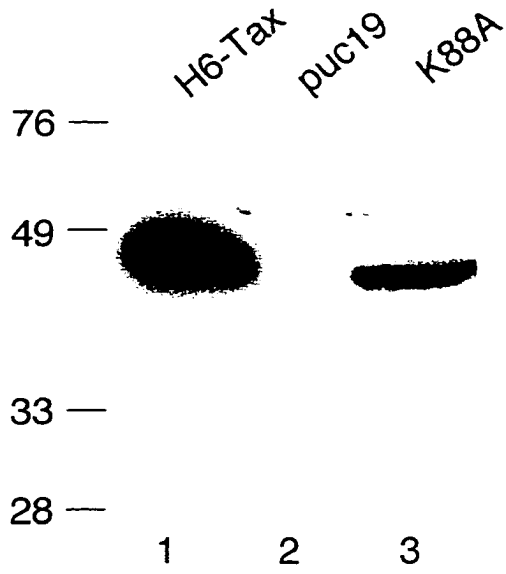

Figure 3.13 Tax mutant K88A is properly expressed in transient transfection assays. Jurkat T-cells were transfected in duplicate with 500 ng of pUC19 (lane 2) or the expression plasmid for Tax K88A (lane 3). Cells were harvested after 24 hours. Duplicates were combined, resuspended in SDS sample buffer, and run on a 12% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with an anti-Tax antibody. Purified recombinant Tax (20 ng) was loaded in lane 1 of the gel as a control.

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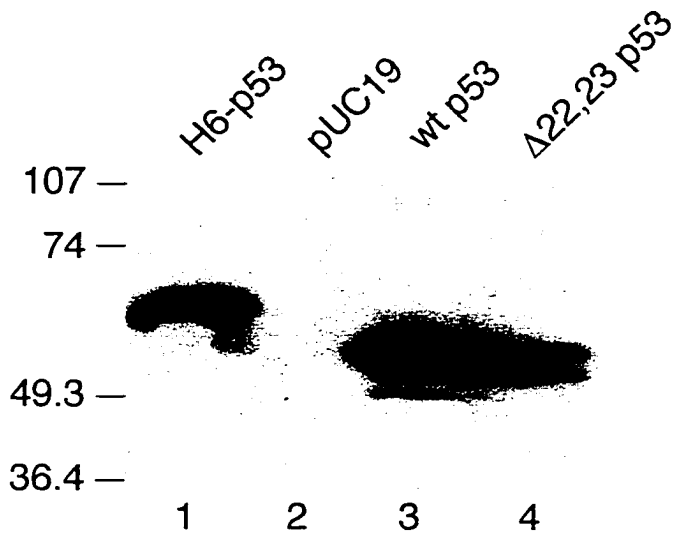

Figure 3.14 p53 Δ22,23 is properly expressed in transient transfection assays. Jurkat T-cells were transfected in duplicate with 500 ng of pUC19 (lane 2), the expression plasmid for wt p53 (lane 3) or the expression plasmid for p53 Δ22,23 (lane 4). Cells were harvested after 24 hours. Duplicates were combined, resuspended in SDS sample buffer, and run on a 12% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with an anti-p53 antibody. Purified recombinant p53 (20 ng) was loaded in lane 1 of the gel as a control.

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CHAPTER 4
TAX TRANSACTIVATION IS MEDIATED THROUGH THE COACTIVATOR CBP IN VIVO

Chapter four is a small collection of functional experiments demonstrating that Tax utilizes CBP to activate transcription in vivo. Figures 4.2A and 4.2B were published along with figures 2.3 and 2.6B as a chapter in a book on the molecular pathogenesis of HTLV-I. Figure 4.3 was published as part of larger study in the journal *Molecular and Cellular Biology*. The citations for the publications appear below.

Jennifer K. Nyborg and Karen Van Orden. 1999. "Binding of HTLV-I Tax to the Coactivator CBP Disrupts the Transcription Activity of c-Jun" in Molecular Pathogenesis of HTLV-I: a current perspective. eds. O. John Semmes and Marie-Louise Hammarskjold, ABI Professional Publications, (USA).

