DISSERTATION

THE HTLV-1 ONCOPROTEIN TAX AND THE TUMOR SUPPRESSOR p53 BIND A COMMON DOMAIN OF CBP/p300 TO MEDIATE TRANSCRIPTIONAL ACTIVATION

Submitted by
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KIRSTEN E. STRICKLER SCOGGIN ENTITLED "THE HLTV-1 ONCOPROTEIN TAX AND THE TUMOR SUPPRESSOR p53 BIND A COMMON DOMAIN OF CBP/p300 TO MEDIATE TRANSCRIPTIONAL ACTIVATION" BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE HTLV-1 ONCOPROTEIN TAX AND THE TUMOR SUPPRESSOR p53 BIND A COMMON DOMAIN OF CBP/p300 TO MEDIATE TRANSCRIPTIONAL ACTIVATION

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent associated with the development of adult T-cell leukemia and encodes a viral transactivator known as Tax. While the precise molecular mechanism by which cellular transformation occurs is unknown, researchers hypothesize that Tax is essential in both viral and cellular gene expression. To activate HTLV-1 transcription, Tax interacts with the cellular protein CREB, and the pleiotropic coactivators CBP/p300. We have characterized a novel and potentially important alternate binding site for Tax on the carboxy-terminal region of CBP/p300 that mediates Tax transcription function. We have determined the minimal binding domain for Tax at the carboxy-terminal binding region, termed CR2, as amino acids 2003 to 2212. A double point mutant of CR2 directed to one of the α-helical motifs in this domain was found to be defective for binding to Tax. We also characterize the region of Tax responsible for interaction with CR2 and show that the previously identified transactivation domain of Tax (amino acids 312 to 319) participates in CR2 binding.

The tumor suppressor p53 also recruits the cellular coactivator CBP/p300 to mediate the transcriptional activation of target genes. Previously, p53 has been shown to interact with several regions of CBP/p300, including the C/H3 and KIX domains. We find that p53 also interacts strongly with a smaller region of the CR2 domain encompassing amino acids 2055 to 2150. We show that the same double point mutation targeted to the first α-helical motif in this domain, defective for interaction with Tax, is also compromised for interaction with p53. The observation that both p53 and Tax bind to overlapping regions of CR2 led us to ask whether or not their binding is mutually exclusive. We show that p53 and Tax compete for binding CR2. Thus, Tax disruption of the p53-CR2 complex may be a contributing molecular event in the HTLV-1 transformation pathway. Together, these studies identify novel Tax-interacting and p53-interacting sites on CBP/p300 and extend our understanding of the molecular mechanism of Tax transactivation.

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Chapter 1

Review of the Literature

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

Human T-cell leukemia virus type 1 (HTLV-1) was the first identified oncogenic human retrovirus, discovered by Gallo and coworkers in 1980 in a T-cell line isolated from a patient with cutaneous T-cell lymphoma (mycosis fungoides) (256). In 1982, a second, highly related virus, isolated from a patient originally diagnosed with hairy T-cell leukemia, was identified as human T-cell leukemia virus type 2 (HTLV-2) (158). HTLV-1 and HTVL-2 are distinct Type C retroviruses that belong to the Deltaretrovirus genus (40). While the viruses share about 65% nucleotide sequence similarity (51, 295), only HTLV-1 is clearly associated with a disease (reviewed in 114). It is estimated that between 10 to 20 million people worldwide are carriers of HTLV-1, with roughly 2% to 5% developing an HTLV-1-associated disease.

1.1a Diseases associated with HTLV-1 infection

Adult T-cell leukemia (ATL), a lymphoproliferative disorder, was first reported in patients with lymphoid neoplasms in 1977 by Takatsuki and coworkers (327). Upon the discovery of HTLV-1 and subsequent epidemiological and genetic studies, HTLV-1 was determined to be etiologically linked to ATL (132, 256, 268, 361). HTLV-1 infection is endemic to southwestern Japan, Africa, northeastern South America, and the Caribbean (327). A recent study suggests that the incidence of HTLV-1 infection is as high as 8% in some parts of Japan as compared with a 0.003% to 0.04% infection rate in the United States (reviewed in 114). Interestingly, there is rather low sequence diversity seen among isolates from different continents (141, 301). HTLV-1 proviral DNA was even found in an Andean mummy, suggesting that this retrovirus has been around for at least roughly 1,500 years (199). Of the 10 to 20 million HTLV-1-infected individuals, a very small percentage (2% to 3%) develops ATL in their lifetime (114). There is a 20 to 30 year latency period between infection and development of malignancy (164, 174).

ATL can be divided into five clinical stages: asymptomatic, pre-leukemic, chronic/smoldering, lymphoma, and acute (296). Of individuals with clinical manifestations of the disease, acute ATL represents the majority of cases with roughly 55%, while 30% of ATL cases are made up of chronic/smoldering type ATL (296, 355). The less aggressive type of ATL, chronic/smoldering, is characterized by few leukemic cells, skin lesions, and hepatosplenomegaly (164). Patients with chronic stage ATL can develop acute ATL within months. Acute ATL is the most aggressive, rapid form of leukemia and patients exhibit an elevated white blood cell count with greater than 5% abnormal T-lymphocytes (with highly convoluted nuclei), skin lesions due to infiltrating leukemic cells, lytic bone lesions, hepatosplenomegaly, and immunodeficiency (114). Immunologic studies of ATL cells have shown that T-cell lymphocytic markers for this virus include CD2+, CD3+, CD5+, CD25+, and CD8-, but most often are of the CD4+ phenotype (65, 111, 135). Tumor cells from ATL patients indicate the disease originates from a single HTLV-1-infected transformed cell, as the HTLV-1 provirus is integrated in a monoclonal or oligoclonal manner (362). Cytogenetic studies have shown that chromosomal abnormalities exist in ATL cells in which an increase in abnormalities is evident in the more severe cases of ATL (90, 276). The prognosis of acute ATL is extremely poor, and the median survival time is six months and the four-year survival rate is 5% (reviewed in 344).

HTLV-1 has also been implicated in the onset of the neurodegenerative disorder known as tropical spastic paraparesis/ HTLV-1-associated myelopathy (TSP/HAM), afflicting 1% to 2% of HTLV-1-infected individuals (98, 99, 147, 270, 324). TSP/HAM is characterized by spasticity and weakness in the lower extremities, sensory loss, and demyelination of the nerves of the spinal cord (reviewed in 363). Damage to the central nervous system (CNS) could be due to infiltration by HTLV-1-infected cells, as high levels of cytotoxic T-lymphocytes have been found in TSP/HAM patients (115, 148, 178). TSP/HAM patients also exhibit abnormal T-lymphocytes; however, they are distinct from those found in the peripheral blood of ATL patients (242). Unlike ATL, TSP/HAM has a shorter latency period and the majority of patients are seropositive for HTLV-1 antibodies (355). TSP/HAM is also three times more likely to affect women than men, whereby ATL is found more often in men (355). Additionally, only a few patient cases have presented with both ATL and TSP/HAM (344). HTLV-1 has also been associated with other diseases such as uveitis (220), infective dermatitis (184) and Sjögren's syndrome (319); however, the exact molecular mechanism for pathogenesis is yet to be determined (reviewed in 344).

Animal models for HTLV-1 infection and diseases have been important for further understanding HTLV-1. Mice transgenic for HTLV-1 developed an inflammatory disease resembling rheumatoid arthritis (146). Rats injected with HTLV-1-infected cells developed neuropathological changes whereby the proviral DNA was detected in the peripheral blood and in the spinal cords of the affected rats (181). Further studies with rat models showed that 15 months post incubation, rats infected with HTLV-1 developed symptoms similar to TSP/HAM with hind limb paraparesis and the presence of proviral DNA in macrophages (162). Severe combined immunodeficient (SCID) mice have also .been used as a small animal model to study HTLV-1 infection (79). SCID mice infected with human peripheral blood lymphocytes from ATL patients developed tumor cells, while mice infected with immortalized HTLV-1-infected cells did not form tumors (79). These results are consistent with those performed in rats and in rabbits transplanted with immortalized HTLV-1-infected cells, in which leukemia did not develop in the infected animals (238).

1.1b HTLV-1 genomic structure and life cycle

HTLV-1 proviral integration occurs at a random location within the host cell genome as no specific integration site(s) have been identified to date (54). The integrated provirus can be spread between cells or be spread to the two daughter cells of an infected cell upon mitotic division (reviewed in 16). A lower rate of mutation is associated with proliferation of cells. Thus, mitotic transmission of the HTLV-1 provirus is thought to be the preferred mode of transmission as there are little observed differences in DNA sequences among HTLV-1 isolates (141, 301). HTLV-1 enters the host cell through cell to cell contacts (although the HTLV-1 receptor has yet to be identified), whereby the viral RNA is reverse transcribed into DNA (355). The double-stranded proviral DNA is then localized to the nucleus and integrated into the host cell genome. The viral genes are then transcribed and translated using the host cell transcription/translation machinery. The HTLV-1 virion is then assembled and released, using viral proteins as well as the host cell proteins (355). The HTLV-1 viral life cycle is then repeated following mitotic or infectious transmission.

The HTLV-1 genome is approximately 9 kilobases of single-stranded RNA and encodes the structural and enzymatic genes of Gag (group-specific antigen), Pol (polymerase), and Env (envelope) as shown in figure 1.1. The Gag proteins are important for membrane targeting, viral assembly, and release of virion particles (reviewed in 186). Following translation into the Gag precursor polyprotein, the polyprotein is cleaved to form three Gag proteins; matrix (MA, 19 kilodalton (kDa)), capsid (CA, 24 kDa), and nucleocapsid (NC, 15 kDa) proteins (114). An ORF located from the 3' end of Gag to the 5' end of Pol encodes the enzymatic activity of HTLV-1 protease, which is responsible for generating mature Gag products and/or proteolytic cleavage of translation products (114). The Pol region encodes the enzymes required for viral replication. Namely, reverse transcriptase is an RNA-dependent DNA polymerase that transcribes viral RNA into DNA, while integrase is responsible for integrating HTLV-1 into the host cell genome (reviewed in 40, 355). Also, the RNA strand in the RNA-DNA duplex during reverse transcription is removed by the viral RNaseH

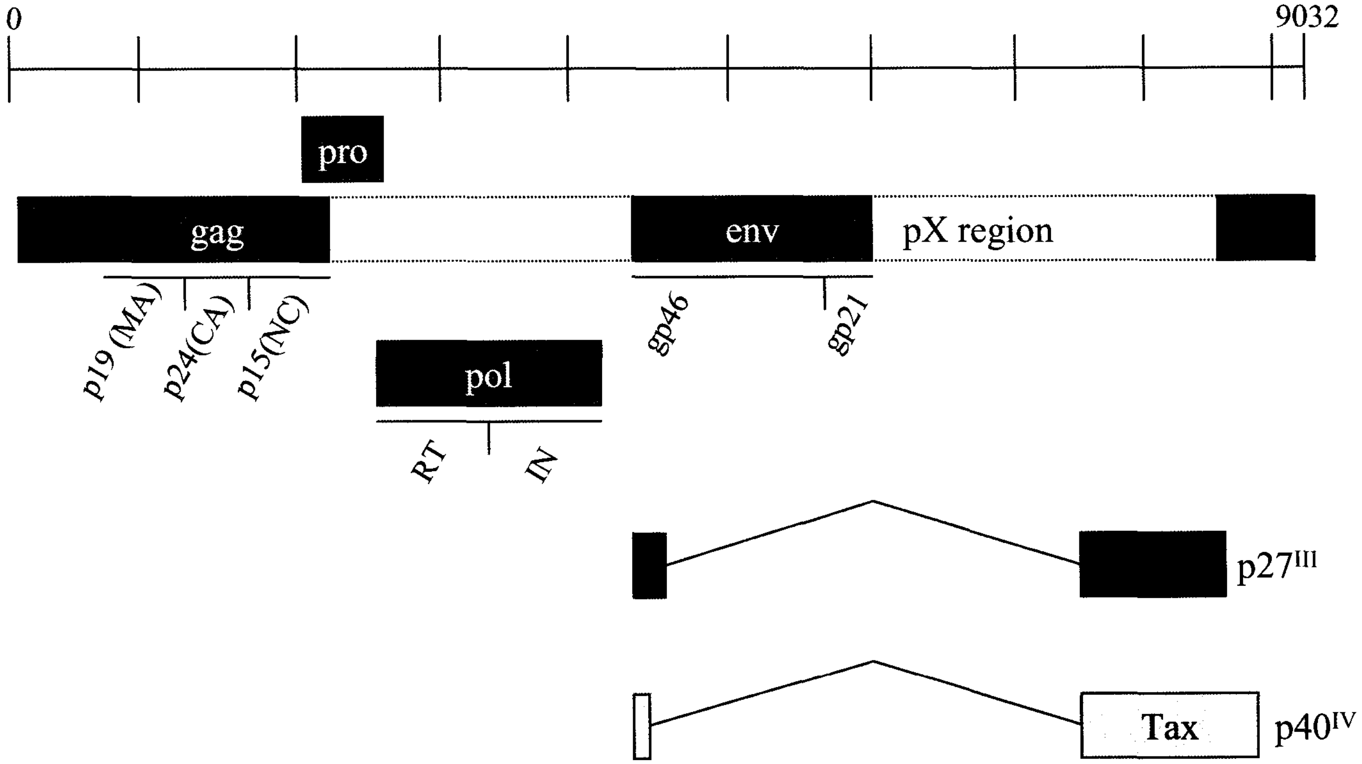


Figure 1.1. **Genomic structure of the human T-cell leukemia virus type 1.** The common viral structures of gag, pro, pol, and env are indicated. The regulatory proteins, Tax and Rex, encoded by the pX region are also indicated. This figure is modified from Grant et al. (111).

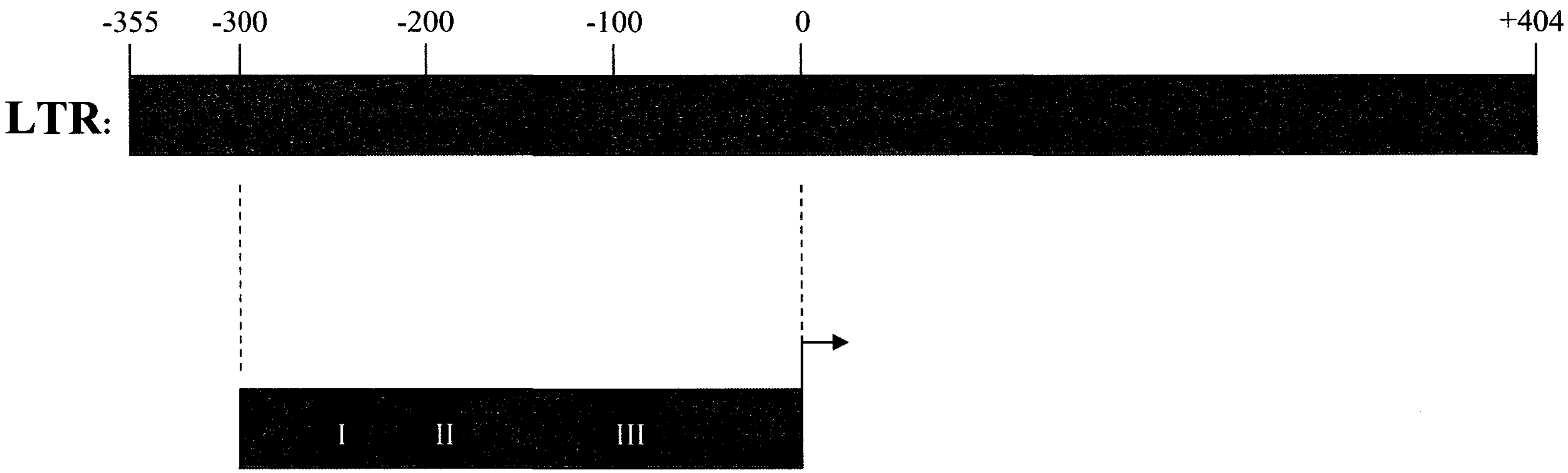
enzymatic activity. Following translation, the Env precursor polyprotein is cleaved into two mature Env products; surface glycoprotein (SU, 46 kDa) and transmembrane protein (TM, 21 kDa), both necessary for forming the outer layer of the viral membrane (114).

HTLV-1 also encodes a unique region in the 3' end of the genome, which generates several distinct regulatory and accessory proteins through alternative splicing and recognition of internal initiation codons. This region, unique to human retroviruses, is known as pX and contains four open reading frames (ORF) (ORF I, II, III, and IV) (58). Figure 1.1 illustrates the structure of the HTLV-1 genome as well as the structural and enzymatic proteins encoded by the HTLV-1 provirus. ORF IV encodes the important regulatory protein Tax, a 40 kDa protein, while ORF III encodes the 27 kDa nuclear phosphoprotein, Rex (229). Tax and Rex are translated from the doubly spliced subgenomic mRNA (50). Tax is responsible for transcriptional activation of the promoter in the U3 long terminal repeat region and will be discussed further in section 1.2. The phosphoprotein Rex also influences HTLV-1 replication and the production of viral structural genes; however, it differs from Tax in that viral gene expression is regulated at the post-transcriptional level (114). More specifically, Rex increases the expression of unspliced viral genomic RNA and singly spliced mRNA from the Env gene (reviewed in 80). Other genes encoded by the HTLV-1 pX region are $p12^{I}$ (ORF I), $p27^{I}$ (ORF I), $p13^{II}$ (ORF II), $p30^{II}$ (ORF II), and $p21^{RexIII}$ (ORF III) (reviewed in 4). Like Tax and Rex, these accessory proteins are generated from alternatively spliced forms of mRNA. $p12^{I}$, $p13^{II}$, and $p30^{II}$ are not necessary for HTLV-1 replication or T-cell immortalization in vitro (71, 267); however, these accessory proteins are important for maintaining high viral loads and viral infectivity (4).

The HTLV-1 genome consists of two long terminal repeats (LTR) that are divided into the U3, R, and U5 regions. The 755 nucleotide 5' LTR contains important sequences essential for viral gene regulation as well as polyadenylation of mRNAs (283). The U3 region contains three semi-conserved 21-basepair repeats, called Tax responsive elements (TREs or viral CREs), which are responsive to Tax transactivation as shown in figure 1.2. The promoter distal repeat (TRE-I) is positioned from -252 to -232, while the promoter central repeat (TRE-II) and the promoter proximal repeat (TRE-III) are located from -204 to -184 and -104 to -84, respectively, all relative to the transcriptional start site (21, 36, 282). These TREs consist of an eight-basepair cyclic-AMP response element (CRE) off-consensus binding site, flanked by GC-rich DNA sequences (273, 282). The TREs are important for Tax transactivation in vitro and in vivo (37, 89, 101, 150, 194, 221, 244). At least two of the three TREs are required for significant Tax transactivation from the viral promoter (36, 88, 110, 150, 230, 248, 271, 295, 306). The U3 region also contains a TATA binding sequence and binding sites for several cellular factors that are necessary for HTLV-1 gene expression and will be described further in section 1.2 (21, 34, 35, 60, 94, 104, 213, 214, 233, 282, 318).

1.1c HTLV-1 transmission and infectivity

HTLV-1 infectivity is inefficient in cell-free environments (59, 257). The most prevalent form of HTLV-1 infection occurs by vertical transmission from mother to child through infected T-lymphocytes in breast milk or through infected maternal lymphocytes from mother to fetus during pregnancy (114). HTLV-1 can also be transmitted by sexual contact as well as contact with HTLV-1 infected blood or blood-products (166, 241). In particular, intravenous drug users can become infected with HTLV-1 through shared



TRE I (Distal Repeat, -252 to -232): 5'-AGGCTC**TGACGTCT**CCCCCC-3'
TRE II (Central Repeat, -204 to -184): 5'-AGGCCC**TGACGTGT**CCCCCT-3'
TRE III (Proximal Repeat, -104 to -84): 5'-AGGCGT**TGACGACA**ACCCCT-3'

Figure 1.2. **Structure of the HTLV-1 LTR.** The HTLV-1 LTR is located both at the 5' end of the viral genome as well as the 3' end. The nucleotide sequence and the positions of the Tax responsive elements (TREs or viral CREs) relative to the transcription start site are shown. The underlined sequences represent the GC-rich flanks and the highlighted sequences constitute the eight-basepair off-consensus CREB binding sites.

needles that contain infected T-lymphocytes (269). For example, one study from 1986 found that HTLV-1 infection was prevalent in 9% of intravenous drug users in New York City as opposed to the 0.003% to 0.04% incidence rate found in the general United States population (reviewed in 114). Routine screening for HTLV-1 infection using enzyme-linked immunoabsorbent assay (ELISA) against HTLV-1 antibodies in the United States did not begin until 1988 (355).

While HTLV-1 has been shown to transform human primary T cells (350), tissue culture cell types of non-lymphoid origin have been shown to be infected with HTLV-1 ex vivo (68, 77, 114, 134, 137). However, productive viral infection in these non-lymphoid cell types does not lead to efficient transformation of these cells, underscoring the importance of T-cell activation and proliferation for immortalization. HTLV-1 DNA is largely found in the CD4+ and CD8+ T cells in HTLV-1 infected individuals (reviewed in 111, 135). While the HTLV-1 receptor required for entry into the host cell is still currently unknown, researchers have found that chromosome 17q21-q23 encodes the gene containing the receptor for HTLV-1 entry (315).

1.1d HTLV-1 immortalization and transformation

Primary human peripheral or cord blood T cells can be immortalized by HTLV-1 in vitro. This continuous growth associated with immortalized T cells requires exogenous interleukin-2. Cells become transformed once they no longer are interleukin-2 dependent (reviewed in 114). These interleukin-2 independent, HTLV-1-infected cells exhibit constitutive activation of the Jak/Stat pathways (218). Additionally, HTLV-1 transformed cells exhibit continuous proliferation, increased expression of cytokines (such as granulocyte-macrophage colony-stimulating factor), and changed expression of surface markers (such as an increased expression of adhesion molecules) (114).

The Tax protein has been implicated as an important factor in the HTLV-1 transformation process of human primary T cells in vivo and in vitro. Tax protein transforms established rat fibroblast cell lines (316, 351) and induces neoplastic transformation of primary rodent fibroblasts through cooperation with the Ras oncogene (258). Primary human T lymphocytes are also immortalized by the expression of Tax (2, 112, 113), while a study of primary T lymphocytes in culture suggests that the viral accessory proteins, $p12^{I}$ (ORF I), $p27^{I}$ (ORF I), $p13^{II}$ (ORF II), $p30^{II}$ (ORF II), are dispensable for immortalization (267). Tax expression was also targeted to the mature T lymphocyte in mice using the human granzyme B tissue-specific promoter and these transgenic mice developed tumors and lymphocytic leukemia (118, 119). Other transgenic mice that expressed the Tax gene by the HTLV-1 LTR developed neurofibromas (131). While the precise mechanism for Tax immortalization of HTLV-1-infected cells remains unknown, it is clear that Tax plays an important role in the oncogenic process.

1.1e Treatment of HTLV-1

Acute ATL patients have a median survival of six months. Treatment of the acute forms of ATL with standard combination chemotherapy regimens, which are typically designed for treatment of acute lymphoblastic leukemia and non-Hodgkin's lymphoma patients, have not been useful in prolonging the lives of these ATL patients (reviewed in 22). Phase II clinical trials in which ATL patients were treated with alpha, beta, and gamma interferon have proven unfruitful (reviewed in 22). However, alpha interferon has also been investigated as a means for treating TSP/HAM patients, in which a few patients exhibited a reduction in their HTLV-1 proviral loads (reviewed in 114). Other therapeutic approaches that appear promising include the combination of zidovudine (AZT) and alpha interferon (23). While this AZT and alpha interferon combination therapy does not cure patients, the median overall survival was increased from six months to ten months for patients in one study (reviewed in 22). A study of five TSP/HAM patients showed a significant decrease in their HTLV-1 proviral load upon treatment with an anti-retroviral agent, lamuvidine (22). Thus, future alternative treatments for acute ATL include combining anti-retroviral agents, such as lamuvidine, with AZT/alpha interferon therapy.

1.2 Tax

The spliced HTLV-1 mRNA, from ORF IV of the pX region of the HTLV-1 genome, is translated into the transcriptional regulatory protein, Tax. Tax is a potent transcriptional activator that interacts with the U3 region of the 5' LTR to increase viral gene transcription (51, 78, 88, 306). The retroviral life cycle is dependent upon Tax, as increased Tax expression leads to a subsequent increase in the synthesis of viral mRNAs that are transcribed, translated, assembled, and released as virions for future adsorption and entry into the HTLV-1 life cycle once again.

1.2a Distinct functional domains of Tax

Tax is a 353 amino acid nuclear phosphoprotein that contains multiple functional and structural domains (see Figure 1.3). Tax localizes to the nucleus of HTLV-1-infected cells (299). The nuclear localization signal of Tax is found in the amino terminal 53

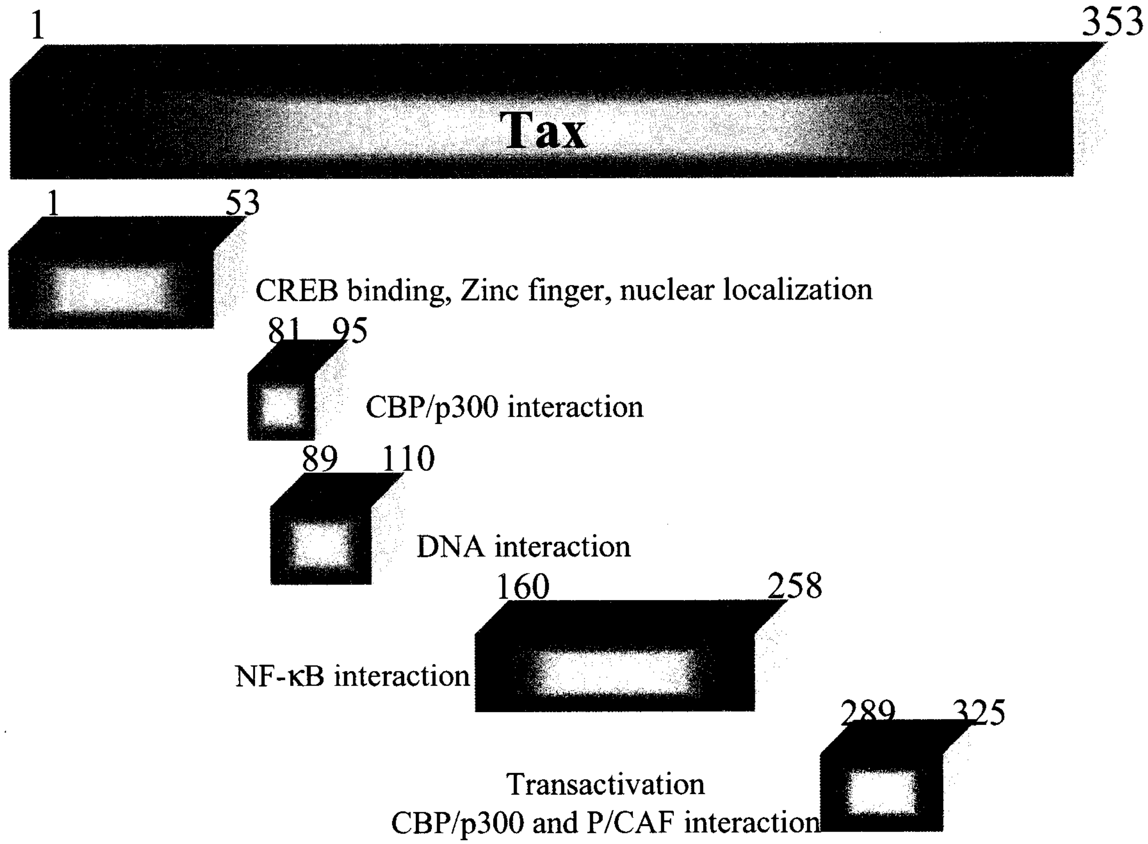


Figure 1.3. **Functional domains of the viral oncoprotein Tax.**

residues (105, 302). The nuclear localization signal consists of a weakly basic domain with a large number of cysteine and histidine residues. This 22 to 53 amino acid cysteine/histidine rich region constitutes the zinc-finger-like motifs within Tax (287). Single point mutations in the zinc-finger domain abrogated transcription from both the HTLV-1 LTR promoter as well as the human immunodeficiency virus type 1 (HIV-1) LTR promoter in vivo (288). Thus, this zinc-binding domain is most likely important for forming protein-protein interactions at the HTLV-1 promoter. The interaction between Tax and CREB is a well-characterized interaction that is also mediated through the amino terminal 59 amino acids of Tax (110). Tax zinc finger mutant proteins and a form of Tax fused to a glutathione-S-transferase tag at its amino terminus were unable to form stable Tax-CREB interactions (1, 110).

Yeast two-hybrid studies suggest that Tax is able to self-associate in vivo (57, 155). The central domain of Tax, located at amino acids 123 to 204, contains the dimerization domain. Point mutations in this centrally located domain abrogated Tax dimerization as well as the ability of Tax to form a complex with viral DNA and with cyclic-AMP response element binding protein (CREB) (154, 321). Tax dimerization is also important for transcriptional activation from the HTLV-1 promoter, as point mutants within this region were unable to support virally-mediated transcription in vivo (154, 321).

After numerous studies suggesting that Tax did not interact with the viral CRE DNA (reviewed in 195), Lenzmeier et al. (194) presented evidence that Tax does directly contact the GC-rich sequences within the viral CREs. Tax binds to the minor groove at specific GC-rich nucleotides within the viral CRE (169, 170, 194, 209). Studies using polyamides, high-affinity synthetic DNA-binding ligands that specifically recognize the GC-rich flanking sequences, suggested that the Tax-DNA interaction was critical for Tax transactivation in vitro (193). Additionally, Kimzey and Dynan (169) localized the domain for Tax interaction with the viral CRE minor groove to a protease-sensitive domain, amino acids 89 to 110 of Tax. The presence of symmetric contacts for Tax binding to the viral CRE further suggests that Tax exists as a dimer when bound to the HTLV-1 promoter.

The DNA-binding domain of Tax has also been reported to bind to CREB-binding protein (CBP) (125). More specifically, single point mutations at amino acids 88 (K88A) and 89 (V89A) were unable to form protein-protein contacts with an amino terminal domain of CBP (described in Section 1.3) (125). Interestingly, we have found that the carboxyl terminal, transactivation domain of Tax is also responsible for an interaction with a domain of CBP (described in Chapter 2) (280). A single point mutation within the putative amphipathic helix at amino acid 312 (Y312E) was unable to support Tax transactivation in vivo (280). The K88A and Y312E Tax point mutants are also compromised for their ability to activate transcription from an HTLV-1 promoter in vitro (193, 280). Thus, two different domains of Tax are responsible for interacting with two separate domains of CBP.

Mutational analysis of Tax determined a minimal transactivation domain in the carboxyl terminal portion of the protein (86). A double point mutation at amino acids 319 and 320 (M47: L319R/L320S) abrogates HTLV-1 transcriptional activation in vivo; however, the M47 Tax mutant remains functional for activation from the HIV-1 promoter (303). A small region from amino acids 315 to 325 was also found to be critical for activation of the HTLV-1 LTR in vivo (288). The entire minimal transactivation domain was later defined as amino acids 284 to 325 and is thought to be responsible for forming important protein-protein contacts with basal transcription factors and/or coactivators (1, 286, 323). Notably, a subdomain of this region, amino acids 312 to 319, contains the sequence aro-X-X-ϕ-ϕ-X-X-ϕ (aro represents phenylalanine or tyrosine, and ϕ represents bulky hydrophobic groups) that is predicted to form the face of an amphipathic helix and mediate protein-protein interactions (260). Further evidence supporting the role of the HTLV-1 transactivation domain in the recruitment of RNA polymerase II transcriptional machinery and/or coactivators lies in another study of the M47 (L319R/L320S) Tax mutant. The M47 Tax mutant was competent for forming a ternary complex with CREB and with the viral CREs; however, this ternary complex was not competent for transcriptional activation from the HTLV-1 promoter (1).

1.2b Tax-mediated transactivation of the HTLV-1 LTR

Tax is a potent activator of HTLV-1 transcription and its expression is necessary for efficient production of the viral mRNA (42, 50, 88, 284). As described above, Tax binds the minor-groove, GC-rich flanking sequences within the viral CREs of the U3 region in the LTR in order to mediate transcriptional activation of the HTLV-1 promoter. Tax also interacts with cellular proteins and these Tax-protein complexes on the HTLV-1 promoter are able to activate viral gene expression (11, 19, 34, 35, 85, 100, 150, 216, 225, 233, 234). Most notably, members of the ATF/CREB family of transcription factors are able to interact with Tax as well as form DNA contacts with the off-consensus CREB binding sequences in the viral CRE (in the presence and absence of Tax) (1, 24, 83, 310, 366). The ATF/CREB family of transcription factors contain a leucine zipper (bZIP) motif (for dimerization and for DNA binding) and a transactivation domain to mediate function (reviewed in 189, 290). Of the members of the ATF/CREB family of transcription factors, much research has focused on CREB. Tax interacts with the bZIP domain of CREB through its conserved basic region (18, 19, 250). Tax enhances the DNA binding affinity of CREB, as well as other bZIP proteins, for the viral CREs (6, 37, 83, 182, 250, 341, 359, 360, 365), potentially by promoting dimerization of the bZIP domain (6, 341). This Tax-induced CREB DNA binding stability is dependent on the core CRE off-consensus sequence as well as the GC-rich flanking sequences (19, 250). The stability of the Tax-CREB complex is also dependent upon the phosphorylation of serine residue 133 of CREB by protein kinase A (101, 182). Thus, the Tax-CREB-viral CRE ternary complex is critical for viral gene expression. Other members of the ATF/CREB family, such as ATF-1 and ATF-2, have been reported to interact with the off-consensus sequences within the viral CREs (18, 297, 365); however, ATF-1 and ATF-2 are unable to form stable Tax-associated complexes (37, 365). Additionally, ATF-1 and ATF-2 were present at the HTLV-1 promoter in HTLV-1-infected T-cells in vivo (191). Recent work by Mesnard and coworkers has focused on ATF-4 (also known as CREB-2) and its role in mediating transcription from the HTLV-1 promoter. Tax enhances the binding of ATF-4 to the viral CREs through its interaction with the bZIP domain (92).

CREB-binding protein (CBP) was first identified to bind the protein kinase A (PKA) serine 133 (Ser133)-phosphorylated form of CREB in 1993 (55). Following this discovery, the amino terminus of CBP was found to be an important activator of CREB-mediated transcription from CREB-responsive genes (183, 246). Studies then showed that Tax could also interact with CBP and recruit CBP to the Tax-CREB-viral CRE ternary complex to activate HTLV-1 transcription (101, 125, 182, 185, 360). The Tax-CREB-CBP-viral CRE quaternary complex was able to form independent of PKA-CREB phosphorylation (101, 182, 185); however, Ser133-phosphorylated CREB exhibited a much higher binding affinity for CBP in the quaternary complex when compared with unphosphorylated CREB in the presence of Tax (101). It is hypothesized that this stable Tax-containing quaternary complex acts as a scaffold to then recruit the RNA Polymerase II (PolII) general transcription machinery to the HTLV-1 promoter for highly active gene expression (101, 185). A schematic of the quaternary complex is shown in figure 1.4. Tax itself has also been reported to directly contact transcription factors located within the RNA PolII general transcription machinery. Tax interacts with the TBP-associated factor (TAF) hTAF$_{II}$28, and TBP to potentiate transactivation (43, 44). Additionally, Tax forms protein-protein contacts with TFIIA in vitro and in vivo and this interaction is important for activating Tax-mediated transcription (61). TAF$_{II}$250, CBP, and p300 are also situated at the viral promoter in HTLV-1-infected cells as evidenced by the use of chromatin immunoprecipitation assays, further supporting the importance of CBP and p300 in the recruitment of general transcription machinery in order to potentiate Tax transactivation (191, 206).

The HTLV-1 LTR also contains sequences other than the viral CREs that are necessary for viral gene expression. A number of other cellular transcription factors other than the ATF/CREB family are able to bind to the HTLV-1 LTR Tax-responsive element 2 (TRE-2), located from -163 to -117 between the central and the proximal viral CRE repeats (214). Members of the Ets proto-oncogene family, Ets-1, Est-2, Elf-1, and TIF-1,

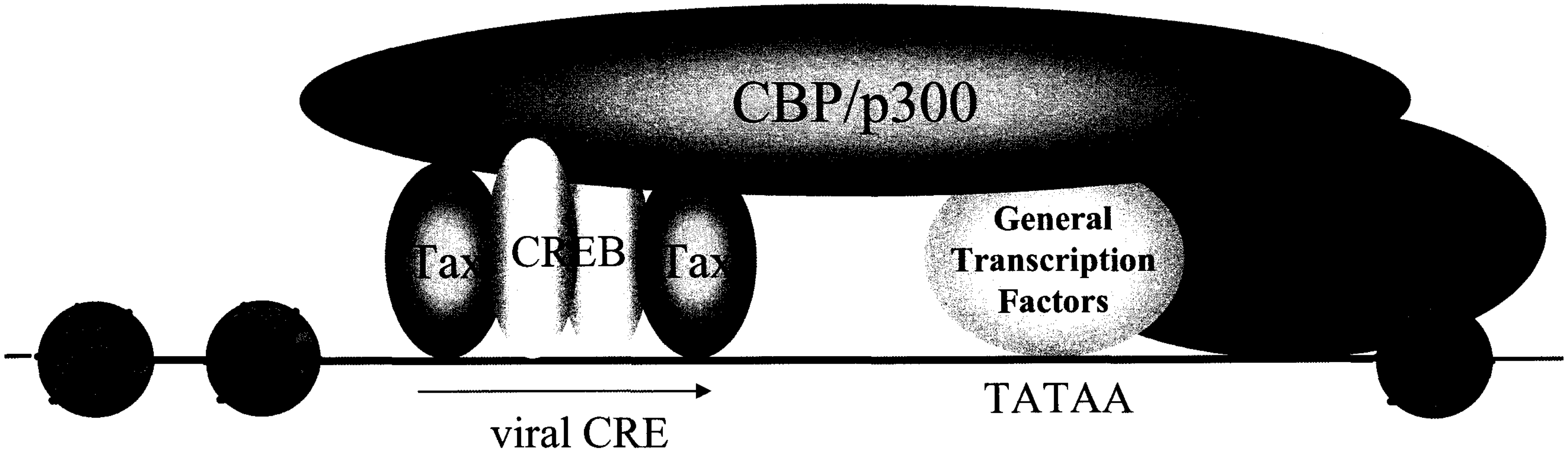


Figure 1.4. **Schematic of the Tax-CREB-CBP/p300-viral CRE DNA quaternary complex.**

can bind directly to TRE-2 to mediate Ets-dependent transcriptional activation (34, 35, 104, 214). In addition, Ets-1 and Ets-2 enhanced Tax-mediated and basal-mediated transcription from an HTLV-1 promoter in vivo, possibly through an Ets interaction with other cellular transcription factors (34, 104). Sp1 can also bind to TRE-2, while Sp1 and Sp3 bind directly to the promoter proximal repeat (TRE-I) (21, 346). Tax has also been shown to interact directly with Sp1 (322). Interestingly, Sp1 and CREB exhibit competitive binding to the promoter proximal repeat; however, it is unclear as to what affect, if any, this competition has on Tax-mediated transactivation in vivo (21). c-Myb also interacts with TRE-2 as well as with five other sequences within the U3 and R regions; however, no interaction between Tax and c-Myb has been reported (35). Lastly, members of the AP-1 family, such as c-Jun and c-Jun/c-Fos heterodimer, appear to be involved in Tax transactivation as they are able to bind the promoter distal, central, and proximal repeats (85, 151, 232). Like the ATF/CREB family proteins, AP-1 family proteins contain a basic leucine zipper motif that is important for dimerization and for DNA binding.

1.2c Tax-mediated transactivation of non-HTLV-1 promoters

While Tax transactivation has predominantly been studied from the HTLV-1 viral promoter, Tax is also able to mediate transcription from cellular promoters. For example, Tax is recruited to a c-Fos promoter, which contains a serum response element. Tax is able to form protein-protein contacts with a serum responsive factor to activate the early response c-Fos gene (5, 84). T-cell transformation may be influenced by Tax-mediated transactivation of the platelet-derived growth factor (PDGF) B promoter, possibly by interactions between Tax and the Ets family of transcription factors (322). Genes involved in the control of T-cell proliferation, such as interleukin-2 (IL-2) and interleukin-2 receptor alpha chain (IL-2R), are also activated by Tax (15, 64, 222). Tax enhancement of the IL-2 and IL-2R genes can positively affect growth factor expression, such as the production of cytokines that could potentially influence T-cell transformation.

The promoters of genes containing NF-κB sites are also positively regulated by Tax through interactions with NF-κB/Rel proteins and their inhibitors (15; reviewed in 28). The NF-κB/Rel transcription factors enhance expression of genes involved in the immune and inflammatory response in activated T cells and also control cell division and apoptosis (reviewed in 41). NF-κB is sequestered to the cytoplasm by NF-κB-specific inhibitors (known as the IκB protein family: IκBα, IκBβ, IκBγ) and as NF-κB precursor proteins (p105 and p100) in the absence of specific stimuli (217). In the cytoplasm, Tax binds to the p105 (133) and p100 (26) NF-κB precursor proteins in order to release active NF-κB subunits and mediate their translocation from the cytoplasm to the nucleus (160, 228, 343). The IκB kinases responsible for site-specific phosphorylation of the NF-κB inhibitors, IκBα and IκBβ, are also constitutively active in the presence of Tax (56, 95, 328). Phosphorylation of these IκB inhibitors leads to their ubiquitination and subsequent degradation, in order to generate active NF-κB subunits to be translocated from the cytoplasm to the nucleus.

Tax also activates NF-κB-dependent transcription in the nucleus of NF-κB-responsive genes (97, 312). Tax increases the dimer formation of NF-κB, which then increases the NF-κB-DNA binding to NF-κB promoters (252). Interestingly, active NF-κB proteins in the nucleus are able to inhibit apoptosis (reviewed in 161) and NF-κB is constitutively active in the nuclei of HTLV-1-infected cells and Tax expressing cells (15). Tax also colocalizes with the p50 and p65 subunits of NF-κB and p300 in discrete nuclear bodies (28, 29). The HIV-1 LTR contains multiple NF-κB-binding sites and Tax point mutants that are defective for transcriptional activation through the NF-κB pathway have been identified using this HIV-1 LTR (288). NF-κB binding sites within the 5' LTR of HIV-1 are critical for Tax-mediated HIV-1 gene expression, as transactivation with only one NF-κB binding site severely reduced this expression (32, 369). Thus, Tax can function in both the cytoplasm and nucleus to regulate NF-κB-responsive genes.

1.2d Tax repression of cellular gene expression

While Tax has largely been studied as an activator of cellular gene expression, it has also been implicated in transcriptional repression of cellular genes. Jeang et al. (152) found that Tax represses transcription of the β-polymerase gene, which encodes a host-cell DNA repair enzyme. It was later discovered that the β-polymerase gene contains a basic-helix-loop-helix (bHLH) transcription factor binding site within its promoter that is necessary for Tax-mediated repression of this gene (329). Tax also mediates repression to the promoters of the pro-apoptotic genes, bax and p53, potentially through the cellular bHLH proteins and the bHLH binding sites (37, 314, 330). The lck gene, encoding a signal transduction protein tyrosine kinase which inhibits cell division, is also repressed by Tax and its expression is altered in Tax-expressing cells through a bHLH binding protein (192). Transcription of p18ink4c, a member of the INK4 family of cyclin-dependent kinase inhibitors, is also repressed by Tax and this repression is mediated through the bHLH binding element of the p18ink4c promoter (313). Semmes et al. (285) found that the bHLH binding sites within the c-Myc gene were necessary for repression by Tax, as Tax was unable to form protein-protein contacts with c-Myc. Recent evidence suggests that Tax is able to form protein-protein contacts with at least one type of bHLH transcription factor, the muscle-specific MyoD, to inhibit the expression of MyoD in muscle cells (264). Thus, Tax is able to exert its repressive effects on promoters containing the bHLH binding sites via an indirect mechanism as well as a direct interaction with bHLH transcription factors.

Most recently, Tax has been shown to inhibit transforming growth factor-β (TGF-β) signaling, which negatively regulates cellular proliferation as part of a tumor suppressor pathway (187). The TATA-less cyclin A promoter is also repressed by the presence of Tax, mediated through the promoter's upstream CREB/ATF binding site (167). Cyclin A is an important factor that interacts with other cellular proteins to regulate cell cycle progression; however, it is unclear as to why Tax represses cyclin A (167). Nuclear hormone transcription, including the glucocorticoid receptor, peroxisome proliferating activated receptor, and retinoid X receptor genes, is also down-regulated in the presence of Tax (72). Thus, Tax repression of cellular genes, such as these involved in DNA repair, in the inhibition of cell division, in nuclear hormone signaling, in tumor suppressor pathways, and in the acceleration of apoptosis, may play an important role in T-cell transformation and leukemogenesis.