Giebler, H. A., J. E. Loring, K. Van Orden, M. A. Colgin, J. E. Garrus, K. E. Escudero, A. Brauweiler and J. K. Nyborg. 1997. Anchoring of CREB binding protein to the human T-cell leukemia virus type-I promoter: a molecular mechanism of Tax transactivation. *Mol. Cell. Biol.* **17**:5156-5164.

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4.1 ABSTRACT

The human T-cell leukemia virus type I (HTLV-I)-encoded Tax protein activates viral transcription and is tightly linked with the malignant transformation of infected T-cells. Tax activates transcription of the viral promoter through interaction with the cellular transcription factor CREB. Although Tax stabilizes the binding of CREB to the Tax-responsive viral CRE's in the HTLV-I promoter, the precise molecular mechanism by which Tax mediates strong transcriptional activation through CREB remains unclear. In this report, we show that the pleiotropic cellular coactivator CBP plays a prominent role in Tax transactivation in vivo. Additionally, we show that transfection of an expression plasmid for one distinct domain of CBP called KIX, serves as a dominant negative and inhibits Tax transactivation in vivo. This suggests that the KIX domain of CBP binds to Tax in vivo, and that CBP is likely a cofactor in mediating Tax stimulation of HTLV-I transcription. Together, these data support a model where Tax anchors CBP to the HTLV-I promoter, with strong transcriptional activation resulting from the CBP-associated activities of nucleosome remodeling and recruitment of the general transcription machinery.

4.2 INTRODUCTION

The human T-cell leukemia virus type I (HTLV-I) is a complex retrovirus responsible for an aggressive and fatal malignancy called adult T-cell leukemia (7, review). The viral genome encodes a unique oncoprotein, called Tax, which is a key regulatory protein that appears to facilitate the transition from viral latency to high levels of virion production in the infected T-cell. Tax mediates the emergence from latency via strong transcriptional activation of the HTLV-I genome. The precise molecular mechanism by which Tax activates viral transcription has been widely studied, but is not fully understood. Tax interacts

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with the host cell protein cAMP-response element binding protein (CREB) (1, 3, 4, 6, 8, 28, 29) to stimulate viral transcription through three 21 base-pair (bp) repeat sequences in the transcriptional control region of the virus (5, 9, 12, 18, 20, 22, 24). The three 21-bp repeats each contain an off-consensus core octanucleotide sequence with similarity to the cyclic-AMP response element (CRE) with a short run of GC-rich nucleotides flanking each side. The CRE and GC-rich flanks together form a critical DNA element (called the viral CRE) that is obligatory for Tax transactivation in vivo (6, 10, 12, 17,18). Tax serves to enhance and stabilize CREB binding to the CRE core (3, 6, 25, 29, 30) and makes specific contacts with the GC-rich flanking sequences (13, 15, 16). These interactions lead to the formation of a stable, Tax-containing ternary complex on the HTLV-I promoter.

To activate transcription, this nucleoprotein complex is believed to serve as a high affinity binding site for the recruitment of CREB binding protein (CBP) (11, 14). CBP is a pleiotropic cellular coactivator protein, which mediates transcriptional activation through a wide variety of structurally unrelated cellular transcription factors (23, review). Tax has been shown to directly interact with a small region of CBP called the KIX domain (amino acids 588-683) in vitro (11, 14, 26). The solution structure of this region of CBP bound to phosphorylated CREB shows that it consists of three interacting alpha helices that form a hydrophobic pocket (21). This suggests that the KIX domain of CBP may serve as a transcription factor binding site which allows CBP to be anchored to target promoters. Once anchored to the promoter, CBP appears to facilitate transcriptional activation through chromatin remodeling and contact with the general transcription machinery (2, 19, 27). Thus the recruitment of CBP to the HTLV-I promoter by Tax most likely serves to activate transcription through the coactivator functions associated with CBP.