1.2e Tax inhibition of the cell cycle regulator, p53

The tumor suppressor protein, p53, is a sequence-specific, DNA-binding transcription factor that is intimately involved in cell cycle regulation. Mutations in the p53 gene are found in approximately 60% of the human malignancies examined (198), supporting an important role for p53 in the maintenance of genomic integrity. Cellular transformation has been strongly linked with the inhibition of p53 function (80). Surprisingly, p53 is only mutated or deleted in approximately 30% of cells from ATL patients and HTLV-1-infected cells (275), while the half-life of p53 in the HTLV-1-infected cells was increased (262). Upon further investigation, it was observed that Tax and p53 do not form protein-protein contacts; however, the amino terminus of Tax is able to stabilize and inactivate p53 function through an indirect mechanism (10, 227, 253-255, 334). Studies by Brady and coworkers suggest that Tax induces hyperphosphorylation of p53 in T cells, thus preventing the recruitment of the RNA PolII general transcription machinery to the p53 promoter for subsequent transactivation of the gene (255). Studies by this group also suggest that Tax inactivation of p53 is dependent on the NF-κB pathway (254), while another model by Nyborg and coworkers suggests that Tax repression of p53 function involves the intracellular competition between p53 and Tax for binding to the coactivator, CBP, to be described in greater detail in section 1.3c (10, 334). Nevertheless, the functional impairment of p53 tumor suppressor functions by Tax might be an important link to HTLV-1 cellular transformation and leukemogenesis.

1.3 CBP/p300

The cellular coactivator protein, CBP, was first identified in 1993 through its interaction with the phosphorylated form of the transcription factor, CREB (55). p300, a paralog of CBP, was later identified through its interaction with the adenoviral-transforming protein E1A (74). CBP and p300 are very large (2,441 and 2,414 amino acids, respectively) cellular proteins conserved from *Caenorhabditis elegans* to *Homo sapiens* that mediate essentially all aspects of gene expression in metazoans. CBP/p300 participates in multiple pathways of gene expression including embryogenesis, programs of differentiation such as hematopoiesis and myogenesis, modulation of cell death, tumor suppressor pathways, signal-dependent and -independent activation, and programs of cellular growth and transformation (reviewed in 102, 103, 109, 335).

1.3a Histone acetyltransferase activity of CBP/p300

CBP/p300 contains a histone acetyltransferase (HAT) domain, located in the central portion of the protein, which is capable of acetylating the amino-terminal tails of all four core histones (H2A, H2B, H3, and H4) (17, 237). CBP/p300 preferentially acetylates lysine residue 5 of histone H2A, lysine residues 12 and 15 of histone H2B, lysine residues 14 and 18 of histone H3, and lysine residues 5 and 8 of histone H4 when assembled into chromatin or free in solution (177, 279). While all core histones are acetylated in vitro and in vivo, the amino-terminal tails of histones H3 and H4 are the preferred substrates (17, 237). CBP/p300 targets nucleosomal histones assembled on specified promoters for hyperacetylation (179, 245), as an open chromatin structure is associated with transcriptionally active genes. Acetylation of the amino-terminal histone tails relieves the repressive effects of chromatin, allowing transcription factor(s), coactivator(s), and/or the RNA PolII general transcription machinery access to the target promoters for subsequent transcriptional activation (107, 188, 340).

Due to its intrinsic acetyltransferase activity, CBP/p300 is also able to acetylate non-histone proteins. The tumor suppressor protein p53 is acetylated to enhance its DNA binding capabilities and to aid in the recruitment of coactivators to nucleosomal p53-responsive promoters (20, 76, 120). CBP/p300 acetylation of lysine residues within hepatocyte nuclear factor-4 (HNF-4) is also crucial for the DNA binding activity of HNF-

4 and its retention in the nucleus (308). CBP/p300 acetylates important transcription factors that regulate immature and mature cells of hematopoietic lineage, including GATA-1, EFKL (erythroid Kruppel-like factor), and c-Myb (138, 277, 364). TFIIEβ and TFIIF, members of the RNA PolII general transcription machinery, are also acetylated by CBP/p300; however, it is still unclear as to what effect this has, if any, on transcription (140). Nuclear receptor coactivators, such as ACTR, SRC-1, and TIF2, are all acetylated by CBP/p300 to potentiate nuclear hormone signaling (48, 49). Other transcription factors that are regulated by CBP/p300 acetylation include the high mobility group (HMG) proteins, HMG1/2/14/17 and HMGI(Y), the muscle differentiation transcription factor MyoD, the HIV-1 virally encoded transcriptional activator Tat, and adenovirus E1A (reviewed in 309). Presently, there is no data to suggest that the viral oncoprotein Tax is acetylated by CBP/p300.

The acetyltransferase activity of CBP/p300 can be regulated by transcription factors binding to the HAT domain. For example, the negative regulator of p53, MDM2, can form a complex with p300 and p53 to inhibit the acetylation of p53 by p300 (173). Other research has shown that a viral interferon regulator factor can bind directly to p300 and inhibit its acetyltransferase activity towards the core histone proteins (200). The bHLH transcription factor, Twist, and the adenoviral protein, E1A, are also able to inhibit the HAT activity of p300 (47, 122). The ability of specific factors to mediate the HAT activity of CBP/p300 may have important consequences for transcriptional activation from certain chromatin-assembled promoters.

1.3b Transcription factor and coactivator interactions with CBP/p300

Since its identification in 1993 as a cellular coactivator of CREB, CBP has been demonstrated to interact with over 40 cellular transcription factors. Recruitment of CBP/p300 to specific promoters by transcription factors is believed to aid in the subsequent recruitment of the RNA PolII transcription machinery to activate transcription. Multiple transcription factor binding sites on CBP/p300 could also act as a means for assembling multi-protein, high molecular weight complexes including non-DNA binding proteins, at target promoters. There is evidence to suggest that CBP/p300 is a component of the RNA PolII holoenzyme (231); thus, recruitment of CBP could be a direct means of recruiting the RNA PolII transcription components to target promoters. Further evidence suggests that CBP/p300 directly interacts with factors, such as TBP and TFIID, found in the general transcription machinery to stabilize the preinitiation complex located at target promoters (66). Figure 1.5 shows CBP/p300 and its transcription factor interactions.

CBP/p300 contains multiple functional domains: the C/H1 domain (amino acids 302 to 451), the KIX domain (amino acids 588 to 683), the bromodomain (amino acids 1,079 to 1,457), the acetyltransferase domain (amino acids 1,232 to 1,712), the CR1 domain (also known as C/H3, TRAM, and TAZ2) (amino acids 1,764 to 1,850), the CR2 domain (also known as IBiD) (amino acids 2,058 to 2,130), and the CR3 domain (amino acids 2,221 to 2,441) (reviewed in 109). The Tax protein was first identified to bind directly to the KIX domain to recruit CBP/p300 to the HTLV-1 promoter (101, 182). By using NMR to study the KIX domain bound to the transactivation domain of CREB, Radhakrishnan et al. (260) determined that the KIX domain consists of three amphipathic

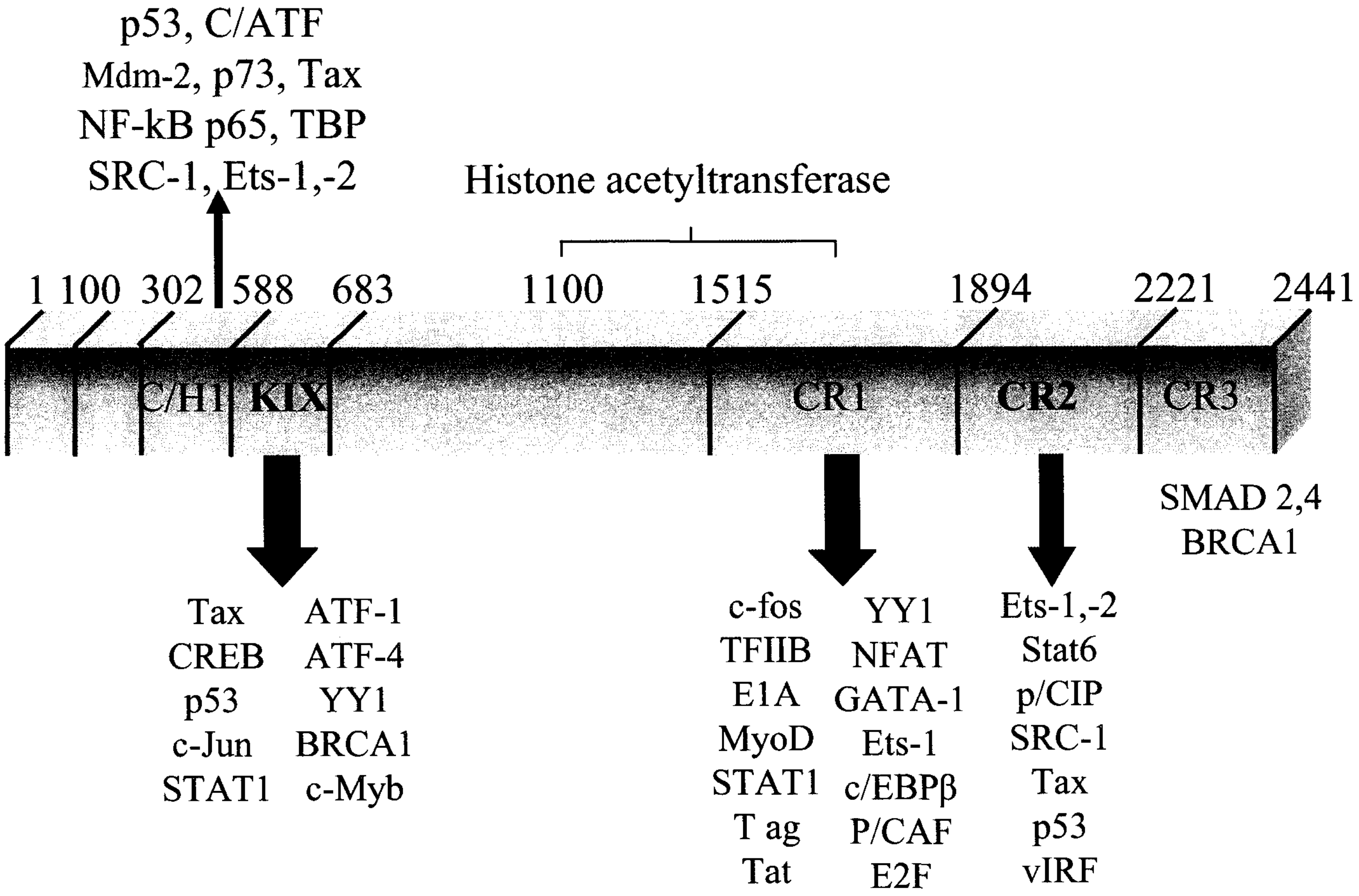


Figure 1.5. **Transcription factor interactions with the cellular coactivator CBP/p300.** The functional domains C/H1, KIX, HAT, CR1, CR2, and CR3 and the amino acid coordinates are noted.

α-helices which form a hydrophobic binding groove. Thus, CREB also interacts with CBP/p300 through the KIX domain; however, specific point mutations within KIX are able to distinguish between the binding of Tax and CREB (352). CREB recognizes the KIX domain from amino acids 586 to 665, while Tax is unable to recognize this smaller domain of KIX, suggesting that Tax and CREB form distinct protein-protein contacts within KIX to mediate transcription (352). Unpublished data from the Nyborg laboratory suggests that the Tax/KIX interaction is enhanced in the presence of CREB and the viral CRE DNA perhaps suggesting the importance of Tax-DNA and Tax-protein contacts for HTLV-1 transcription.

Tax has recently been shown to interact with two other domains within CBP/p300. The C/H1 domain is directly adjacent to the KIX domain and is able to interact with Tax (190). The close proximity of the C/H1 and KIX domains may provide a large binding surface for Tax. Tax also interacts with the CR2 domain to mediate HTLV-1 transcription function as discussed in chapter 2 (280). The tumor suppressor protein p53 is another transcription factor of note that interacts with multiple domains of CBP/p300. p53 interacts with the C/H1 domain and the KIX domain to enhance p53-mediated transcription (334). p53 has also been shown to interact with the CR1 domain (235) and a large carboxyl-terminal domain of CBP/p300 (120). This ill-defined carboxyl-terminal domain was later defined as the CR2 domain and shown to enhance coactivator-mediated transcription as described in chapter 3 (205).

The coactivator p300/CBP-associating factor (P/CAF) also interacts with CBP/p300 through a minimal domain of CR1 (amino acids 1801 to 1850) (261, 354). Like CBP/p300, P/CAF also contains acetyltransferase activity; thus, the P/CAF-

CBP/p300 interaction may be a means of recruiting multiple histone acetyltransferase activities to target promoters to activate transcription (reviewed in 27, 38). The steroid receptor coactivator-1 (SRC-1) is a nuclear receptor coactivator that interacts with CBP/p300, through the CR2 domain (159). SRC-1 also contains acetyltransferase activity, and the CBP/p300-SRC-1 multi-HAT complex stimulates ligand-dependent activation by a multitude of nuclear receptors (reviewed in 196). Thus, CBP/p300 is able to exert its pleiotropic functions ranging from embryogenesis to signal-dependent transcription through its ability to interact with a multitude of transcription factors and coactivators.

1.3c Competition between Tax and transcription factors for binding to CBP/p300

Tax repression of cellular gene expression is also mediated by direct competition for binding to CBP/p300. The levels of CBP/p300 protein expressed intracellularly are at limited concentrations (251); thus, competition between transcription factors binding to CBP/p300 is one means of regulating gene expression. As shown in figure 1.5, numerous unrelated transcription factors bind to similar, overlapping domains within CBP/p300. Moreover, several studies by Nyborg and coworkers have provided evidence that Tax and transcription factor interactions with CBP/p300 can be mutually exclusive; thus, competition for limiting amount of CBP/p300 is one mechanism for disruption of transcriptional activity (62, 190, 205, 280, 334, 336). Colgin and Nyborg (62) found that Tax represses the transcriptional activity of the proto-oncoprotein, c-Myb, in vivo and that overexpression of c-Myb was capable of disrupting Tax binding to the KIX domain of CBP/p300 in vitro. Tax also represses the cellular proto-oncoprotein c-Jun, which plays an important role in cellular proliferation and transformation (336). Tax and c-Jun compete for binding to the KIX domain and these proteins reciprocally repress transcriptional activity from the c-Jun and the HTLV-1 promoters, respectively (336). The transcriptional activity of the p53 gene family member p73 is also repressed in the presence of Tax (190). In HTLV-1-infected cells, the transcription function of p73β is decreased while its stability is increased, suggesting that Tax is responsible for disruption of p73 gene function (190). Tax and p73β compete for binding to the C/H1 domain of CBP/p300 and this mutually exclusive binding may lead to the decrease in p73β transcriptional activation. As described in section 1.2e, Tax represses the transcription function of the tumor suppressor protein p53. In addition to Tax repression of p53-mediated transcription through indirect interactions with the bHLH binding site, Tax also competes with p53 for binding to various domains of CBP/p300. Tax and p53 exhibit mutually exclusive binding to the KIX and CR2 domains of CBP/p300 in vitro (10, 205, 314, 334). Additionally, p53 represses Tax transactivation in vivo (334). Direct coactivator competition could explain why the p53 present in HTLV-1-infected cells is transcriptionally inactive, but has a low mutation frequency.

Tax also interferes with the transactivation of other transcription factors, such as Smad and MyoD. Smad-dependent transcription is repressed in the presence of Tax and the suppression of transactivation is reversed when CBP/p300 is overexpressed in the cell (224). Smad is involved in the transforming growth factor-β signaling pathway; thus, Tax interference with Smad-coactivator recruitment may be an important link to leukemogenesis (224). MyoD-dependent transcription is also repressed in the presence of Tax; however, the repression is non-reciprocal (263). Tax prevents binding of this muscle specific transcription factor important for cell proliferation and differentiation to the KIX domain; however, MyoD is unable to inhibit Tax transactivation (263).

Tax protein constitutes 0.15% of the total cell protein during its intermittent expression in HTLV-1-infected cells (300). The abundance of Tax protein in these infected cells suggests that Tax may have better access and stronger binding affinity to the limiting amounts of CBP/p300. During these high levels of Tax expression, Tax may occupy the functional domains of CBP/p300 and prevent other transcription factor interactions at these sites. This coactivator-dependent inactivation of cellular transcription factor activity may be an important step in cellular transformation and leukemogenesis.

1.3d CBP/p300 and disease

CBP and p300 are highly homologous proteins that exhibit overlapping expression patterns; however, there are differences in the expression and subcellular localization of each protein during embryogenesis and development. Partanen et al. (247) studied the pattern of CBP and p300 expression in the developing mouse and found that both proteins were required for development. While the CBP and p300 expression patterns overlapped in certain cell types, each protein was also present in distinct cell types such as the developing lung (247). Homozygous p300 knockout mice and homozygous CBP knockout mice are embryonically lethal, dying between embryonic days 9 and 11.5 (357). The results using gene knockouts suggests that CBP and p300 have some discrete functions during embryogenesis such that a full complement of CBP is not able to compensate for a lack of p300 (or vice versa). Furthermore, homozygous mice expressing a truncated form of CBP, lacking the HAT domain, were also lethal between embryonic days 9.5 and 10.5 as they developed hematopoietic and vasculoangiogenesis defects, indicating that CBP is an important regulator of hematopoiesis (240).

Developmental abnormalities also result from haploinsufficiency of CBP or p300, indicating that even a slight reduction in the total amount of CBP or p300 in the cell can result in aberrant gene expression. Rubinstein-Taybi syndrome (RTS) is a human developmental disorder that results from haploinsufficiency of the CBP allele (251). RTS is characterized by facial abnormalities, mental retardation, and broad thumbs and toes (219). Patients also exhibit a predisposition to certain types of cancers with hematopoietic malignancies such as acute lymphocytic leukemia and acute myelogenous leukemia occurring less frequently (219). Mouse models have also been used to examine haploinsufficiency of CBP or p300 in animals whereby only 50% of mice heterozygous for the CBP ($CBP^{+/-}$) gene were born alive (180). Inactivation of a single CBP allele in mice results in similar clinical features to RTS such as facial abnormalities and growth retardation (180, 239, 317). Other phenotypic characteristics of heterozygous CBP mice include hematopoietic differentiation defects and splenomegaly (180). As the $CBP^{+/-}$ mice aged, 39% of them developed hematopoietic malignancies, including primary lymphocytic leukemia (180). Tumors of primary myelogenous leukemia origin also developed in sublethally irradiated wild-type mice transplanted with cells originally from tumor-free $CBP^{+/-}$ mice. Interestingly, mice heterozygous for the p300 gene did not exhibit the same defects as the $CBP^{+/-}$ in their hematopoietic system. The haploinsufficient p300 mice that died early had neural tube closure defects, similar to the defects found in the embryonic lethal nullizygous p300 mice (357). Thus, p300 appears to play an important role in neural development. Crossbred mice that are heterozygous for both the CBP and p300 alleles die at an early age and exhibit similar phenotypic characteristics as the nullizygous mice (357).

Chromosomal translocations disrupting the CBP and p300 genes by fusion of their sequences with other genes have been implicated in various forms of leukemia. The translocation of the acetyltransferase gene encoding MOZ (monocytic leukemia zinc finger) fused to the amino terminus of CBP is associated with a distinctive subtype of acute myeloid leukemia (33). This t(8;16)(p11;p13) translocation is relatively rare, but individuals with this translocation have a poor survival rate. The MOZ-CBP fusion protein inhibits transcription and subsequent cell differentiation associated with the hematopoietic cell-specific gene, AML1, linking disruption of MOZ acetyltransferase activity with leukemogenesis (171). Translocations of the MLL (mixed lineage leukemia) gene with CBP (t(11;16)(q23;p13.3)) are observed in other cases of acute myeloid leukemia and in all chemotherapy (topoisomerase II inhibitors)-related acute leukemias (13, 274, 304). The MLL gene is fused in-frame to the CBP gene, retaining the HAT activity of CBP. The MLL-CBP chimeric protein causes deregulation of normal MLL function, possibly by promoting aberrant chromatin accessibility to MLL target genes (13). Acute myeloid leukemia translocations involving MLL and p300 (t(8;22)) have also been documented; however, they are less common than the MLL-CBP fusion protein (46, 139). These in-frame deletions lead to a gain of function or a loss of function in the CBP or p300 genes, resulting in aberrant expression of genes involved in differentiation and in controlling passage through the cell cycle, thus contributing to malignant transformation and leukemogenesis.

Understanding the role of CBP/p300 in the molecular mechanism of Tax transactivation is important for additional insight regarding the link between Tax and leukemogenesis. In the following studies, we identify a different site of interaction for Tax within CBP/p300, discrete from the KIX domain. We also found that the tumor suppressor p53 is able to bind to an overlapping site within this domain of CBP/p300. We found that this alternate binding site within CBP/p300 was important in vivo for Tax transactivation and p53 transactivation. We also found that Tax competes with the nuclear receptor coactivator, steroid receptor coactivator-1 (SRC-1), for binding to this site. Additionally, Tax and p53 also compete for binding to this domain. Thus, we have identified another example of Tax competition with a transcription factor (and coactivator) for binding to limiting amounts of CBP/p300. This mutually exclusive binding to CBP/p300 may have implications for deregulation of one or several of the pathways that are regulated by CBP/p300 leading to leukemogenesis.

Chapter 2

The Oncoprotein Tax Binds the SRC-1-Interacting Domain of CBP/p300 to Mediate Transcriptional Activation

This chapter was published in *Molecular and Cellular Biology.* The text of this manuscript is presented as it appeared in this journal. The figures in this manuscript are presented exactly as they appeared in the publication, and the data not shown experiments are presented as supplemental figures. Aida Ulloa, a co-author on this paper, carried out the experiment represented in figure 2.5b. The reference for this chapter is shown below.

Scoggin, K.E.S., A. Ulloa, and J.K. Nyborg. 2001. The Oncoprotein Tax Binds the SRC-1-Interacting Domain of CBP/p300 To Mediate Transcriptional Activation. Mol. Cell. Biol. **21**(16): 5520-5530.

2.1 Abstract

Oncogenesis associated with human T-cell leukemia virus (HTLV) infection is directly linked to the virally-encoded transcription factor Tax. To activate HTLV-1 transcription Tax interacts with the cellular protein CREB, and the pleiotropic coactivators CBP/p300. While extensively studied, the molecular mechanisms of Tax transcription function and coactivator utilization are not fully understood. Previous studies have focused on Tax binding to the KIX domain of CBP, as this was believed to be the key step in recruiting the coactivator to the HTLV-1 promoter. In this study, we identify a carboxy terminal region of CBP (and p300) that strongly interacts with Tax and mediates Tax transcription function. Through deletion mutagenesis, we identify amino acids 2003 to 2212 of CBP, which we call carboxy-terminal region 2 (CR2), as the minimal region for Tax interaction. Interestingly, this domain corresponds to the steroid receptor coactivator 1 (SRC-1)- interacting domain of CBP. We show that a double point mutant targeted to one of the putative α-helical motifs in this domain significantly compromises the interaction with Tax. We also characterize the region of Tax responsible for interaction with CR2 and show that the previously identified transactivation domain of Tax (amino acids 312 to 319) participates in CR2 binding. This region of Tax corresponds to a consensus amphipathic helix, and single point mutations targeted to amino acids on the face of this helix abolish interaction with CR2 and dramatically reduce Tax transcription function. Finally, we demonstrate that Tax and SRC-1 bind to CR2 in a mutually exclusive fashion. Together, these studies identify a novel Tax-interacting site on CBP/p300 and extend our understanding of the molecular mechanism of Tax transactivation.

2.2 Introduction

The human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus etiologically linked to an aggressive and often fatal malignancy called adult T-cell leukemia (ATL) (344). It is estimated that between 5 and 20 million people are HTLV-1 carriers worldwide; however, only a small percentage of these infected individuals develop ATL (80). ATL develops after a prolonged latency period of up to 30 years post infection (305) and is characterized by lytic bone lesions, skin abnormalities, and greater than 5% abnormal T cells (355). HTLV-1 infection is also associated with the neurodegenerative disorder known as tropical spastic paraparesis or HTLV-1 associated myelopathy (211, 344, 363). The disorder is often characterized by demyelination of the nerves of the spinal cord resulting in weakness or paralysis in muscles of the lower extremity. The molecular basis of HTLV-1-associated diseases is strongly linked to the expression of the virally-encoded Tax protein (195).

Tax is a potent transcriptional activator that stimulates HTLV-1 viral gene expression, which leads to high-level virion production in the infected T cell. Three highly conserved 21-bp repeat enhancer elements, located in the HTLV-1 transcriptional control region, are critical to Tax-activated transcription. These elements, which are referred to as viral cyclical response elements (CREs), carry a central binding site for the transcription factor CREB, flanked by highly conserved GC-rich DNA sequences. Tax associates with the HTLV-1 promoter through protein-protein interactions with CREB (83, 365) and protein-DNA interactions with the minor groove GC-rich sequence (169, 170, 194, 209). The formation of this Tax-containing promoter bound complex appears to be critical in the recruitment of the multifunctional cellular coactivators CBP and p300.

Several previous studies indicate that Tax specifically binds to the KIX and C/H1 domains of CBP/p300 to recruit the coactivators to the HTLV-1 promoter, resulting in strong transcriptional activation of the virus (101, 163, 182).

CBP and p300 are very large (2,441 and 2,414 amino acids, respectively), highly conserved coactivator proteins that serve as mediators in the regulation of gene expression in metazoans (109, 335). Numerous pathways of regulated gene expression converge at CBP and its paralog p300. These include signal dependent and independent activation, programs of differentiation, embryogenesis, modulation of apoptotic pathways and pathways involved in transcriptional regulation (102, 103, 109, 335). Although CBP was originally named following its identification as a coactivator for Ser133-phosphorylated CREB, the acronym is a misnomer, as CBP is utilized by numerous cellular transcription factors and is likely involved in the transcriptional regulation of nearly all protein-coding genes in the cell. In addition, many viral activator proteins have evolved strategies to take advantage of CBP's coactivator properties (9, 75, 182, 354).

Alterations in CBP expression levels appear to influence the onset of certain types of cancers, as evidenced by Rubinstein-Taybi patients. Rubinstein-Taybi syndrome is caused by mutations or rearrangements in one CBP allele, underscoring the importance of proper intracellular CBP concentrations in normal cellular processes and tumor suppression (251). Translocations into the genes that encode CBP and p300 have also been strongly correlated with the development of treatment-related (chemotherapy) acute myeloid leukemia. Patients with this ailment carry an in-frame translocation of the mixed lineage leukemia (MLL) gene to the CBP gene, suggesting that dysregulation of CBP plays a role in leukemogenesis (274, 304). Furthermore, defects in primitive hematopoiesis were observed in the mice nullizygous for the CBP gene (240, 357). Although about 50% of mice heterozygous for the CBP gene are born alive, a significant percentage exhibit hematopoietic defects and malignancies (180).

CBP recruitment by Tax to the HTLV-1 promoter has been studied extensively in recent years, and the region of Tax interaction with CBP has been mapped to the amino-terminal C/H1 and KIX domains (101, 125, 182, 352). In this report we show that Tax also interacts strongly with another region of CBP. This region, which we call carboxy-terminal region 2 (CR2), is located between amino acids (aa) 2003 and 2212. CR2 is conserved in both CBP and p300, and not surprisingly, we show that Tax binds strongly to the CR2 domain present in both proteins. We identify a carboxy-terminal region of Tax that is predicted to form an amphipathic helix and show that a specific amino acid on the surface of this helix (aa Y312) is responsible for the interaction with CR2. This CR2-interacting carboxy-terminal region of Tax is distinct from the previously identified KIX-interacting domain of Tax, which has been shown to reside between amino acids 81 and 95 (125). Transient-cotransfection assays provide functional evidence supporting the relevance of the Tax-CR2 interaction. Specifically, Tax Y312→E is defective for transactivation in vitro and in vivo, and two additional point mutations in this region of Tax (I315→E and L319→R) are defective for Tax transactivation in vivo. Interestingly, the CR2 region of CBP corresponds to the steroid receptor coactivator 1 (SRC-1)-interacting domain that is critical to coactivator utilization by liganded nuclear hormone receptors (106, 196). Tax has previously been shown to strongly repress transcription mediated through steroid and retinoid receptors (72). We show in this report that Tax competes for SRC-1 binding to CR2 in vitro, suggesting that the mechanisms of Tax repression of nuclear hormone signaling may be mediated through coactivator competition. Together, these data indicate that Tax recruits CBP/p300 through binding to multiple independent sites, illustrating the complex nature of activator-coactivator interactions in transcriptional regulation.

2.3 Materials and Methods

2.3a Cloning, expression, and purification of recombinant proteins. The expression and purification of GST-C/H1-KIX$_{aa302-683}$ and GST-CR2$_{aa1894-2221}$ has previously been described (334). The six glutathione *S*-transferase (GST)-CR2 deletion mutants were prepared by PCR amplification of the mouse CBP cDNA sequence (pRC/RSV-CBP) (208) corresponding to aa 1894 to 2150, 1894 to 2100, 1894 to 2003, 2003 to 2212, 2003 to 2150, 2100 to 2212, and 2055 to 2150. These PCR fragments were inserted into the *Bam*HI and *Eco*RI sites of pGEX2T (Amersham Pharmacia Biotech) and transformed into *Escherichia coli* BL21(DE3)pLysS cells, and the GST fusion proteins were purified by glutathione-agarose chromatography. The four double point mutants of GST-CR2$_{aa2003-2212}$ were made by PCR amplification of the CBP cDNA sequence using the QuickChange site-directed mutagenesis kit (Stratagene). The PCR products were cloned, and the proteins expressed and purified as described above. Histidine-tagged CR2 (His$_6$-CR2) was made by PCR amplification of the CBP cDNA sequence corresponding to amino acids 2003 to 2212 with *att*B and *att*R sites at their respective 5' and 3' ends for cloning into the Gateway system (Life Technologies, Inc.). This PCR fragment was inserted into the *att*B and *att*R sites of pDonr206 (Gateway) and subsequently cloned into pDest17 (Gateway), a His$_6$ fusion vector for expression and purification by nickel chelate chromatography as previously described (101). The CR2 region from human p300, encompassing aa 1970 to 2193 (corresponding to mouse CBP CR2 aa 2003 to 2212) was cloned by PCR amplification of the relevant sequence with *att*B and *att*R sites at their respective 5' and 3' ends and insertion into pDonr206 (Gateway), then into pDest15 (Gateway), a GST fusion vector. The final clone was transformed in BL21(DE3)pLysS cells, expressed and purified by glutathione-agarose chromatography. All purified proteins were dialyzed against TM buffer (50mM Tris-HCl [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA [pH8.0], 1mM dithiotrhreiotol, 0.1% [vol/vol] Tween-20, 20% [vol/vol] glycerol) and stored at -70°C.

CREB (83) and Tax-His_6 (366) were expressed and purified as previously described (101). CREB was serine-133 phosphorylated by protein kinase A for use in the in vitro transcription assays as previously described (101). Tax Y312→E was made using the QuickChange site-directed mutagenesis kit (Stratagene) in the pTaxHis_6 background (366) and purified by nicked chelate chromatography. Tax K88→A was expressed and purified as previously described (125). GST-Tax (full length) and GST-$Tax_{aa286\text{-}337}$ (63) were made by appropriate PCR amplification of the Tax cDNA sequence and inserted into the *Bam*HI site of pGEX2T (Amersham Pharmacia Biotech). The GST-Tax proteins were purified using glutathione-agarose chromatography as described above. Purified proteins were stored at -70°C in TM buffer. Full-length SRC-1 (291) was transcribed and translated using the TNT Quick-coupled transcription/translation system (Promega). SRC-1 was labeled with [^{35}S]methionine during the in vitro transcription-translation reaction.

2.3b GST pulldown assays. All GST pulldown experiments were performed using 12.5 μl of glutathione-agarose beads equilibrated in pulldown buffer (20mM HEPES [pH7.9], 12.5 mM $MgCl_2$, 10μM $ZnSO_4$, 25mM KCl, 0.5mM EDTA [pH8.0], 10% [vol/vol] glycerol, 0.05% [vol/vol] Nonidet P-40). The purified GST proteins were incubated with the equilibrated beads for 2 hours at 4°C, washed, and incubated with the second protein overnight at 4°C. The reactions were washed three times with pulldown buffer, and bound proteins were separated by electrophoresis sodium dodecyl sulfate-12% polyacrylamide gel (SDS-12% PAGE), transferred to nitrocellulose, and analyzed by Western blot. The following antibodies were used in the GST pulldown experiments: anti-His antibody (H-15; Santa Cruz Biotechnology), anti-GST antibody (Sigma), and anti-Tax antibody (epitope corresponding to the 13 carboxy-terminal aa). The GST pull-down competition assays were carried out by incubating 5 pmol GST or GST-$CR2_{aa2003-2212}$ with 12.5 μl of glutathione-agarose beads equilibrated in pulldown buffer for 2 hours at 4°C, washed, and incubated with radiolabeled SRC-1 (0.1 μl) and increasing amounts of wild-type Tax or Tax Y312→E overnight at 4°C. The reactions were washed four times with pulldown buffer, and bound proteins were separated by electrophoresis on SDS-12%PAGE. Bound, labeled SRC-1 was detected by PhosphorImager analysis. Tax binding was detected by Western blot analysis using an anti-His_6 antibody (H-15; Santa Cruz Biotechnology).

2.3c Yeast two-hybrid assay. Full-length Tax (p$TaxHis_6$) (366) was cloned into a *TRP1*-marked Gal4 activation domain construct, pDest22 (Gateway). The KIX domain (aa 588 to 683) and the CR2 domain (aa 2003 to 2212) were generated by PCR amplification of their respective domains from the CBP cDNA sequence (pRC/RSV-CBP) and subsequently cloned into a *LEU2*-marked Gal4 DNA-binding domain construct, pDest21 (Gateway). Different fusion constructs and a promoter containing the *HIS3* gene with four upstream Gal4 binding sites were transformed into *Saccharomyces cerevisiae* strain MaV103 (relevant genotype: *MAT***a** *leu2-3, 112 trp1-901 his3Δ200 gal4Δ gal80Δ GAL1::LacZ GAL1::HIS3@lys2 SPA-L10::Ura3)* for two-hybrid analysis. Protein-protein interactions were detected by cell growth phenotypes in the presence of aminotriazole on plates lacking histidine (130).

2.3d Mammalian expression plasmids, cell culture, and transient-cotransfection assays. The Tax expression plasmid, pHTLV-I Tax (36), the c-*fos* expression plasmid RSV-c-*fos* (8), and the luciferase reporter plasmids viral CRE-Luc (101) and AP-1-Luc (336) have been described previously. The cytomegalovirus (CMV) expression plasmids for $\mathrm{CR2}_{\mathrm{aa}\ 2003\text{-}2212}$ and $\mathrm{CR1}_{\mathrm{aa}\ 1515\text{-}1895}$ were prepared by PCR amplification of the CBP cDNA sequence corresponding to their respective amino acids, incorporating the *att*B and *att*R sites on the ends of the PCR fragment. The $\Delta\mathrm{CR2}_{\mathrm{aa}\ 2003\text{-}2212}$ L2068→A/L2071→A CMV expression plasmid was prepared by PCR amplification of the $\mathrm{GST\text{-}CR2}_{\mathrm{aa2003\text{-}2212}}$ double point mutant plasmid, incorporating the *att*B and *att*R sites on the ends of the PCR fragment. These fragments were cloned into pDonr206 (Gateway), and then into the CMV promoter-driven expression vector pDest26 (Gateway). The Gal4-Tax point mutants Y312→E, I315→E, and L319→R were made in the Tax cDNA sequence in the pGal4-Tax-S expression plasmid (63) using the QuickChange site-directed mutagenesis kit (Stratagene). Transient-cotransfection assays were performed in the HTLV-I-negative human T-lymphocyte cell line Jurkat. Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin. Transient cotransfections were performed with 10^6 cells in unsupplemented medium, a constant amount of DNA, and Lipofectamine (Life Technologies, Inc.) for 5 h. Supplemented medium was then added to each transfection and incubated for an additional 24 h. Cells were lysed and assayed for luciferase activity using a Turner Designs model TD 20-e luminometer. The *Renilla* luciferase plasmid was used as an internal control.

2.3e Recombinant plasmids and in vitro transcription assays. The 4TxRE G-less cassette carries four copies of the third 21-bp Tax-responsive elements (TxRE) located upstream of the HTLV-1 core promoter (7). Preinitiation complexes were formed on 100ng of DNA template in TM buffer supplemented with 10 μM acetyl-coenzyme A [Sigma] by the addition of the indicated amounts of CREB and/or Tax and 70 μg CEM nuclear extract (73) in a final reaction volume of 30 μl. The reactions were incubated for 60 minutes at 30°C. RNA synthesis was initiated by the addition of 250 μM of ATP, CTP, UTP, and 12 μM GTP plus 0.07 μM [α-^{32}P] UTP (3000 Ci/mmol) and incubated for an additional 35 minutes at 30°C. RNaseT1 was then added to the reaction for an additional 20 minutes at 37°C. The reactions were terminated by the addition of stop solution (133mM NaCl, 0.5% [vol/vol] SDS, 3.3mM Tris-HCl [pH7.9]) with recovery standard. RNA was purified with phenol-chloroform, precipitated with ethanol, and analyzed by urea 6.5% denaturing PAGE. Gels were dried and visualized by PhosphorImager analysis.

2.4 Results

2.4a Identification of a carboxy-terminal region of CBP that interacts with Tax

Several previous studies have shown that Tax physically interacts with the C/H1 and KIX domains of CBP to activate transcription (101, 125, 182). We were interested in testing whether additional regions of CBP may participate in Tax transactivation. To test this hypothesis, several GST-CBP fusion proteins spanning the carboxy-terminal half of the coactivator were bound to glutathione-agarose beads, and used in a GST pulldown assay with full-length purified, recombinant Tax. All of these GST-CBP fusion constructs are illustrated in figure 2.1.A. Tax bound strongly to one of the three carboxy-terminal regions of CBP (Fig. 2.1.B, lane 5). This region, which we call carboxy-terminal region 2 (CR2), encompasses CBP aa 1894 to 2212. As positive controls, we also tested the binding of Tax to the amino-terminal C/H1 domain (aa 302 to 409), and to a region that encompasses both C/H1 and KIX (aa 302 to 683) (Fig. 2.1.B, lanes 2 and 3). We observed markedly stronger Tax binding to CR2 than to these other CBP regions. A Coomassie-stained gel showing the GST proteins used in the pulldown assay is shown in figure 2.1.C. The interaction between Tax and CR2 was also confirmed using the yeast two-hybrid assay (Fig. 2.1.D).

2.4b Critical CR2 amino acids required for Tax binding

We next determined the minimal region of CR2 competent for Tax binding. Progressive deletions of CR2 revealed that aa 2150 (Fig. 2.2.A, lane 3) represents the carboxy-terminal border, and aa 2003 (Fig. 2.2.A, lane 6) represents the amino-terminal border competent for wild type interaction with Tax. Further deletions from either terminus significantly reduced the CR2 interaction with Tax (Fig. 2.2.A). These data

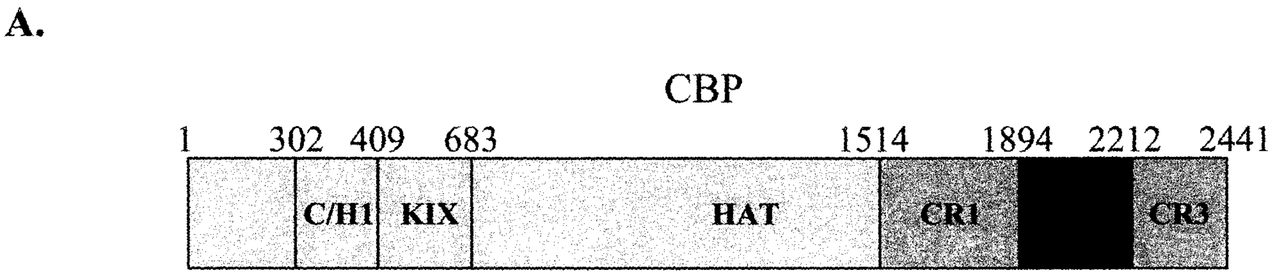


Figure 2.1. **Tax binds to both the CR2 and C/H1-KIX domains of CBP.** (A) Schematic representation of the 2,441-aa cellular coactivator CBP. The regions tested for Tax binding are indicated. HAT, histone acetyltransferase domain.

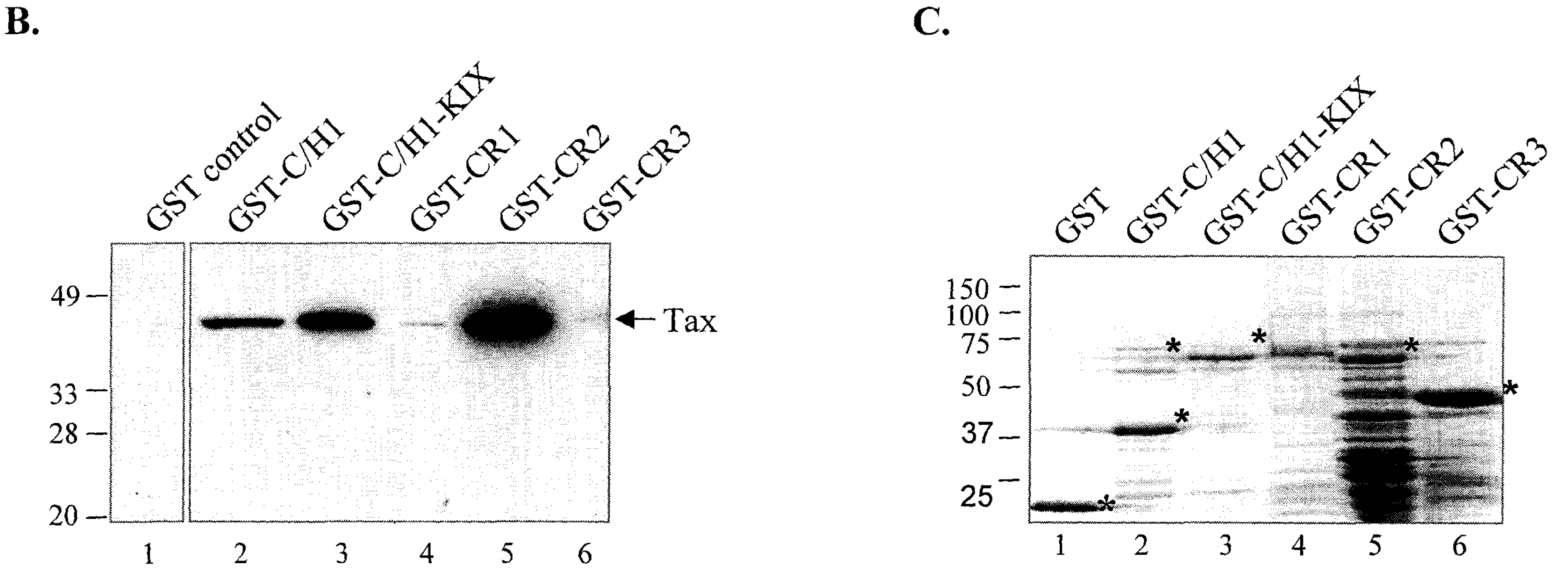


Figure 2.1. (B) Tax binds to the CR2 domain in vitro. Purified recombinant Tax (50 pmol) was incubated with GST alone or the indicated GST-CBP fusion protein (50 pmol each). As controls, Tax binding to GST-C/H1$_{aa302-409}$ and GST-C/H1-KIX$_{aa302-683}$ was also assayed. The bound proteins were separated by SDS-12% PAGE, transferred to nitrocellulose, and detected using an anti-His$_6$ antibody. The positions of bound Tax and protein molecular size standards are indicated (in kilodaltons). (C) Coomassie-stained SDS-12% PAGE showing the GST fusion proteins used in panel B. Asterisks denote GST fusion proteins, and protein molecular size standards are indicated in kilodaltons.

D.

DNA-Binding Domain	Activation Domain	10mM AT	20mM AT
DB alone	AD alone	+	-
DB alone	AD-Tax	+	-
DB-CR2	AD alone	+	-
DB-CR2	AD-Tax	+	+
DB-KIX	AD alone	+	-
DB-KIX	AD-Tax	+	+

Figure 2.1. (D) Tax binds to the CR2 domain in vivo. Growth phenotypes of the designated DNA-binding (DB) and activation domain (AD) constructs were assayed by streaking cells on plates lacking histidine and containing 10 or 20 mM aminotriazole (AT). Plates were analyzed following 5 days of incubation at 30°C.

indicate that the minimal region of CR2 competent for interaction with Tax should reside between aa 2003 and 2150. Unexpectedly, this region in isolation (GST-CR2$_{aa2003-2150}$) was not competent for Tax binding (Fig. 2.2.A, lane 8). Rather, a larger region, encompassing aa 2003 to 2212, was required for Tax binding (Fig. 2.2.A, lane 6). This observation suggests that amino acids near either end of this domain participate in Tax binding, and that while removal of one has no detectable effect on the interaction, removal of both termini abolishes Tax binding. This hypothesis fits with secondary structural analysis that predicts that this region of CBP has the potential to form multiple α-helices (291). It is possible that a critical number of interacting motifs are required for Tax binding, and that the strength of the Tax-CR2 interaction is a function of the absolute number of helical motifs present in the fragment.