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Although the molecular interactions between Tax and CBP have been well characterized biochemically (11, 14, 26), we were interested in directly testing the effect of CBP on transcriptional activation mediated through Tax in vivo. Here we show that in transient cotransfection assays CBP strongly potentiates Tax transactivation and that this effect is specific for the viral CRE DNA element. Additionally, cotransfection of an expression plasmid for just the KIX domain of CBP inhibits Tax activation of viral CRE-dependent transcription. These data suggest that cotransfected KIX can act as a competitive inhibitor of Tax transactivation by occupying the CBP binding site on Tax. Together, these data support a model where Tax functions to directly recruit CBP to the viral promoter. The stable association of CBP with the viral promoter results in HTLV-I transcriptional activation achieved through the CBP-associated activities of nucleosome remodeling and recruitment of the general transcription machinery.

4.3 RESULTS AND DISCUSSION

4.3a CBP potentiates Tax transactivation through the viral CRE in vivo.

To further characterize the mechanism of Tax transactivation through CREB and CBP, we have utilized functional assays to investigate the interactions between these molecules in vivo. Transient transfection assays were performed in HTLV-I-negative Jurkat T-cells using a luciferase reporter construct carrying three copies of the Tax-responsive viral CRE cloned upstream of the thymidine kinase minimal promoter. As a control, we also tested the same reporter but with three copies of the cellular CRE element

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cloned upstream of the minimal promoter. The cellular CRE element contains a consensus CREB binding site but does not have the GC-rich flanking sequences necessary for Tax recognition and is therefore unresponsive to Tax (Fig. 4.1). Figure 4.2A shows that cotransfection of the viral CRE luciferase reporter and an expression plasmid for Tax produced a 9-fold increase in luciferase activity over reporter alone (compare lanes 1 and 4). Cotransfection of increasing amounts of a CBP expression plasmid in the Tax-containing reactions significantly increased viral CRE promoter activity, producing up to a 25-fold increase in luciferase activity as compared to Tax alone (Fig. 4.2A, lanes 4-6). Interestingly, the dramatic effect of CBP was dependent upon Tax in the reaction, as CBP produced a negligible effect on luciferase activity from the viral CRE promoter in the absence of Tax (Fig. 4.2A, lanes 1-3). These results are in marked contrast to the results obtained with the cellular CRE luciferase reporter, which is unresponsive to Tax. Figure 4.2B shows that CBP had only a modest effect on transcription from this reporter construct, and Tax did not further enhance CBP-mediated transcriptional activation (lanes 7-12). This modest effect of CBP on the cellular CRE reporter is most likely mediated through endogenous phosphorylated CREB which binds CBP and the cellular CRE element to activate transcription. Together, these data provide strong evidence that CBP plays a fundamental role in mediatingTax transactivation of HTLV-I transcription in vivo.

4.3b The KIX domain of CBP inhibits Tax Transactivation in vivo.

Previous studies carried out in the lab have demonstrated that Tax can efficiently recruit just the KIX domain of CBP to CREB and the viral CRE in vitro. These data, together with the preceeding functional experiment, suggest that the Tax transactivation in vivo is dependent on interaction with the KIX domain

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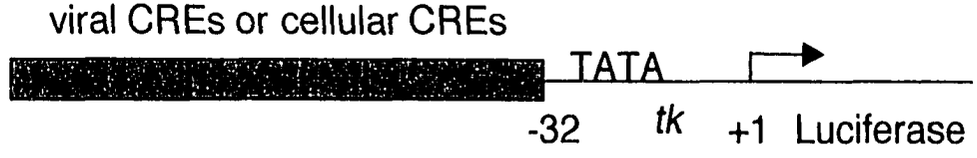

Figure 4.1 Schematic illustration of the viral CRE and cellular CRE luciferase reporter constructs used in transient cotransfection assays. Note that the two constructs are identical except for the transcription factor binding sites cloned upstream of the thymidine kinase (*tk*) minimal promoter.