In an effort to identify critical amino acids within the CR2 region responsible for the interaction with Tax, we prepared and characterized double point mutations. The amino acids selected for point mutagenesis were chosen based on homology between mouse and human CBP as well as homology between CBP and p300. We also used sequence gazing to select leucine residues that had the potential to form α-helices (and thus the potential to form protein-protein contacts). The selected residues were changed to alanines, as they are the least disruptive to secondary and tertiary structure. Four CR2 constructs were prepared, each carrying two point mutations. These double point mutations were F2101→A/I2102→A, L2068→A/L2071→A, L2072→A/L2075→A, and L2140→A/L2143→A. Figure 2.2.B shows that only the double point mutation L2068→A/ L2071→A had a significant effect on Tax binding (lane 5). Interestingly, this

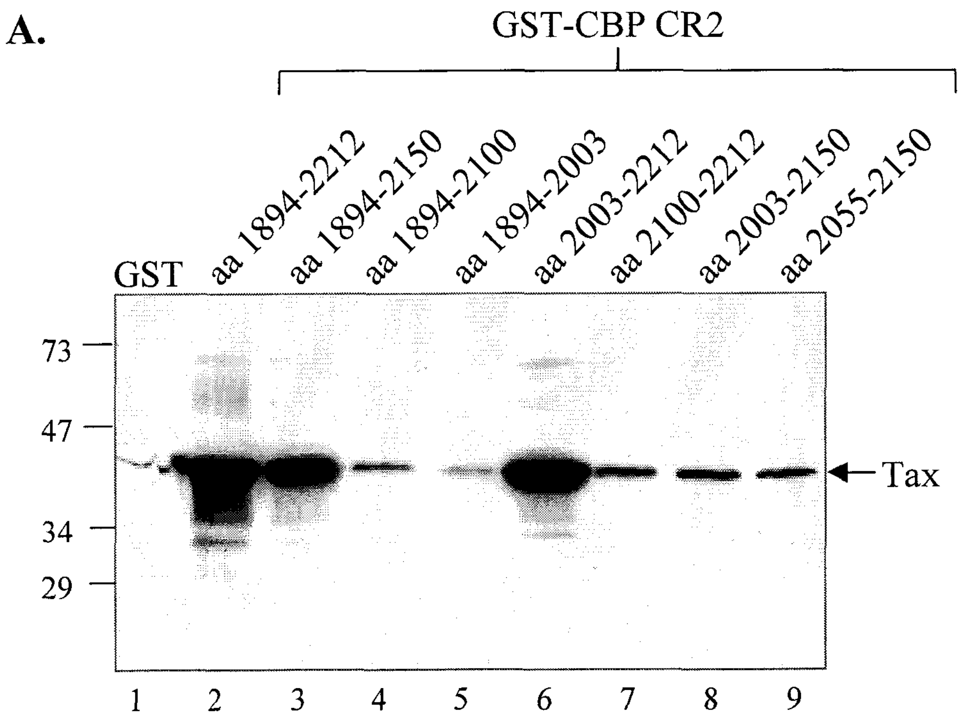


Figure 2.2. **Identification and characterization of minimal CR2 domain.** (A) Tax interacts with aa 2003 to 2212 of CBP in vitro. Purified recombinant Tax (50 pmol) was incubated with GST alone or the indicated GST-CR2 deletion mutants (50 pmol). As a positive control, Tax binding to the full-length region of GST-CR2$_{aa1894-2212}$ was also tested. The bound proteins were separated by SDS-12% PAGE, transferred to nitrocellulose, and detected using an anti-His$_6$ antibody. Positions of bound Tax and protein molecular size standards are indicated (in kilodaltons). The Western blot was stripped and reprobed with anti-GST to ensure that equal amounts of GST fusion protein were used in the assay.

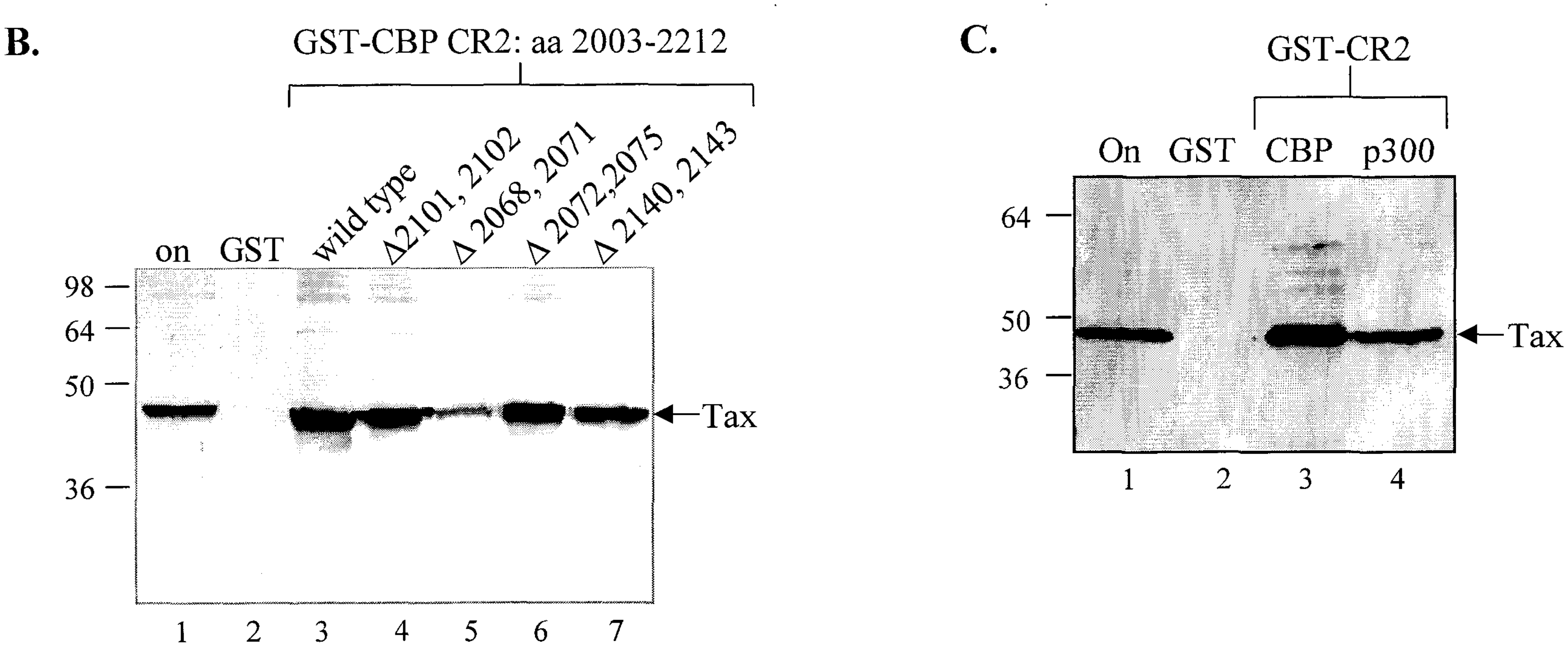


Figure 2.2. (B) Tax is defective for an interaction with the double point mutant ΔCR2 L2068$\rightarrow$A/L2071$\rightarrow$A. Purified recombinant Tax (50 pmol) was assayed for its ability to bind to GST alone or the GST- CR2$_{aa2003\text{-}2212}$ double point mutants F2101$\rightarrow$A/I2102$\rightarrow$A, L2068$\rightarrow$A/L2071$\rightarrow$A, L2072$\rightarrow$A/L2075$\rightarrow$A, and L2140$\rightarrow$A/L2143$\rightarrow$A (50 pmol each). Tax binding to wild-type GST-CR2$_{aa2003\text{-}2212}$ was tested as a positive control. The bound proteins were separated by SDS-12% PAGE, transferred to nitrocellulose, and detected using an anti-His$_6$ antibody. Bound Tax and protein molecular size standards are indicated (in kilodaltons). Five percent of the Tax onput is shown in lane 1. (C) Tax binds equally well to the CR2 domains derived from CBP and p300. Purified recombinant Tax (50 pmol) was incubated with GST alone or the GST-CR2 region from CBP (aa 2003 to 2212), or p300 (aa 1970 to 2193) (50 pmol each). The bound proteins were electrophoresed on SDS-12% PAGE, transferred to nitrocellulose, and detected using an anti-His6 antibody. Bound Tax and protein molecular size standards are indicated. Asterisks indicate the double point mutant (in kilodaltons) in GST ΔCR2 L2068$\rightarrow$A/L2071$\rightarrow$A. Bound Tax was quantified using ImageQuant, and the results indicated that the intensities were nearly equal.

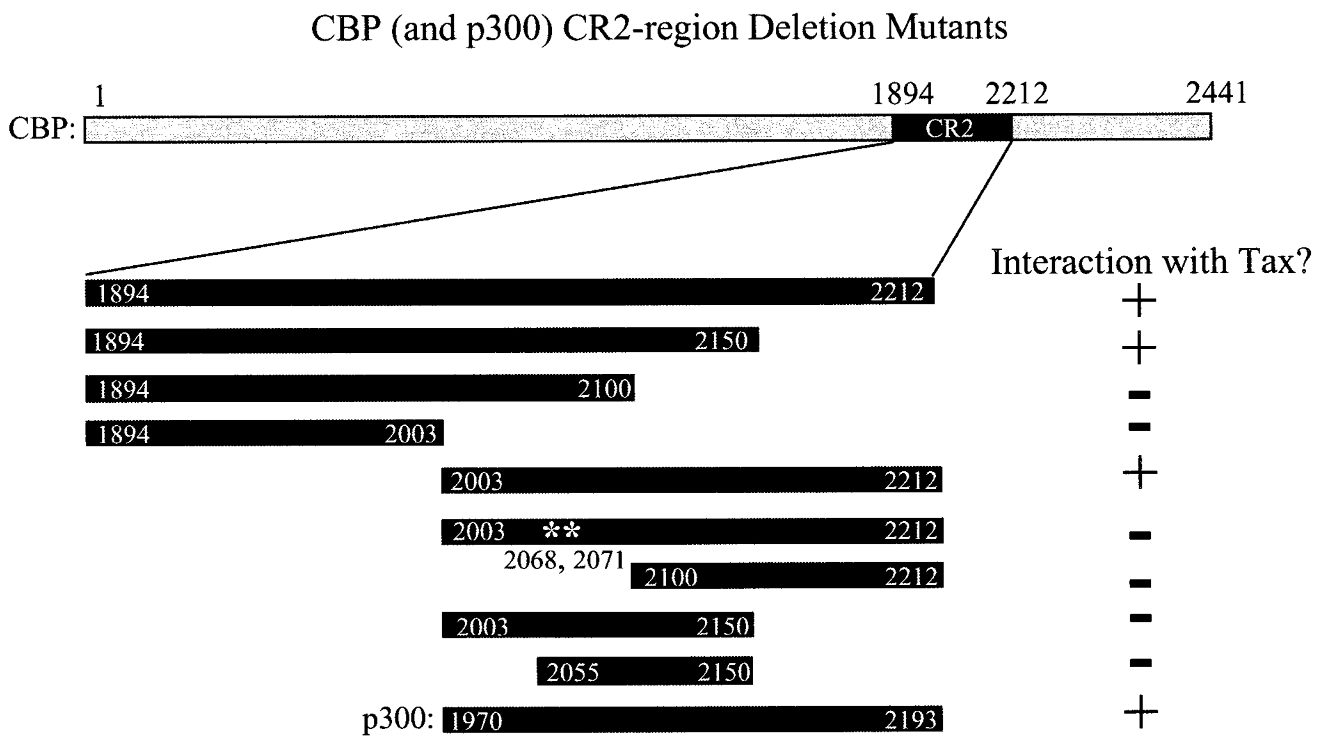


Figure 2.2. (D) Summary of the CR2-Tax interactions.

mutation disrupts one of the 4 α-helices that have been predicted to reside within this region (291). Although we cannot rule out the possibility that this CR2 double point mutation disrupts the structure of the full CR2 domain, the data provide further evidence for a specific interaction between Tax and CR2.

Although CBP and p300 are highly homologous, it is unclear whether these two proteins are functionally redundant. CBP and p300 have domains of high amino acid sequence homology (e.g., KIX, >90%), whereas other regions, such as CR2, are more divergent (~50%). We were therefore interested in testing whether Tax interacts with the corresponding CR2 region present in p300. To address this question, we cloned the corresponding region of p300 (aa 1970 to 2193), fused it to GST, and tested Tax binding in a GST pulldown assay. Figure 2.2.C shows that Tax binds comparably to the CR2 regions from both CBP and p300 (lanes 3 and 4). A summary of the Tax interaction with the various CR2 constructs from CBP/p300 is shown in figure 2.2.D.

2.4c CR2 domain represses Tax transactivation in vivo

The observation that Tax efficiently binds to the CR2 domain of CBP in vitro led to the hypothesis that expression of CR2 in vivo might compete with active CBP/p300 in the cell, thus inhibiting Tax transactivation. To test this idea, we performed transient-cotransfection assays in HTLV-1-negative Jurkat T cells using a reporter plasmid carrying three copies of the Tax-responsive viral CRE (viral CRE-Luc) driving expression of the luciferase gene (Fig. 2.3.A) (101). We measured Tax transactivation in the presence of increasing amounts of an expression plasmid carrying the CR2 region (aa 2003 to 2212) under the control of the CMV promoter. As expected, expression of Tax strongly activated transcription from the Tax-responsive promoter (Fig. 2.3.B, lanes 1

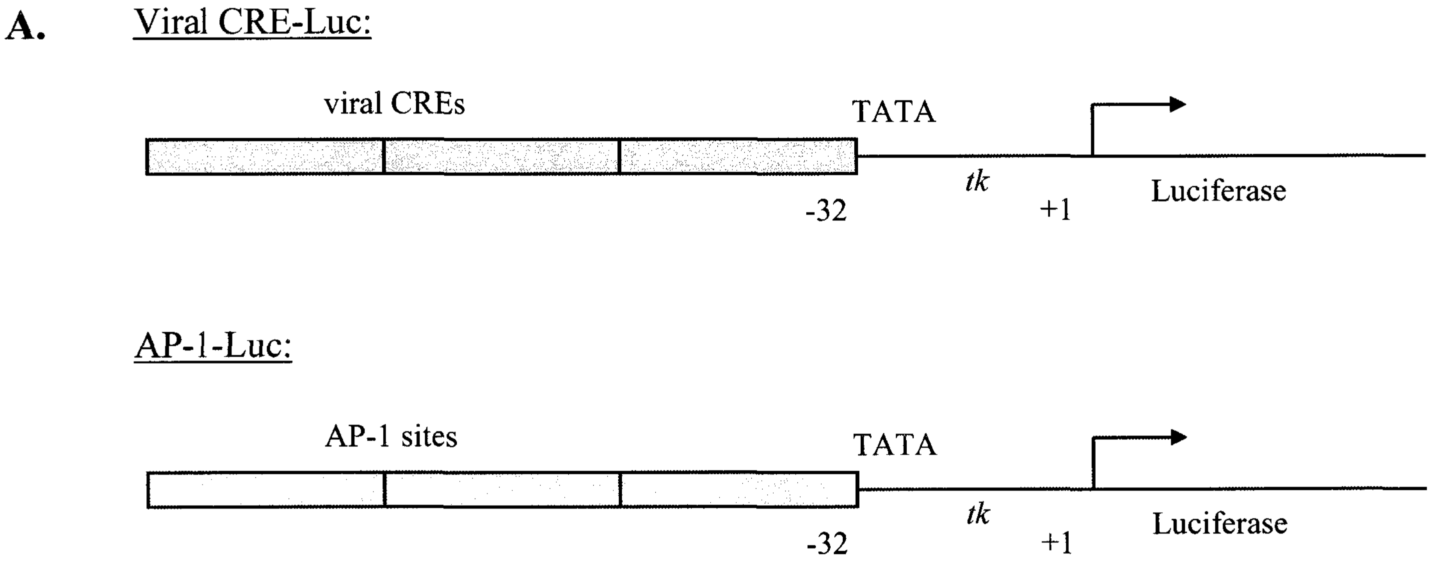


Figure 2.3. **CR2aa2003-2212 inhibits Tax-activated transcription in vivo**. (A) Schematic illustration of the viral CRE and AP-1-luciferase reporter constructs used in the transient-cotransfection assays. Transient-cotransfection assays were performed in HTLV-1-negative human Jurkat T cells.

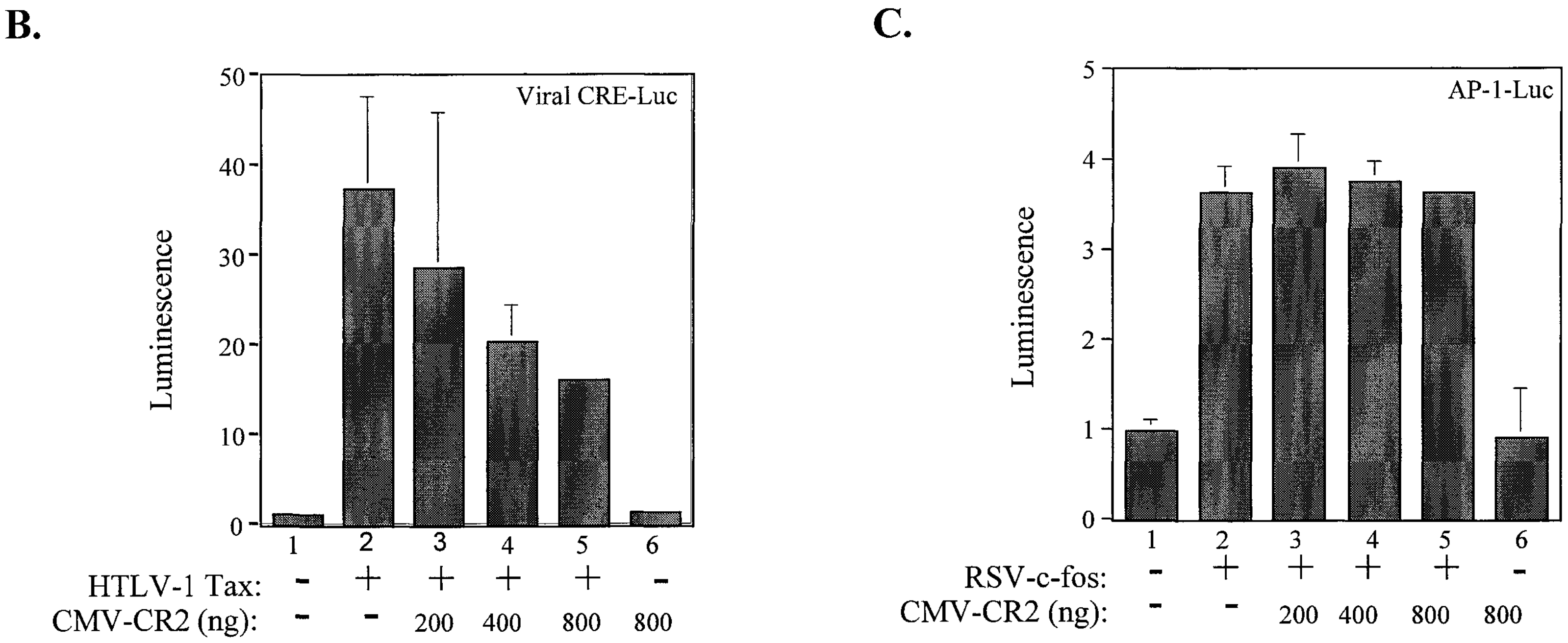


Figure 2.3. (B) CMV-CR2aa2003-2212 expression inhibits Tax transactivation. The Tax-responsive viral CRE-Luc reporter plasmid (100ng) (14) was cotransfected with a constant amount of the HTLV-1 Tax expression plasmid (6) (200ng) (lanes 2 to 5) and an increasing amount of an expression plasmid for CMV-CR2aa2003-2212 (lanes 3 to 6), as indicated. (C) CMV-CR2aa2003-2212 expression does not affect c-*fos*-activated transcription in vivo. Transient-cotransfection assays were again performed in Jurkat T cells; however, the AP-1-Luc reporter plasmid (400ng) (14, 53) was cotransfected with a constant amount of the RSV-c-*fos* (3) (400ng) (lanes 2 to 5), and an increasing amount of the CR2 expression plasmid, CMV-CR2aa2003-2212 (lanes 3 to 6), as indicated.

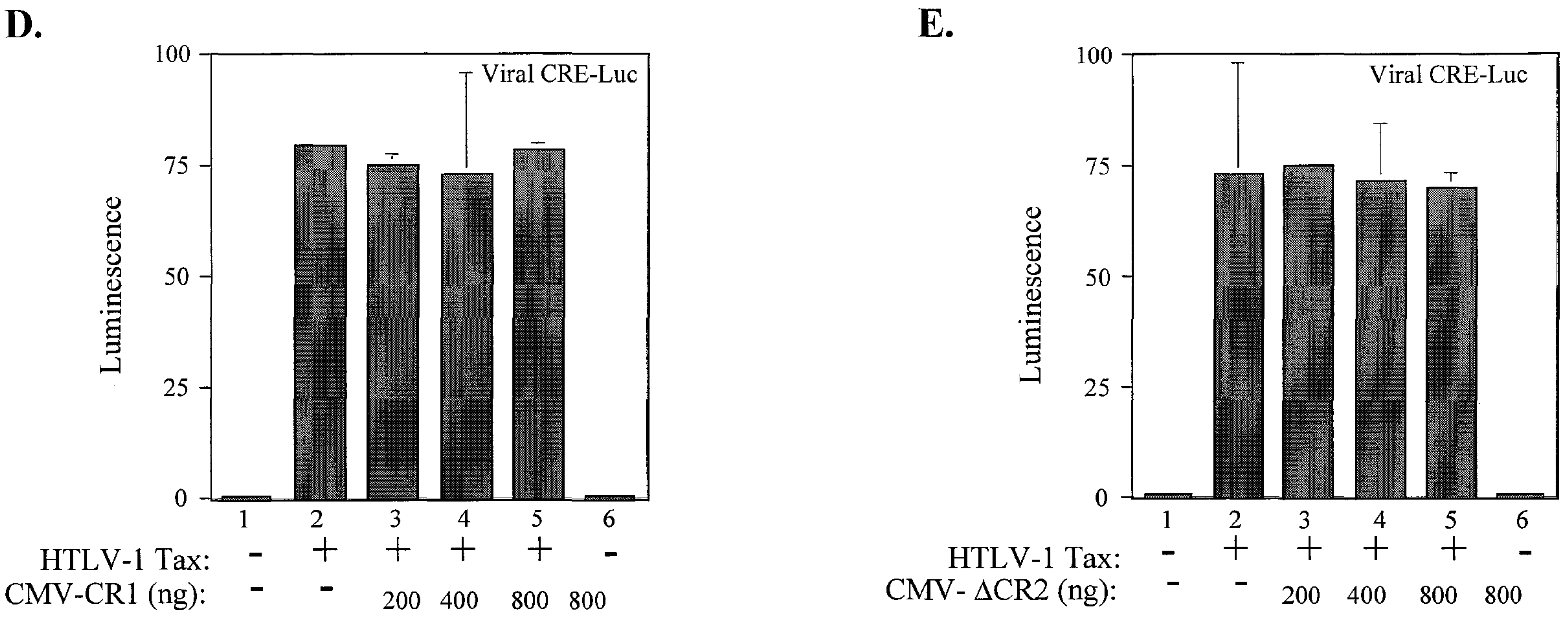


Figure 2.3. (D and E) CMV-CR1aa1515-1895 expression and CMV-ΔCR2aa2003-2212 expression of the L2068→A/L2071→A double point mutation does not affect Tax-activated transcription in vivo. The viral CRE-Luc reporter plasmid (100ng) was cotransfected with a constant amount of the HTLV-1 Tax expression plasmid (6) (200ng) (lanes 2 to 5) and an increasing amount of the expression plasmid for CMV-CR1aa1515-1895 (panel D, lanes 3 to 6), as indicated, or CMV-ΔCR2aa2003-2212 carrying the L2068→A/L2071→A double point mutation (panel E, lanes 3 to 6) as indicated,. In all experiments, a constant amount of the *Renilla* luciferase reporter plasmid (10ng) was added to each reaction as an internal control. Luminescence was quantitated with a luminometer, and activation was quantitated relative to expression from the viral CRE-Luc reporter plasmid in the absence of Tax or from the AP-1-Luc reporter plasmid in the absence of c-*fos*. The values shown are the mean fold activation (in duplicate) +/- the standard deviation. The experiments are representative of at least three independent experiments. Western blot analysis using anti-His6 antibody confirmed expression of CMV-CR2 and CMV-ΔCR2 following transfection (see supplemental Fig. 2.7).

and 2). Cotransfection of increasing amounts of the CR2 expression plasmid repressed Tax transactivation in a dose-dependent fashion (Fig. 2.3.B, lanes 3 to 5). While the highest amount of CR2 (800 ng) strongly repressed Tax transcription function, it had no effect on reporter plasmid expression in the absence of Tax, suggesting that the effect of CR2 was specific to Tax and that CR2 expression was not toxic to the cells (Fig. 2.3.B, lane 6). It is noteworthy that in these experiments, CR2 may be directly affecting Tax protein levels, as the expression plasmid uses the Tax-responsive HTLV-1 promoter to drive Tax synthesis. Therefore, the repressive effect of CR2 may occur through both reductions in Tax levels and repression of the viral CRE-luciferase reporter plasmid. As a control, we tested CR2 expression on c-*fos*-dependent transcription from an AP-1-luc reporter plasmid (Fig. 2.3.A). Figure 2.3.C shows that increasing concentrations of CR2 had no effect on c-*fos*-dependent transcription (lanes 3 to 5), suggesting that CR2 disruption of transcriptional activity is specific to proteins that bind to the CR2 domain. As additional controls, we also tested expression of two CBP molecules that are negative for an interaction with Tax. We have shown that Tax does not interact with CR1 (Fig. 2.1.B) or a form of CR2 carrying the L2068→A/L2071→A double point mutation (ΔCR2) (Fig. 2.2.B). As expected, cotransfection of plasmids expressing these molecules had no effect on Tax transactivation (Fig. 2.3.D and E).

2.4d Critical Tax amino acids required for CR2 binding

To further characterize the Tax-CR2 interaction, we were interested in determining the region of Tax that is involved in CR2 binding. We prepared deletion mutants of Tax, fused them to GST, and tested their interaction with purified His_6-

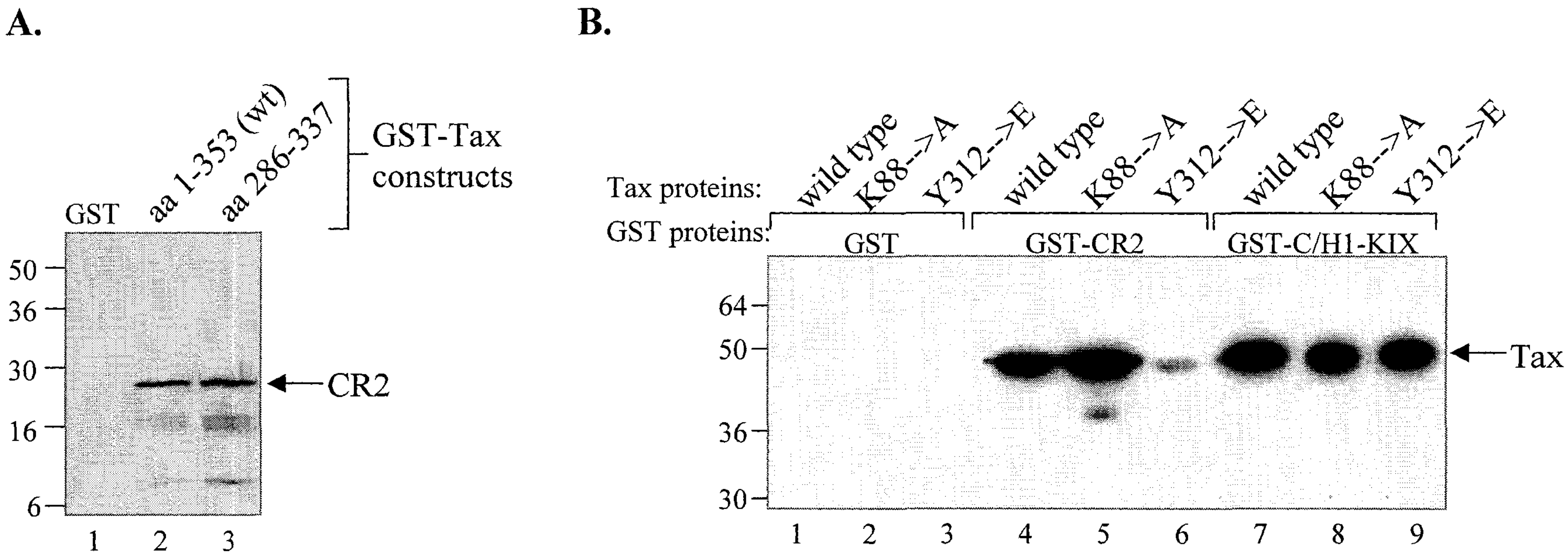


Figure 2.4. **Carboxy-terminal activation domain of Tax interacts with CR2**. (A) CR2 binds to the putative transactivation domain of Tax. Purified, recombinant His6-CR2aa2003-2212 (50 pmol) was incubated with GST alone or the indicated GST-Tax fusion constructs (50 pmol). The bound proteins were separated by SDS-10% PAGE, transferred to nitrocellulose, and detected using an anti-His6 antibody. The positions of bound His6-CR2aa2003-2212 and protein molecular size standards are indicated (in kilodaltons). (B) The Tax Y312→E point mutant is defective for interaction with CR2. Purified recombinant wild-type Tax (lanes 1, 4, and 7), Tax K88→A (lanes 2, 5, and 8), or Tax Y312→E (lanes 3, 6, and 9) (50 pmol each) were incubated with GST alone or the indicated GST- CR2aa2003-2212 or GST-C/H1-KIX aa302-683 fusion proteins. The bound proteins were separated by SDS-12% PAGE, transferred to nitrocellulose, and detected using an anti-His6 antibody. Bound Tax and protein molecular size standards are indicated (in kilodaltons).

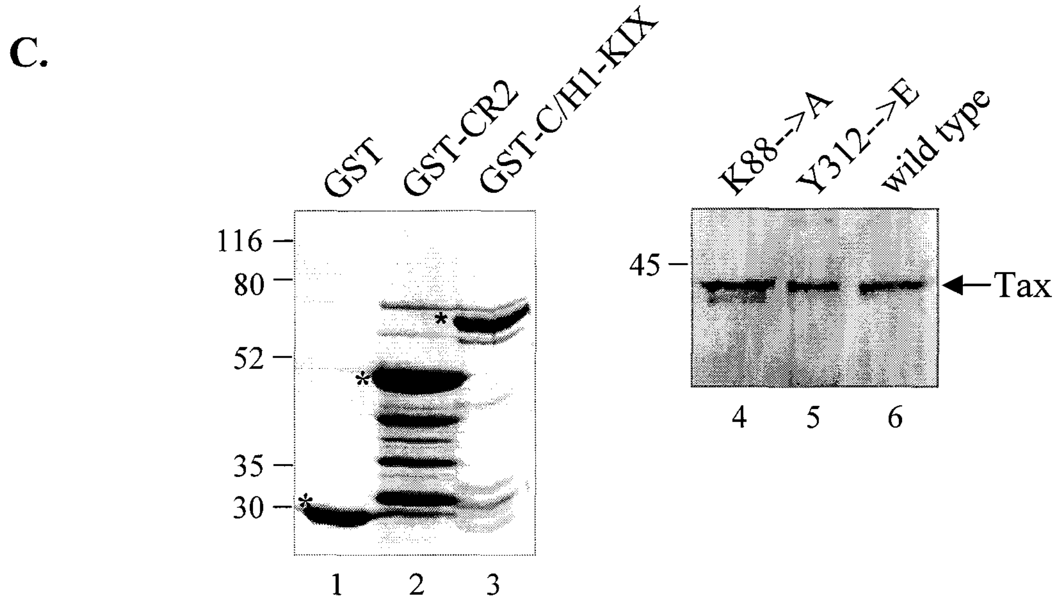


Figure 2.4. (C) Coomassie-stained gels of the purified proteins used in the experiment shown in panel 2.4.B. The left panel shows the GST fusion proteins; asterisks denote the relevant protein band. The right panel shows both the wild-type and mutant forms of Tax used in the experiment. Protein molecular size standards are indicated (in kilodaltons).

$CR2_{aa2003-2212}$. Figure 2.4.A shows that CR2 interacted comparably with both full-length Tax and a carboxy-terminal fragment of Tax encompassing aa 286 to 337, suggesting that the CR2-interacting region of Tax resides near the carboxy terminus of the protein. Interestingly, several previous studies have identified several point mutations in this region that significantly reduce or abolish Tax transactivation of HTLV-1 transcription in vivo, suggesting that this region of Tax may serve as an activation domain (286, 288, 303). Since one of the prominent functions of an activation domain is coactivator recruitment, we reasoned that specific amino acids in this region might contact CR2. To address this possibility, we created a point mutation specifically within a subdomain of this region (Tax aa 312 to 319) that we predicted would participate in amphipathic helix formation. This prediction is based on the sequence, aro-X-X-ϕ-ϕ-X-X-ϕ (aro represents F or Y, and ϕ represents bulky hydrophobic groups), where the hydrophobic and aromatic amino acids are predicted to form the face of an amphipathic helix, and mediate protein-protein contacts (260). This sequence has been identified in the activation domains of several transcription factors, including CREB and p53 (260). The Tax sequence $_{312}$-Y-T-N-I-P-I-S-L-$_{319}$ matches this conserved sequence, suggesting that it might participate in coactivator binding. To determine whether this sequence in Tax is involved in CR2 binding, we targeted the critical tyrosine at aa 312 and replaced it with a glutamic acid. Purified full-length Tax protein carrying the Y312→E mutation was tested in a GST pulldown assay with GST-$CR2_{aa2003-2212}$. As a control, we compared the binding of Tax Y312→E with Tax K88→A, a mutant form of Tax that has been reported to be defective for KIX binding (125). Figure 2.4.B shows that Tax protein carrying the Y312→E point mutation was significantly compromised for an interaction with CR2 (lane 6), whereas

Tax K88→A was not (lane 5). Tax Y312→E bound GST-C/H1-KIX with apparent wild-type affinity, indicating that the mutation is not globally disruptive to the structure of Tax (Fig. 2.4.B, lane 9). Interestingly, we observed near wild-type binding of Tax K88→A to our GST-C/H1-KIX construct. Although this binding was unexpected, it is likely due to additional contacts between Tax and the C/H1 portion of GST-C/H1-KIX, as we have previously observed strong binding of Tax to this region (190). Coomassie-stained gels of Tax and the GST fusion proteins used in the GST pulldown assay are shown in figure 2.4.C.

2.4e Functional studies of Tax-CR2 interaction

We were then interested in testing whether point mutations in Tax that disrupted the CR2 interaction also disrupted Tax transactivation. We first examined Tax Y312→E activity in an in vitro transcription assay, using a plasmid carrying a Tax-responsive promoter driving synthesis of two tandem G-less cassettes. The G-less cassette allows synthesis of a defined 380-nucleotide (nt) guanineless transcript from the closed circular template. The Tax-responsive promoter, which drives the G-less cassette, carries four viral CRE elements cloned immediately upstream of the core HTLV-1 promoter (7). A schematic representation of the promoter construct is shown in figure 2.5.A. We performed in vitro transcription assays using nuclear extracts prepared from the HTLV-1-negative human T-cell line CEM. Figure 2.5.A shows the results of the in vitro transcription experiment. As expected, the addition of wild-type Tax alone and in combination with the Ser133-phosphorylated form of CREB produced an increase in transcription from the 4TxRE/G-less template (8- and 15-fold, respectively) (Fig. 2.5.A, lanes 1 to 3). However, the addition of the same amount of Tax protein carrying the

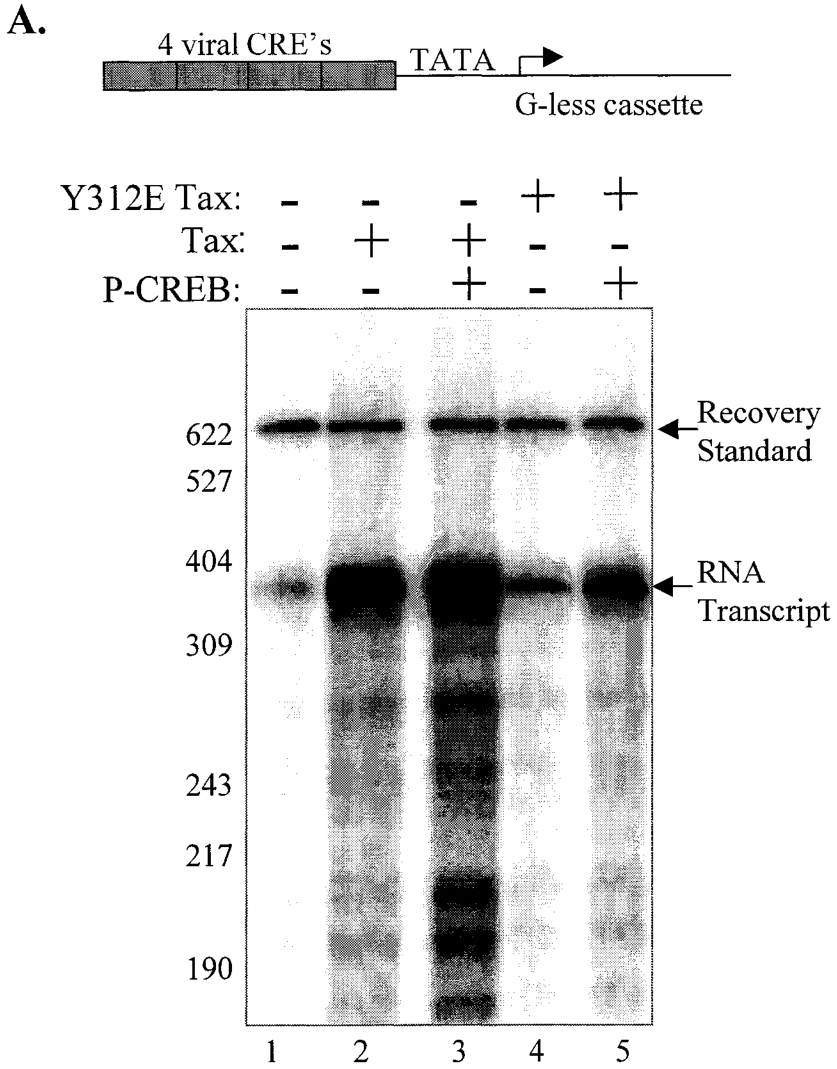


Figure 2.5. **Tax Y312→E is defective for transactivation both in vitro and in vivo**. (A) Tax Y312→E is defective for transcriptional activation from a Tax-responsive element promoter. The in vitro transcription assay was performed on a 4TxRE G-less cassette template that carries four tandem copies of the third (promoter-proximal) viral CRE driving expression of a 380-nt RNA (shown schematically above panel 5A). Transcription reaction mixtures contained the 4TxRE G-less template (100ng), nuclear extract (70 µg of CEM, a human T-cell line) (lanes 1 to 5), purified recombinant wild-type Tax (100ng) (lanes 2 and 3), purified recombinant Tax Y312→E (100ng) (lanes 4 and 5), and pCREB (50ng) (lanes 3 and 5). The positions of the full-length 380-nt RNA transcript, labeled DNA recovery standard, and molecular size markers are indicated (in nucleotides). This experiment is representative of three independent experiments.

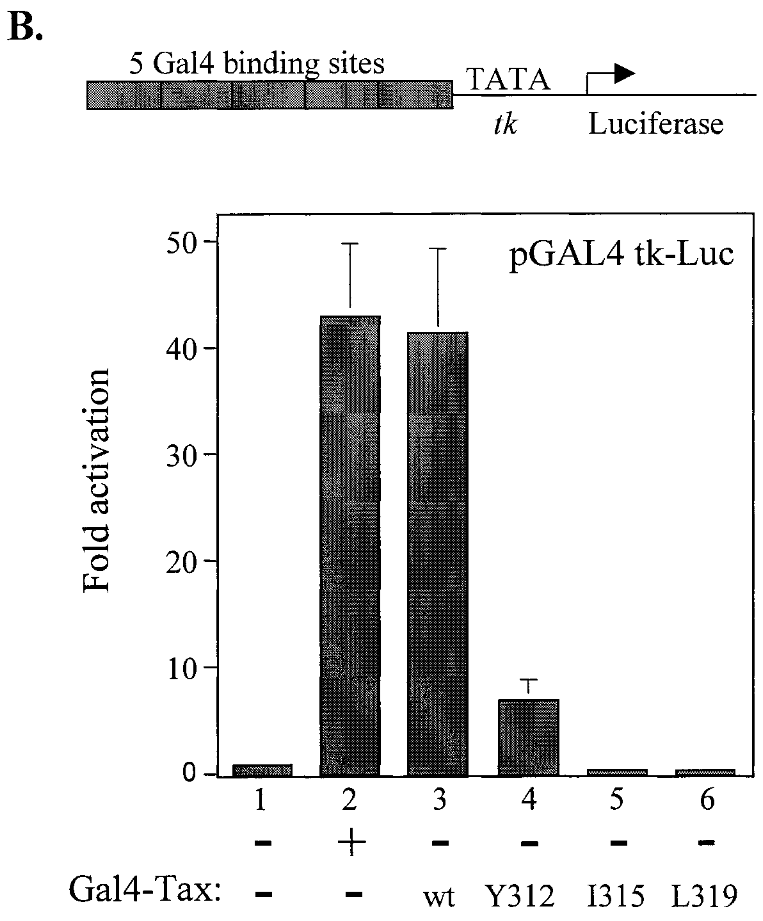


Figure 2.5. (B) Tax Y312→E is defective for transactivation in vivo. Transient-cotransfection assays were performed in HTLV-1-negative human T-lymphocyte Jurkat cells. The Gal4-luciferase reporter plasmid (400ng) (shown schematically above panel B) was cotransfected with Gal4-p53 (aa 1 to 42) (200ng) (lane 2) or the indicated wild-type or mutant Gal4-Tax expression plasmid (200ng) (lanes 3 to 6) and a constant amount of *Renilla* luciferase reporter plasmid (10ng). The Gal4-Tax point mutations used in this study are Y312→E, I315→E, and L319→R. Luminescence was quantitated with a luminometer, and activation was calculated relative to the activation from the Gal4 reporter plasmid alone in the absence of Tax or p53. The values shown are the mean fold activation of duplicates +/- the standard deviation. The experiment shown is representative of three independent trials.

Y312→E point mutation produced only a very modest increase in RNA synthesis (twofold) (Fig. 2.5.A, lane 4). The addition of Ser133-phosphorylated CREB to the Tax Y312→E reactions only partially rescued transcriptional activation (fivefold) (Fig. 2.5.A, lane 5). In an attempt to directly evaluate an effect of the Tax Y312→E on coactivator utilization, we tested the effect of full-length p300 in these experiments. We did not, however, observe enhanced Tax transactivation upon p300 addition (see supplemental Fig. 2.8). This observation is consistent with previous reports showing that p300 does not activate transcription in vitro in the absence of chromatin (176, 179).

To further examine the role of the CR2-interacting domain in Tax transactivation, we performed transient-cotransfection assays. For these experiments, we used an expression plasmid carrying the full-length Tax protein fused to the DNA-binding domain of Gal4 (63). In this background, we introduced the Y312→E point mutation and two additional point mutations in the activation domain region of Tax. These two new mutations, I315→E and L319→R, were also targeted to the putative amphipathic helix in Tax that likely plays a role in HTLV-1 transcription (see above). Figure 2.5.B shows, as expected, that cotransfection of Gal4-wtTax strongly activated transcription from a reporter plasmid carrying 5 copies of the Gal4 DNA-binding site (Gal4-Luc; Fig. 2.5.B, compare lanes 1 and 3). Each of the Gal4-Tax point mutants, Y312→E, I315→E and L319→R, were significantly compromised for Tax transactivation (Fig. 2.5.B, lanes 4 to 6). As a control for promoter activity, we also tested the activation domain of p53 (aa 1 to 42) fused to the Gal4 DNA-binding domain (GaL4-p53$_{\mathrm{aa1\text{-}42}}$), in the transient-cotransfection assay (Fig. 2.5.B, compare lanes 1 and 2). These data further support a functional role for this region of Tax in the activation of transcription.