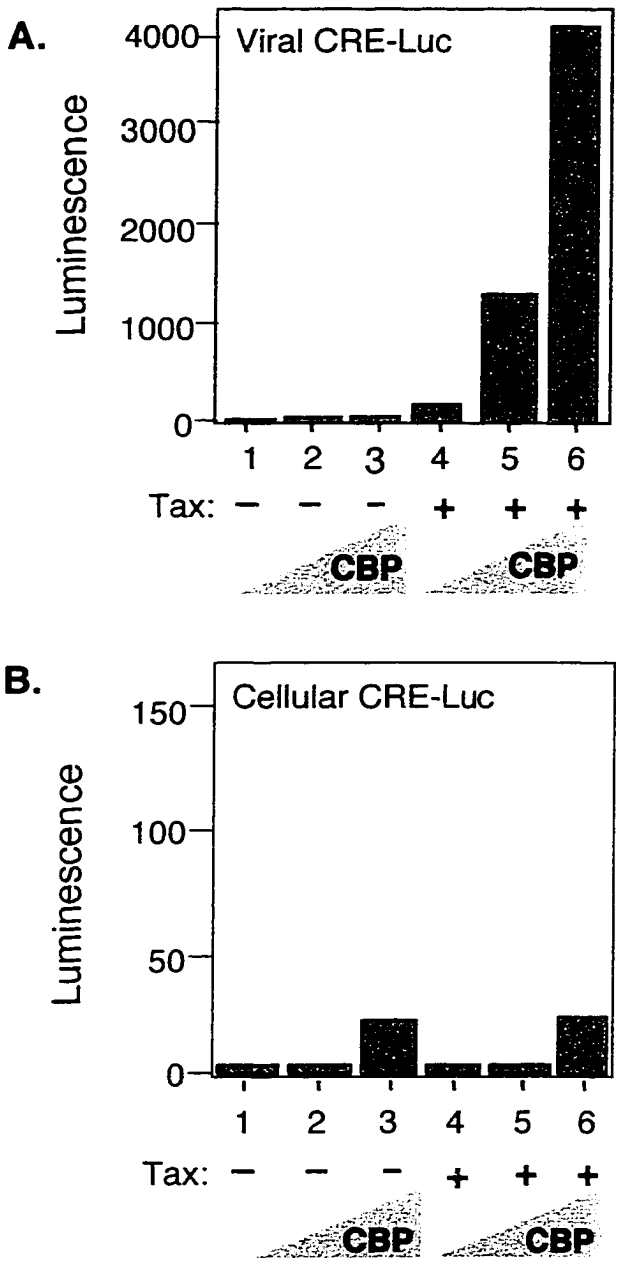

Figure 4.2 CBP mediates Tax transactivation in vivo. Transient cotransfection assays were performed using HTLV-I negative Jurkat T-lymphocytes. (A) Cells were transfected with 400 ng of the viral CRE-Luciferase reporter plasmid. The Tax expression plasmid (50 ng) and/or increasing amounts (0, 400 or 800 ng) of the CBP expression plasmid were cotransfected as indicated. (B) Cells were transfected with the cellular CRE-Luciferase reporter plasmid. The Tax expression plasmid (50 ng) and/or increasing amounts (0, 400 or 800 ng) of the CBP expression plasmid.were cotransfected as indicated. The luminescence values shown are the average of duplicate samples.

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of CBP. If this is true, then the addition of a KIX expression plasmid to transient cotransfection assays should compete with active CBP in the cell, thus inhibiting Tax transactivation in vivo. To test this hypothesis, transient transfection assays were performed using the viral CRE luciferase reporter construct and expression plasmids for Tax and the KIX domain of CBP. Figure 4.3 shows that addition of the KIX expression plasmid to the chimeric promoter carrying the viral CRE's had a negligible effect on luciferase activity, indicating that the plasmid was not toxic to the cells. When the Tax expression plasmid was transfected into the cells, a predicted increase in luciferase activity was observed. However, cotransfection of the KIX expression plasmid decreased Tax transactivation in a dose-dependent fashion. The observation that cotransfection of the KIX expression plasmid inhibited Tax stimulation on the viral CRE suggests that KIX may compete with endogenous CBP for association with Tax on the promoter. These data provide evidence that Tax interaction with the KIX domain of CBP is functional and necessary for Tax transactivation in vivo.