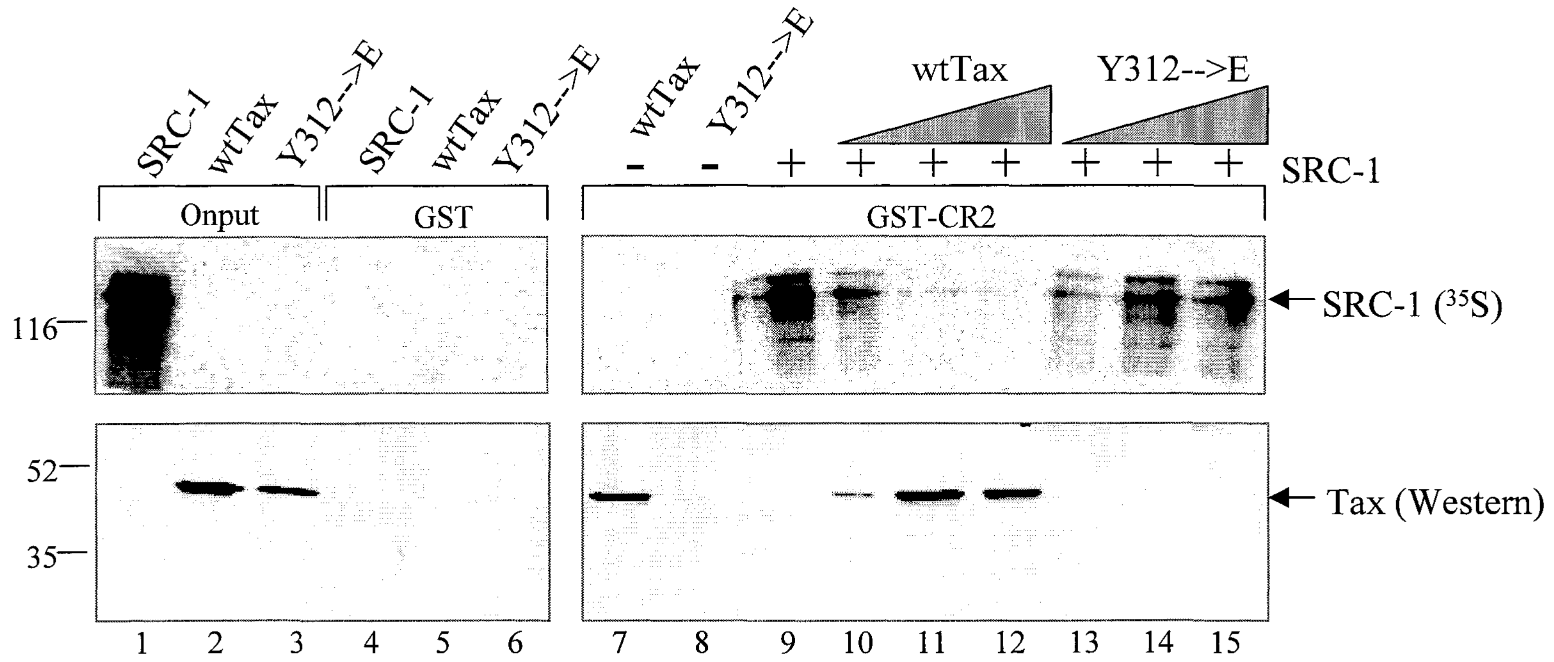


Figure 2.6. **Tax and SRC-1 binding to CR2aa2003-2212 is mutually exclusive**. SRC-1 [^{35}S]-labeled transcription-translation product (0.1μL) was incubated with GST alone or GST-CR2$_{aa2003-2212}$ (5 pmol) (lanes 4 and 9 to 15) in the presence of increasing amounts of wild-type Tax protein (5, 50, and 100 pmol) (lanes 10 to 12) or Tax Y312→E (5, 50, and 100 pmol) (lanes 13 to 16). Wild-type (wt) and Y312→E Tax (50 pmol) were also incubated with GST alone (5 pmol) (lanes 5 and 6) or GST-CR2$_{aa2003-2212}$ (5 pmol) (lanes 7 and 8). Bound proteins were separated by SDS-12% PAGE. Top, PhosphorImager analysis of bound SRC-1. Bottom, Western blot analysis using anti-His$_6$ antibody. The positions of SRC-1 onput (100%) (lane 1), wild-type Tax (5 pmol) (lane 2), and Tax Y312→E (5 pmol) (lane 3) and molecular size markers are indicated (in kilodaltons).

2.4f Tax and SRC-1 compete for CR2 binding in vitro

In addition to binding Tax, the CR2 region also corresponds to the SRC-1-interacting domain of CBP that is critical to coactivator utilization by liganded nuclear hormone receptors (106, 196). Previously, Tax has been shown to strongly repress transcription mediated through steroid and retinoid receptors (72). This observation, together with the observation that both Tax and SRC-1 physically interact with CR2, led to the hypothesis that their binding may be mutually exclusive. To directly test this idea, we examined whether increasing concentrations of Tax can displace SRC-1 from CR2 in vitro. As a control, we also tested Y312→E Tax, which is defective for CR2 binding. Glutathione beads were bound with GST-$CR2_{aa2003\text{-}2212}$ and then incubated with in vitro-transcribed and translated full-length SRC-1. Increasing amounts of wild-type Tax or Tax Y312→E were included in the binding reactions containing SRC-1, and the resulting protein-protein interactions were detected by PhosphorImager and Western blot analysis. Figure 2.6 shows that increasing amounts of Tax dramatically reduced SRC-1 binding to CR2, with a concomitant increase in Tax binding (lanes 10 to 12). As expected, increasing amounts of Tax Y312→E had no effect on SRC-1 binding to CR2 (lanes 13 to 15). Together, these data indicate that the binding of SRC-1 and Tax to CR2 is mutually exclusive in vitro.

2.5 Discussion

The results presented in this report indicate that the HTLV-1 Tax protein interacts with multiple domains of the pleiotropic coactivators CBP and p300. In addition to the interactions with the C/H1 and KIX domains, we show here that Tax strongly interacts with a carboxy-terminal region of CBP, encompassing CBP aa 2003 to 2212. Tax also binds strongly to the corresponding CR2 region in p300. Expression of the CBP-CR2 region alone in vivo represses Tax transactivation function, suggesting that Tax binding to the isolated CR2 region prohibits recruitment of the full-length coactivators. We identify a carboxy-terminal region of Tax, encompassing aa 312 to 319, that participates in the interaction with CR2. A point mutation in this region, Y312→E, is defective for CR2 binding and Tax transactivation. We show that other point mutations (I315→E and L319→R) in this region also strongly compromise Tax transactivation function. The data in this report suggest that the 312 to 319 region of Tax forms a potent activation domain and that this region functions to contact CR2 and thus participate in the recruitment of the coactivator to the HTLV-1 promoter. Further evidence supporting a role for the Tax activation domain in coactivator interaction comes from a recent report showing that M47 Tax (L319→R, L320→S) is partially defective for interaction with full-length p300 in an electrophoretic mobility shift assay (125). Together, these data strengthen previously published reports showing that a variety of point mutations in this region strongly repress Tax transcription function. These include double point mutations at aa 310 to 311, 315 to 316, and 319 to 320 and single point mutations at aa 316, 317, 318, and 320 (288, 303).

We have begun to delineate the molecular basis of the Tax-CR2 interaction. A short sequence in the Tax activation domain conforms precisely to a consensus sequence found in the activation domains of several other transcription factors (260). This consensus sequence, aro-X-X-ϕ-ϕ-X-X-ϕ- is predicted to form an amphipathic helix and therefore participate in protein-protein interactions. The Tax sequence $_{312}$-Y-T-N-I-P-I-S-L-$_{319}$ matches the reported consensus sequence precisely. Furthermore, mutation of the critical tyrosine residue (Y312→E), which is predicted to reside on the face of the helix, dramatically reduced CR2 binding in vitro, and Tax transactivation in vitro and in vivo. Mutation of additional residues (I315→E and L319→R) also predicted to reside on the face of the helix also dramatically reduced Tax transactivation in vivo. These data strongly suggest that the activation domain of Tax forms an amphipathic helix-binding surface which mediates CR2 interaction. Previous research has also shown that the M47 Tax mutant is defective for transcriptional activation from the HTLV-1 long terminal repeat (LTR) while completely functional for transactivation from the human immunodeficiency virus type 1 LTR (1, 286). The other half of the activator-coactivator interaction is contributed by CR2 aa 2003 to 2212. Interestingly, this minimal CR2 region carries four predicted α-helical domains (291), which may be critical for interaction with the amphipathic helix of Tax. Consistent with this idea, disruption of a single α-helix by point mutagenesis (L2068→A/ L2071→A) significantly reduced Tax binding to CR2. These data define the Tax-CR2 interface and provide a framework for further studies on the molecular structure of the Tax-CR2 interaction.

The observation that Tax contacts the CR2 region of CBP and p300 indicates that at least three distinct sites on the coactivator may participate in mediating Tax transactivation. Several previous studies have shown that Tax also binds to the KIX and C/H1 domains of CBP and that these interactions may be relevant in Tax transactivation (101, 125, 182, 352). It is interesting that distinct regions of Tax (defined by the K88→A and Y312→E point mutations) make independent contacts on CBP/p300, suggesting that discrete coactivator contacts can be made simultaneously. Functional studies suggest that independent coactivator contacts contribute to Tax function, as KIX and CR2, when expressed in isolation, each repressing Tax transactivation approximately 50% (101). This evidence suggests that the distinct Tax-CBP interactions occur simultaneously and perhaps cooperate to enhance coactivator-mediated transcriptional activation. It is not currently known whether Tax binding simultaneously at multiple sites in CBP/p300 leads to more efficient coactivator recruitment or whether the individual interactions promote dissimilar effects on Tax-activated transcription. It is interesting that other transcription factors bind to multiple domains of CBP which could be important for reconfiguring the CBP molecule for chromatin remodeling and/or recruitment of the RNA polymerase II transcriptional machinery (149, 334). Most notably, the tumor suppressor p53 also binds to the KIX domain as well as a C-terminal domain of CBP, whereby Tax and p53 exhibit mutually exclusive binding to the KIX domain (121, 334). The fact that Tax binds as a dimer on the HTLV-I promoter in the nucleoprotein complex also provides further potential for multiple interactions with CBP and p300, as well as with other components of the transcription machinery (155, 321).

It is interesting that the CR2 region of CBP defined in this study precisely overlaps with the SRC-1 interaction domain of CBP (159, 176, 356). SRC-1 is a prominent member of a family of coactivators that bind to this specific region of CBP and mediate transcriptional activation of nuclear hormone receptors (106, 196). Given the overlap of the amino acids required for Tax binding and SRC-1 binding (aa 2003-2212 and 2058 to 2130, respectively), it not surprising that the binding of these two proteins to CR2 in vitro is mutually exclusive. These data are consistent with a recent study showing that Tax is a potent inhibitor of nuclear receptor-activated transcription mediated through SRC-1 (72). Interestingly, a double point mutation in Tax, at aa 319 and 320 (M47), (303), relieved the repression of nuclear hormone signaling. This observation is not unexpected, as this double point mutation likely disrupts the amphipathic helix in Tax and thus the Tax-CR2 interaction. Reduced Tax binding to CR2 would give SRC-1 greater access to the limiting coactivator and relieve the repression. Although the report did not identify a mechanism of Tax repression of nuclear hormone transcription function, the authors did suggest that CBP may be involved. These data provide strong corroborating evidence for a functionally relevant Tax-CR2 interaction in vivo.

In summary, the studies presented herein define a new domain on CBP/p300 involved in Tax transactivation and demonstrate the complexity of activator-coactivator interactions in mediating gene regulation. Several previous studies have provided extensive evidence for Tax binding to the KIX domain, and more recently to the C/H1 domain, resulting in competition with other transcription factors that bind to these regions (10, 62, 314, 334, 336). Our demonstration here that Tax binds to an additional region of CBP/p300 has further implications for Tax derailment of CBP coactivator function in the HTLV-1-infected cell. These studies provide the biochemical foundation for future work on the molecular interactions at the CR2-Tax interface, as well as the biological consequences of this novel interaction.

2.6 Acknowledgments

We thank D. Heery (University of Leicester) for the pSG5-hSRC1e expression plasmid and Karen Van Orden, Jeanne Mick, Melissa Gonzales, and Raji

Edayathumangalam for their significant contributions to this research. We also thank Steve McBryant and Holli Giebler for critical reading of the manuscript.

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Supplemental Figures for Chapter 2

The following figures were cited as “data not shown” in the text.

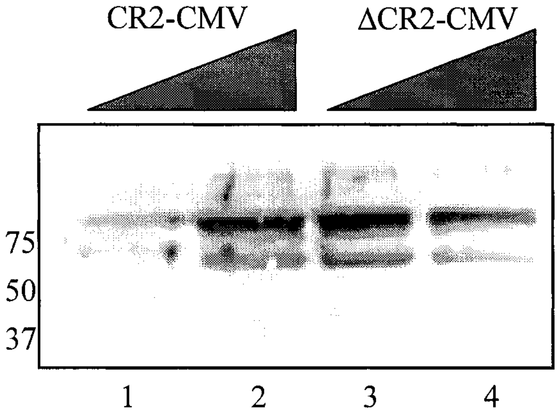


Figure 2.7. **Expression of CR2-CMV and ΔCR2-CMV in Jurkat T cells.** The CR2-CMV expression vector (1 and 2 μg) was cotransfected into Jurkat cells (lanes 1 and 2) and the ΔCR2-CMV expression vector (1 and 2 μg) was also cotransfected into Jurkat cells (lanes 3 and 4). After 24 hours, cells were analyzed by Western blot on a SDS-15% PAGE using an anti-His_6 antibody. Protein molecular size standards are indicated (in kilodaltons).

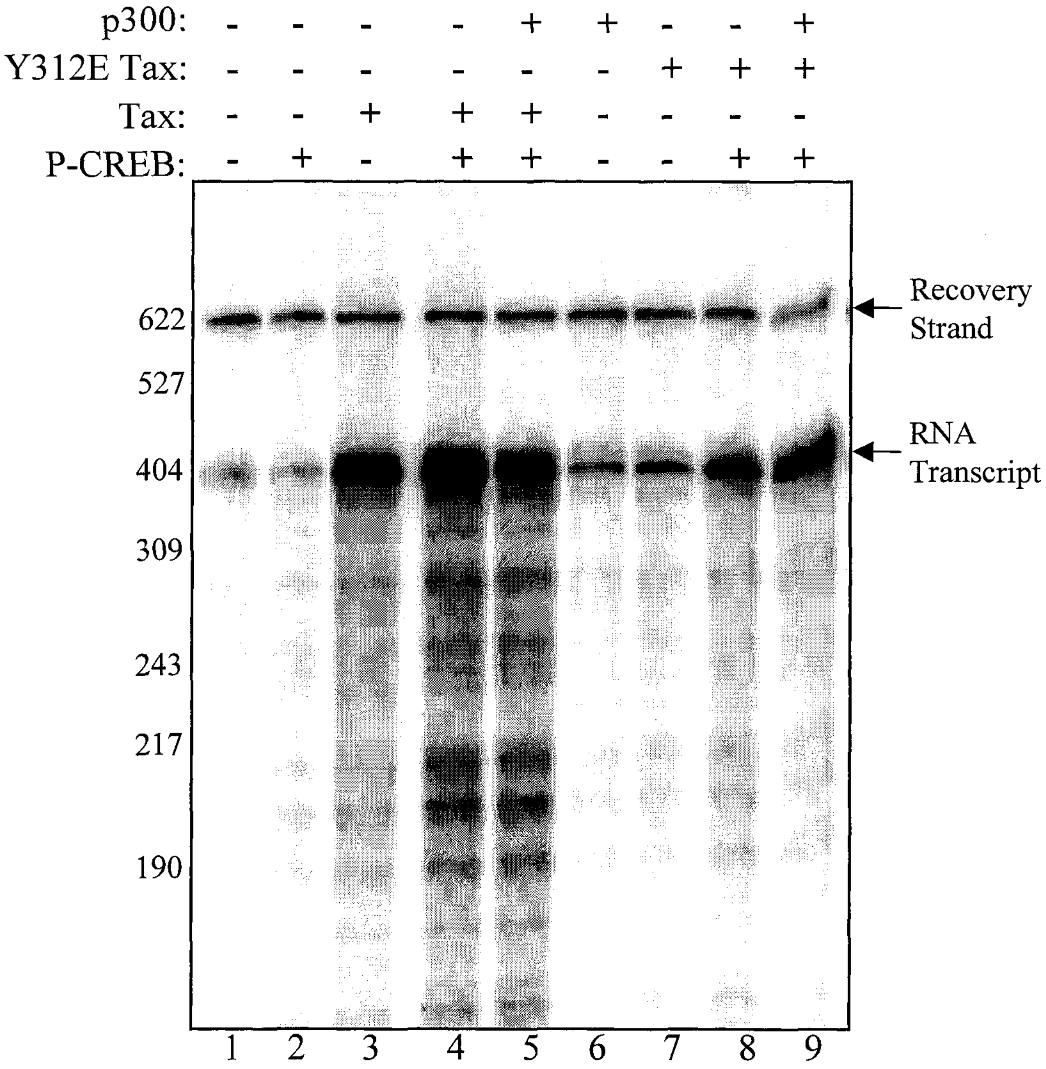


Figure 2.8. **p300 does not enhance Tax transactivation in vitro.** p300 is defective for transcriptional activation from a Tax-responsive element promoter. The in vitro transcription assay was performed on a 4TxRE G-less cassette template that carries four tandem copies of the third (promoter-proximal) viral CRE driving expression of a 380-nt RNA. Transcription reaction mixtures contained the 4TxRE G-less template (100ng), nuclear extract (70 μg of CEM, a human T-cell line) (lanes 1 to 9), purified recombinant wild-type Tax (100ng) (lanes 3 to 5), purified recombinant Tax Y312→E (100ng) (lanes 7 to 9), pCREB (50ng) (lanes 2, 4, 5, 8, and 9), and p300 (100ng) (lanes 5, 6, and 9). The positions of the full-length 380-nt RNA transcript, labeled DNA recovery standard, and molecular size markers are indicated (in nucleotides).

Chapter 3

p53 Transcriptional Activity Is Mediated Through the SRC1-Interacting Domain of CBP/p300

This chapter was published in *The Journal of Biological Chemistry.* The text of this manuscript is presented as it appeared in this journal. All the figures that appeared in this manuscript are presented exactly as they appeared in this journal and the data not shown experiments are presented as supplemental figures. Jill Livengood and I are first co-author on this publication. My experiments are shown as figures 3.2b, 3.3a, and 3.6. I also cloned the CR2 construct used in figure 3.5d and provided the GST fusion proteins used in figure 3.4. The reference for this chapter is shown below.

Livengood[†], **J.A., K.E.S. Scoggin**[†], **K., Van Orden, S.J., McBryant, R.S., Edayathumangalam, P.J., Laybourn, and J.K. Nyborg**. 2002. p53 Transcriptional Activity Is Mediated through the SRC1-interacting Domain of CBP/p300. J. Biol. Chem. **277**(11): 9054-9061.

3.1 Abstract

The tumor suppressor p53 recruits the cellular coactivator CBP/p300 to mediate the transcriptional activation of target genes. In this study, we identify a novel p53-interacting region in CBP/p300, which we call CR2, located near the carboxyl terminus. The 95-amino acid CR2 region (amino acids 2055-2150) is located adjacent to the C/H3 domain and corresponds precisely with the minimal steroid receptor coactivator 1 (SRC1)-interacting domain of CBP (also called IBiD). We show that the region of p53 that participates in the CR2 interaction resides within the first 107 amino acids of the protein. p53 binds strongly to the CR2 domain of both CBP and the highly homologous coactivator p300. Importantly, an in-frame deletion of CR2 within the full-length p300 protein strongly compromises p300-mediated p53 transcriptional activation from a chromatin template in vitro. The identification of the p53-interacting CR2 domain in CBP/p300 prompted us to ask if the human T-cell leukemia virus (HTLV-1) Tax protein, which also interacts with CR2, competes with p53 for binding to this domain. We show that p53 and Tax exhibit mutually exclusive binding to CR2 region, possibly contributing to the previously reported Tax repression of p53 function. Together, these studies identify and molecularly characterize a new p53 binding site on CBP/p300 that participates in coactivator-mediated p53 transcription function. The identity of the p53·CR2 interaction indicates that at least three distinct sites on CBP/p300 may participate in mediating p53 transactivation.

3.2 Introduction

CBP and the highly related protein p300 are very large, highly conserved coactivator proteins that serve to mediate the regulation of gene expression in metazoans. Many transcriptional regulatory pathways converge at CBP and p300 (102, 103, 109, 294). These include pathways required for development and differentiation, response to hormonal stimulation, apoptosis, and tumor suppression. A significant number of transcription factors, such as Mdm2, BRCA1, HTLV-1 Tax, and SRC1, have been demonstrated to interact with CBP/p300, with several binding at multiple sites on the coactivators (335). The functional significance of these multivalent activator/coactivator interactions is currently unknown.

p53 is a sequence-specific, DNA-binding transcription factor that induces apoptosis or cell cycle arrest in response to genotoxic stress, thus blocking the transmission of DNA mutations to progeny cells (172). Loss of p53 activity has been identified in 60% of the human malignancies examined (123, 198), consistent with its critical role in the suppression of malignant transformation. The tumor suppressor functions of p53 are directly linked to its ability to mediate transcriptional activation. To stimulate transcription, p53 binds as a tetramer to specific response elements located in the transcriptional control regions of p53 target genes (172, 197). This step initiates the assembly of the complex transcriptional apparatus that initiates RNA synthesis. This critical early step in transcriptional activation is believed to be facilitated by the ability of p53 to simultaneously bind the specific DNA sequences and recruit CBP/p300 to the p53-responsive promoters. CBP/p300 recruitment appears to concomitantly bring RNA polymerase II to the target promoters (231), increasing the rate of preinitiation complex assembly (358). There is also evidence that, following promoter association, CBP/p300 may also recruit or stabilize components of the general transcription machinery, including TFIIB and TBP (66, 183). CBP/p300 also facilitates transcriptional activation through nucleosome and transcription factor acetylation. The coactivators have been shown to directly acetylate lysine residues present within the amino-terminal tails of the four core histones (279). Acetylation appears to increase the accessibility of the nucleosomal DNA to transcription factor binding, a critical step in gene activation (188, 340). Interestingly, CBP and p300 have also been shown to acetylate p53 at lysine residues 373 and 382 (120). Although acetylated p53 binds short fragments of DNA with a higher affinity than the unacetylated form, this modification does not appear to significantly affect p53 DNA binding activity on chromatin assembled templates (76).

These observations serve to illustrate a prominent role for CBP/p300 in mediating the tumor suppressor functions of p53. However, the molecular details of the physical interaction between the activator and coactivator remain elusive. Several previous studies have indicated that p53 specifically binds to multiple sites on the coactivator, including the KIX domain (334), and an ill-defined carboxyl-terminal region of CBP/p300 (12, 121, 201, 281). The amino-terminal activation domain of p53 has been shown to participate in each of these coactivator interactions (67, 334). In studies that attempted to elucidate the precise carboxyl-terminal region of CBP/p300 involved in p53 binding, only the C/H3 domain of CBP (approximate aa1764-1850; also called TAZ2 and TRAM) has emerged as a site of p53 interaction (235). However, a recent study using heteronuclear NMR methods to monitor the intermolecular interactions between the activation domain of p53 and C/H3 showed that the binding affinity was weak (K_D=300 μM) (67). This result suggests that p53 may make additional contacts within the carboxyl-terminal region of CBP/p300.