4.4 MATERIALS AND METHODS

4.4a Recombinant plasmids, cell culture and transfections. The luciferase reporter plasmid viral CRE-Luc contains exactly the same promoter sequences as previously reported (constructed from pminCAT-21 bp repeat and pminCAT-CRE, see 6), however the promoter fragment has been cloned into the pGL2Basic (Promega) luciferase reporter plasmid. The Tax expression plasmid, pHTLV-I Tax has been previously described (5). The RSV-KIX plasmid was constructed by inserting a PCR fragment corresponding to amino acids 459 to 679 of mouse CBP into a modified pRc/RSV plasmid.

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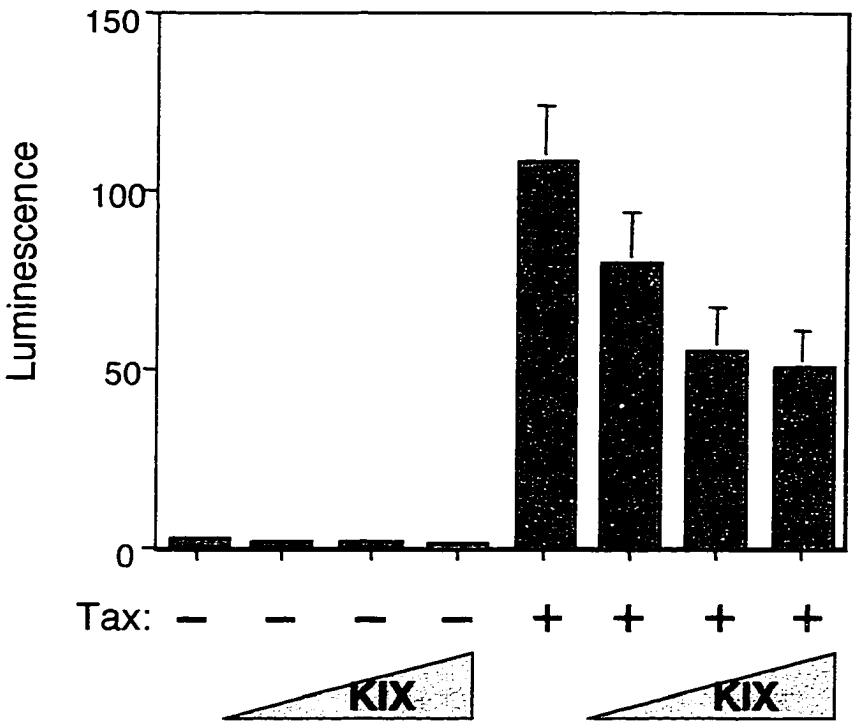

Figure 4.3 KIX represses Tax transactivation of the viral CRE in vivo. Transient cotransfection assays were performed in the HTLV-I-negative human Jurkat T-cells. Cotransfections were performed with 400 ng of the viral CRE promoter luciferase reporter plasmid, 50 ng of the Tax expression plasmid, and/or the indicated amount of the KIX expression plasmid. Luminescence was quantitated using a luminometer. Values shown are the mean luminescence ±SE from two independent experiments performed in triplicate.

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The HTLV-I-negative Jurkat human T-lymphocyte cell line was maintained in Iscoves medium (IMDM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. Transient co-transfection assays were performed in Jurkat T-cells, grown to a density of $1x10^6$ cells/ml. The cells were transfected with lipofectamine (Life Technologies) and a constant amount of DNA and assayed for luciferase activity according to the manufacturer's directions. A Turner Designs Model TD 20-e Luminometer was used to measure luminescence.

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CHAPTER 5
Future Directions

The data presented here support a model for Tax deregulation of host cell gene expression and possibly transformation through sequestration of the cellular coactivator CBP. We show that CBP, and more specifically the KIX domain of CBP, plays a critical and functional role in Tax transactivation through the viral CRE DNA sequence in vivo. As a consequence of this interaction other transcription factors are denied access to the KIX domain leaving them unable to activate transcription of target genes and leading to deregulation of host cell gene expression. Future studies will focus on confirming that the Tax-KIX interaction plays a critical role in the cellular transformation process. Furthermore, we would like to test our model of coactivator competition as a possible mechanism for c-jun oncogenesis. Experiments designed to answer these questions are outlined below.

5.1 Does Tax disrupt KIX interactions in vivo?

We would like to show that in fact KIX interactions are disrupted or altered in vivo. This information could be elucidated through the use of

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immunoprecipitation assays. We will utilize HTLV-I infected T-cell lines as well as a Tax inducible cell line to make extracts for these experiments. Immunoprecipitation of CBP from these different extracts will allow analysis of the associated proteins through western blot analysis. We predict that interactions mediated through the KIX domain of CBP will be disrupted in the presence of Tax. The effect of Tax on interactions with other regions of CBP is currently unknown. Based on our studies of Tax and p53, and the observation that Tax does not disrupt p53 interaction with the C/H1 domain of CBP, we predict that interactions with other regions of CBP will remain intact. Similar experiments were initiated in the past and the experimental techniques have been worked out. However, we found that inadequate amounts of CBP protein were immunoprecipitated from cell extracts. This may have been due to the dilute CBP antibody we used for these experiments. We will have a new CBP antibody made that is more concentrated and in large enough quantity that we could couple the antibody to the protein A beads used in the procedure. This should increase the efficiency with which we can immunoprecipitate CBP and allow us to examine the proteins complexed with CBP in vivo.

5.2 Can a Tax mutant defective for interaction with KIX transform cells?

We, and others, have shown that the Tax K88A mutant is defective for interaction with KIX. This mutant is also unable to compete with other transcription factors for the KIX domain of CBP. Consequentially, we hypothesize that this mutant is unable to disrupt interactions with KIX in vivo and unable to deregulate CBP mediated transcription. To test this hypothesis we could stably transfect a cell line with the mutant Tax gene and express Tax K88A using an inducible expression system. Immunoprecipitation experiments

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as describe above could then be performed to analyze CBP complex formation in vivo in the presence of Tax K88A. Based on our previous findings we predict that this mutant would have no effect on protein interactions with the KIX domain.

It would also be interesting to determine if the Tax K88A mutant has lost transformation potential as a result of its inability to interact with KIX. This could be tested using techniques similar to those used to demonstrate that wild type Tax can transform cells. These assays involve transfection of Rat embryo fibroblasts with a Tax expression plasmid. Cellular transformation is then measured by looking for anchorage-independent cell growth and colony formation on soft agar.

5.3 Is competition for the KIX domain of CBP the mechanism of c-jun mediated oncogenesis?

Though the oncogenic potential of c-Jun is well established, the mechanism of c-Jun oncogenesis is unknown. Based on our findings with Tax, it would be reasonable to speculate that oncogenic c-Jun may aberrantly bind to the KIX domain of CBP. The oncogenic form of c-Jun may be overexpressed or may bind to KIX with heightened affinity. Either of these two scenarios would lead to inappropriate occupation of the KIX binding site. This may, in effect, transcriptionally inactivate other KIX binding transcription factors, and specifically may inhibit p53 transcription function. Thus, the c-Jun oncogenic pathway may mimic that observed with Tax. This hypothesis is based in part on the preliminary observation that overexpression of c-Jun causes inhibition of p53 function. Experiments to investigate this question could mimic those carried out in chapter three in our study of Tax inhibition of p53 function. These involve transient transfection assays and in vitro binding assays to demonstrate

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competition for the KIX domain as a mechanism for repression of transcription function.

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