In this study, we set out to further characterize the interaction between p53 and the carboxyl-terminal half of CBP/p300. We were interested in determining whether another carboxy terminal site on CBP/p300, alone or in conjunction with C/H3, might account for the observed tight binding of p53 to this region (12, 121, 201, 281). We have identified a new p53-interacting domain on CBP (aa 2055-2150) and p300 (aa 1970-2193), which we have named CR2. This region corresponds precisely with a domain present on both CBP and p300 that is utilized by steroid receptor coactivator 1 (SRC1) in activated transcription by liganded nuclear hormone receptors (159, 177, 291). Furthermore, this region has been shown to be an important interaction site for numerous transcription factors, including IRF-3 and HTLV-I Tax (202, 280). Recently, the solution structure of this domain (IBiD) was solved using heteronuclear NMR, and was shown to be composed of three tightly compacted α-helices (202). A mutation in CBP that resides in the first of the three α-helices in the CR2 region significantly reduces the interaction with p53. We also show that p53 binds to the CR2 domain present in the highly homologous coactivator p300. Importantly, deletion of this region in full-length p300 strongly compromises p53-mediated transcriptional activation in vitro from a template carrying the Mdm2 promoter assembled into chromatin. We identify the first 107 amino acids of p53, which carries the tripartite activation domain, as those involved in the CR2 interaction. Finally, we show that p53 and the HTLV-1 Tax protein compete for interaction with CR2 in vitro, possibly contributing to the previously reported Tax repression of p53 transcription function (3, 227, 253, 334).

3.3 Materials and Methods

3.3a Cloning, expression, and purification of recombinant proteins. The expression and purification of GST-C/H1-KIX$_{aa302\text{-}683}$, GST-KIX$_{aa588\text{-}683}$, GST-CR1$_{aa1514\text{-}1894}$, GST-CR2$_{aa1894\text{-}2221}$, and GST-CR3$_{aa2212\text{-}2441}$ have previously been described (334). The GST-CR2 deletion and point mutants and the CR2 region from human p300 (encompassing aa 1970-2193; corresponding to mouse CBP CR2 aa 2003-2212) have also been previously described (280). These purified proteins were dialyzed against TM buffer (50mM Tris-HCl (pH 7.9), 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA (pH8.0), 1mM dithiothreitol, 0.1% (v/v) Tween-20, 20% (v/v) glycerol) and stored at -70°C. Full-length His_6-tagged p53 and the His_6-tagged double point mutant of p53 (L22Q/W23S) were expressed and purified as previously described (334). Tax was expressed from the $pTaxH_6$ expression plasmid (366), and purified as previously described (101).

In the experiments presented in Fig. 3A, full-length p53 and the amino-terminal fragment of p53 (amino acids 1-107) were transcribed and translated using the TNT Quick-Coupled in vitro transcription/translation system (Promega). Full-length p53 and p53 (aa 1-107) were labeled with [^{35}S]methionine during the in vitro transcription/translation reaction. Because of differences in methionine incorporation (12 methionines in full-length p53 *versus* four methionines in the amino-terminal p53 fragment), we used three times the amount of the amino-terminal fragment of p53 in vitro transcription/translation product (6μl) in the GST pull-down assay. The in vitro transcription/translation products were incubated with 10 pmol of each GST fusion protein. The amino-terminal fragment of p53 (aa 1-107) was cloned by PCR amplification of the full-length, wild-type p53 cDNA (p53-H-19) (123). The PCR product was inserted into the *NdeI/BamHI* site of pET15b (Novagen).

Drosophila core histones were purified as previously described (39). The yeast NAP-1 cDNA (87) was cloned into pGEX-2T (Amersham Biosciences, Inc.), and the GST-yNAP-1 fusion protein was expressed in *Escherichia coli* and purified by glutathione-agarose affinity chromatography and Q-Sepharose. We coexpressed FLAG-tagged ISWI and Acf1 from baculovirus and purified the complex by anti-FLAG affinity batch binding and elution as previously described (145). His$_6$-tagged wild-type p300 and p300ΔSRC proteins were expressed from recombinant baculoviruses and purified as previously described (177).

3.3b GST pull-down assay. All GST pull-down experiments were performed as previously described (334). Anti-p53 (DO-1 (epitope corresponding to aa 11 to 25), Santa Cruz Biotechnology), anti-Tax (epitope corresponding to the 13 carboxyl-terminal amino acids) and anti-His$_6$ (H-15, Santa Cruz Biotechnology) antibodies were used in the GST pull-down experiments.

3.3c Electrophoretic mobility shift assay. The singly-end labeled p53 consensus site double-stranded oligonucleotide probe (0.4 nM) was incubated with purified His$_6$-p53 (0.15 μM), and increasing amounts of GST-CR1$_{aa1514-1894}$, GST-CR2$_{aa2055-2150}$, GST-CR3$_{aa2212-2441}$, or GST-KIX$_{aa588-683}$ protein (0.20 μM, 0.50 μM, 0.65 μM) for 45 min on ice as previously described (101). Protein·DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel.

3.3d Transcription template. The p53-responsive Mdm2 P2 G-less plasmid DNA used in the assembly reactions carried the two p53 response elements from the Mdm2 P2 intragenic promoter (349). Briefly, a 567 bp fragment carrying the p53 response elements, TATA sequence and start site was PCR-amplified and cloned immediately upstream of a 190-bp G-less cassette. The identity of the Mdm2 P2 G-less construct was confirmed by restriction analysis.

3.3e Chromatin assembly and topological assay. Nucleosomes were assembled on the Mdm2 P2-G-less plasmid as previously described (96, 144). Following the addition of the DNA, ATP (3 mM), creatine phosphokinase (1 μg/ml), and phosphocreatine (30 mM) were added in a 70-μl reaction containing 10 mM HEPES (K^+) (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, and 5% (v/v) glycerol. Briefly, histone octamers were preassembled with GST-yNAP-1 (8:1 GST-yNAP-1/core histones) on ice for 30 min. Assembly reactions were incubated for 2 h at 27°C. DNA topological assays were performed as previously described (96). The samples were analyzed on a 1% agarose gel, and the degree of supercoiling was visualized by Sybr Gold (Molecular Probes) staining.

3.3f In vitro transcription assay. The supercoiled Mdm2 P2 G-less plasmid was assembled into chromatin using GST-yNAP-1, Acf1/ISWI, and *Drosophila* histones, at a 1.1:1.0 histone:DNA ratio. Following chromatin assembly, preinitiation complexes were formed on the equivalent of 200 ng of the plasmid DNA in the absence or presence of p53 (160 nM), p300 (20 nM), and/or p300ΔSRC (20 nM). All reactions contained 100 μM acetyl CoA (United States Biochemical). Nuclear extract (70 μg) (73), prepared from CEM cells (a mutant p53 human T lymphocyte cell line), was added immediately following the addition of the activator and/or coactivator. Following a 60-min preincubation reaction at 30°C, RNA synthesis was initiated by the addition of 250 μM ATP, GTP, CTP, and 12 μM UTP plus 0.8 μM [^{32}P]UTP (3000 Ci/mmol, PerkinElmer

Life Sciences). Transcription reactions were processed and analyzed as previously described (193). Molecular weight markers (radiolabeled *Hpa* II-digested pBR322) were used to estimate the size of the RNA products.

3.3g p300 acetylation assay. The acetyltransferase activity of p300 and p300ΔSRC was assayed by incubation of p53 (235 nM) with p300 (30 nM) or p300ΔSRC (30 nM) and [^{14}C]acetyl CoA (100 pmol, 57mCi/mmol, Amersham Biosciences, Inc.) in a solution containing 50 mM Tris (pH 8), 10% glycerol, 10 mM sodium butyrate, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated at 30°C for 60 min, proteins were separated by 18% SDS-PAGE and fixed, and acetylation was visualized by PhosphorImager analysis.

3.3h Mammalian expression plasmids, cell culture, and transient cotransfection assays. Jurkat T-cells (a p53-negative human T lymphocyte cell line) were cultured in Isocove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, and penicillin-streptomycin. For transient cotransfection assays, cells were grown to a density of 10^6 cells/ml and transfected with Lipofectamine (Invitrogen) and a constant amount of DNA for 5 h. The cells were allowed to recover for 19 h before harvest. Cells were lysed, and luciferase activity was measured using the Dual-Luciferase reporter assay system with a Turner Designs model TD 20-e luminometer. Luciferase activity was normalized to pRL-TK vector (Promega), which encodes the *Renilla* luciferase from the HSV-TK promoter, as an internal control.

Expression plasmids for p53 (pC53-SN3) (14), CMV-CR2 (280), and pRC/RSV-CBP (208) have already been described. The luciferase reporter plasmid pG13-Luc (165) has also been described.

3.4 Results

3.4a Identification of the p53-interacting CR2 region of CBP by GST pull-down assay

We began this study by testing three large regions of CBP spanning the carboxyl-terminal half of the coactivator (Fig. 3.1.A). Each of these CBP regions were cloned and expressed as GST fusion proteins and tested in GST pull-down assays with purified, recombinant, full-length p53. p53 binding to the KIX domain (aa 588-683) served as a positive control (Fig. 3.1.B, lane 6). We found that p53 bound strongly to only one of the three carboxyl-terminal regions of CBP (Fig. 3.1.B, lane 4). This region, which we call carboxyl-terminal region 2 (CR2), encompasses CBP amino acids 1894-2221. Consistent with previous studies (67, 235), we found that p53 also bound to C/H3 (TRAM/TAZ2), because our carboxyl-terminal region 1 protein (CR1, aa 1514-1894) encompasses this domain (Fig. 3.1.B, lane 3; see Fig. 3.1.A). However, p53 binding to this region was significantly less than that observed with either CR2 or KIX in our GST pull-down assay. These data indicate that, at least in our assay, p53 interacts most strongly with the region of CBP, encompassing amino acids 1894-2221. This observation was confirmed using the yeast two-hybrid assay (see supplemental Fig. 3.7). Interestingly, the CR2 domain (aa 1894-2221) corresponds closely to the steroid receptor coactivator 1 (SRC1)-interacting domain of CBP (aa 1982-2163) (159, 291, 356). SRC1 is a prominent member of a family of coactivators that utilize CBP/p300 to mediate transcriptional activation of nuclear hormone receptors (106, 196).

CBP and p300 are highly homologous proteins, while their precise role in the regulation of gene expression mediated by either protein is unclear. Because CBP and

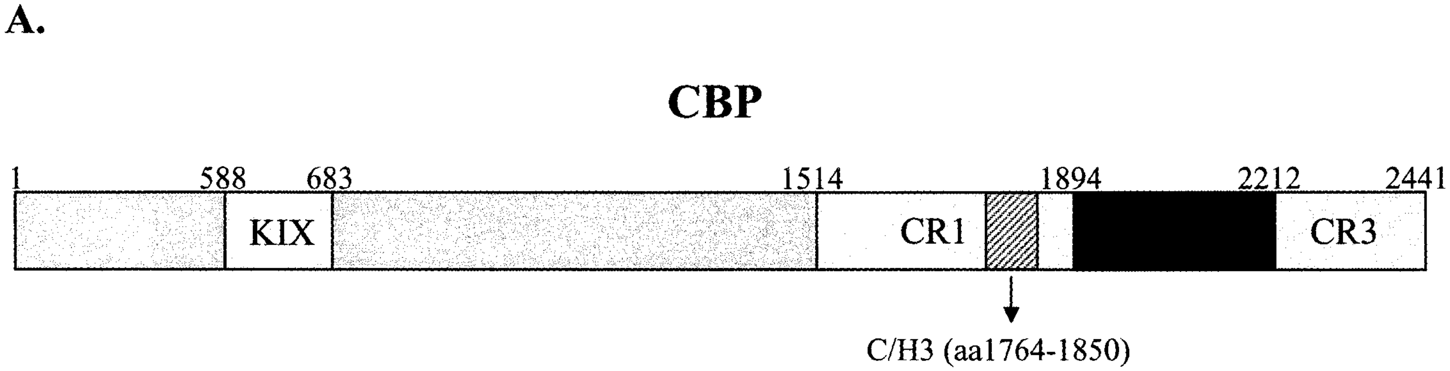


Figure 3.1. **p53 binds strongly to the CR2 domain of CBP.** (A) Schematic representation of the 2,441-amino acid cellular coactivator CBP. The regions tested for p53 interaction are indicated.

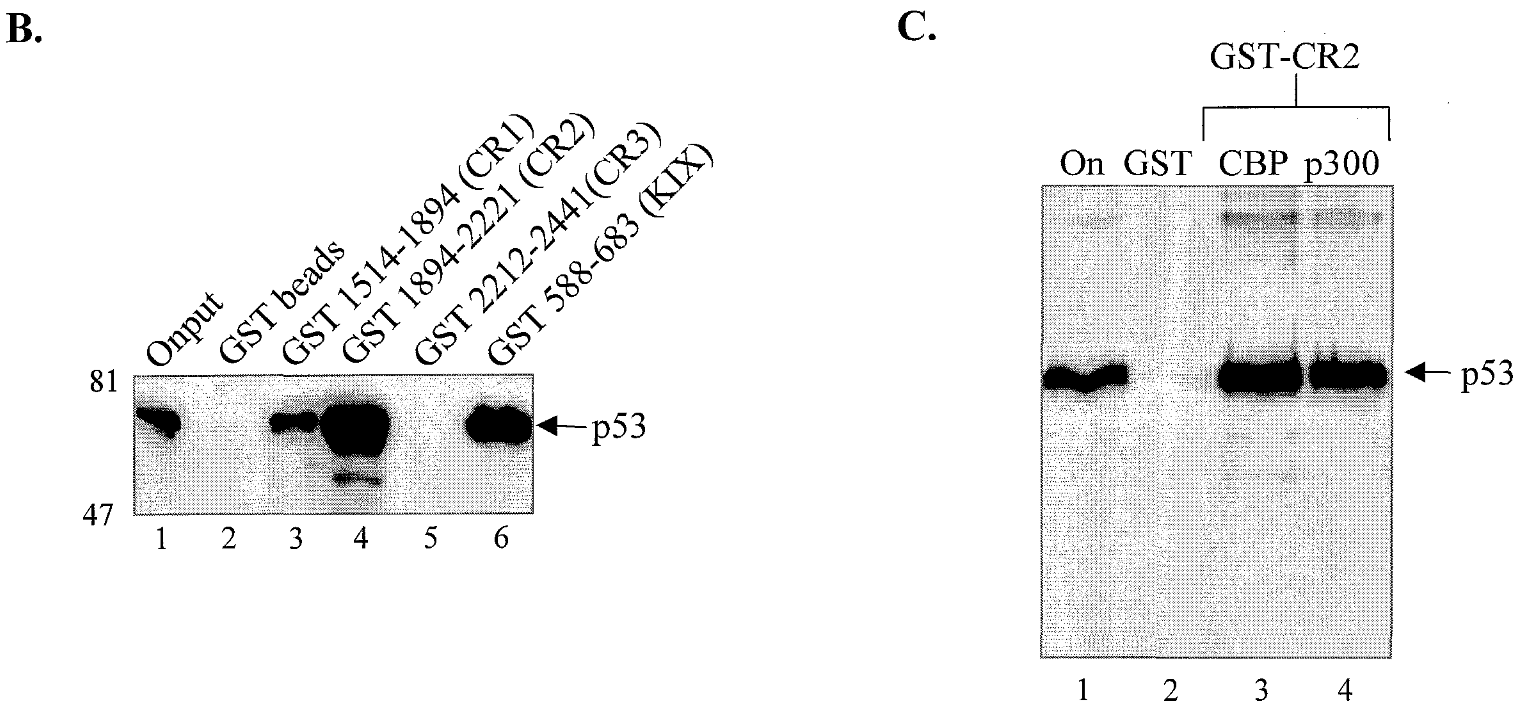


Figure 3.1. (B) p53 binds to the CR2 domain in vitro. Full-length, purified, recombinant p53 (10 pmol) was incubated with GST alone (lane 2) or the indicated GST-carboxyl-terminal region fusion proteins (10 pmol each) (lanes 3-5). As a positive control, we also tested p53 binding to GST-KIX$_{aa588\text{-}683}$ (lane 6). p53 was detected using an anti-p53 antibody. Onput p53 (5%) is shown (lane 1). Bound p53 and protein molecular weight standards are indicated. (C) p53 binds equally well to the CR2 domains derived from CBP and p300. Purified p53 (20 pmol) was incubated with GST alone or the GST-CR2 region from CBP (aa 2003-2212) or p300 (aa 1970-2193) (20 pmol each). p53 was detected using an anti-p53 antibody. Onput p53 (5%) is shown (lane 1). Bound p53 is indicated.

p300 share roughly 50% homology within the CR2 region, we were interested in testing whether p53 also recognizes the CR2-like domain found in p300. To address this question, we cloned the p300 CR2 region (aa 1970-2193) fused to GST and tested p53 binding in a GST pull-down assay. Figure 3.1.C shows that p53 binds comparably to the CR2 regions from both CBP and p300 (lanes 3 and 4).

3.4b Fine mapping and mutational analysis of the minimal p53-interacting region of CR2

We were next interested in mapping the minimal region of CR2 competent for p53 interaction. For these studies, we analyzed p53 binding to a series of deletion mutants of CR2 using the GST pull-down assay. Progressive carboxyl-terminal deletions of GST-CR2 revealed that amino acid 2150 represents the carboxyl-terminal border competent for wild type interaction with p53 (GST-CR2$_{aa1894-2150}$) (Fig. 3.2.A, lanes 3-6). Progressive amino-terminal deletions of CR2 revealed that amino acid 2055 represents the amino-terminal border competent for wild type interaction with p53 (GST-CR2$_{aa2055-2150}$) (Fig. 3.2.A, compare lane 3 with lanes 7-10). These data show that the minimal region of CR2 competent for interaction with p53 resides within a 95-amino acid fragment, bordered by residues 2055 and 2150 (Fig. 3.2.A, lane 10). This region precisely overlaps with the minimal CBP sequence (aa 2058-2130) required for interaction with SRC-1 (291).

To identify critical amino acids within CR2 responsible for interaction with p53, we prepared and characterized a series of double point mutations. The amino acids targeted for mutagenesis were chosen based on conservation between CBP and p300, as well as conservation between the mouse and human CBP. We targeted specific leucine residues

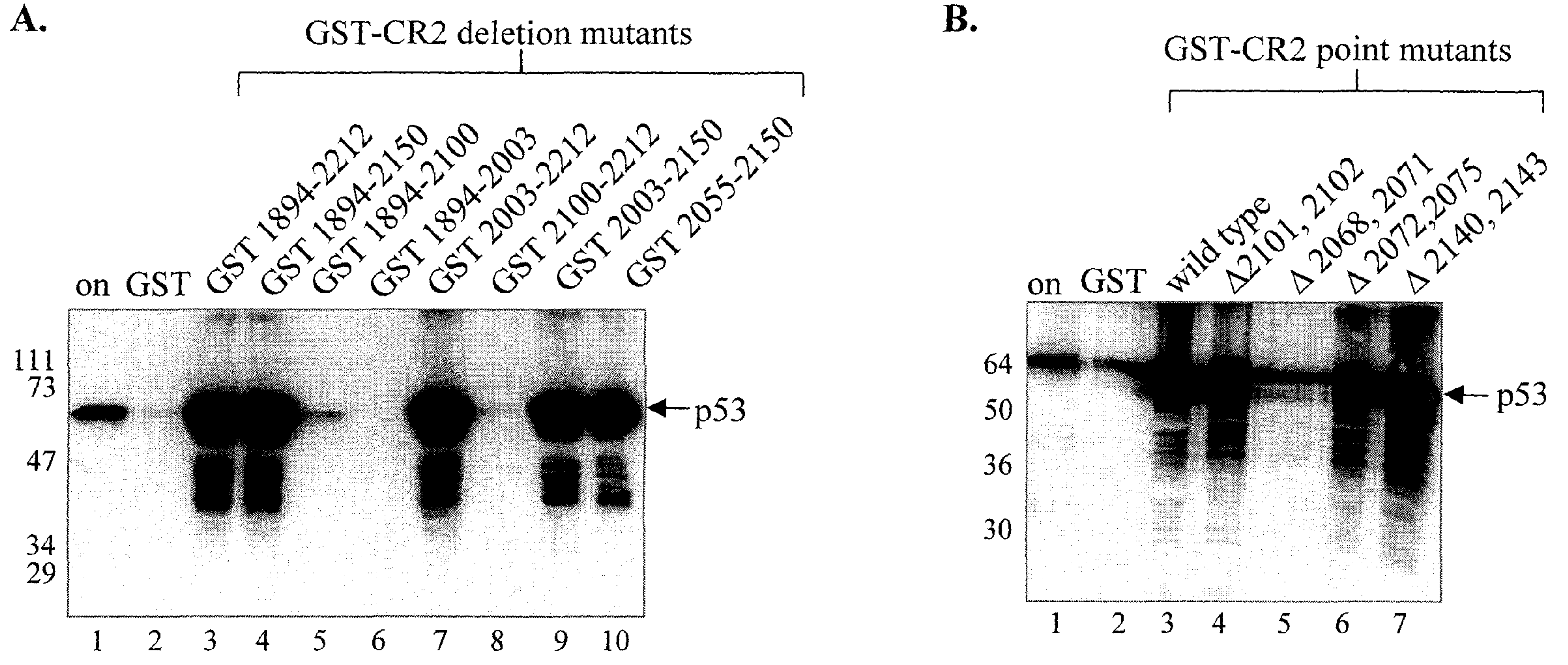


Figure 3.2. **Identification of the minimal region of CR2 competent for p53 binding.** (A) p53 interacts with amino acids 2055-2150 of CBP in vitro. Purified p53 (10 pmol) was incubated with GST alone (lane 2) or the indicated GST-CR2 deletion mutants (10 pmol) (lanes 4-10). As a positive control, p53 binding to full-length GST-CR2$_{aa1894-2221}$ was also tested (lane 3). p53 was detected using an anti-p53 antibody. Onput p53 (5%) is shown (lane 1). Bound p53 and protein molecular weight standards are indicated. (B) p53 is defective for an interaction with the CR2 double point mutant L2068A/L2071A. Purified p53 (25 pmol) was assayed for its ability to bind to GST alone or the GST-CR2$_{aa2003-2212}$ double point mutants: F2101A/I2102A, L2068A/L2071A, L2072A/L2075A, or L2140A/L2143A. (25 pmol). p53 binding to wild-type GST-CR2$_{aa2003-2212}$ was tested as a positive control (lane 3). Onput p53 (5%) is shown (lane 1). Bound p53 and protein molecular weight standards are indicated.

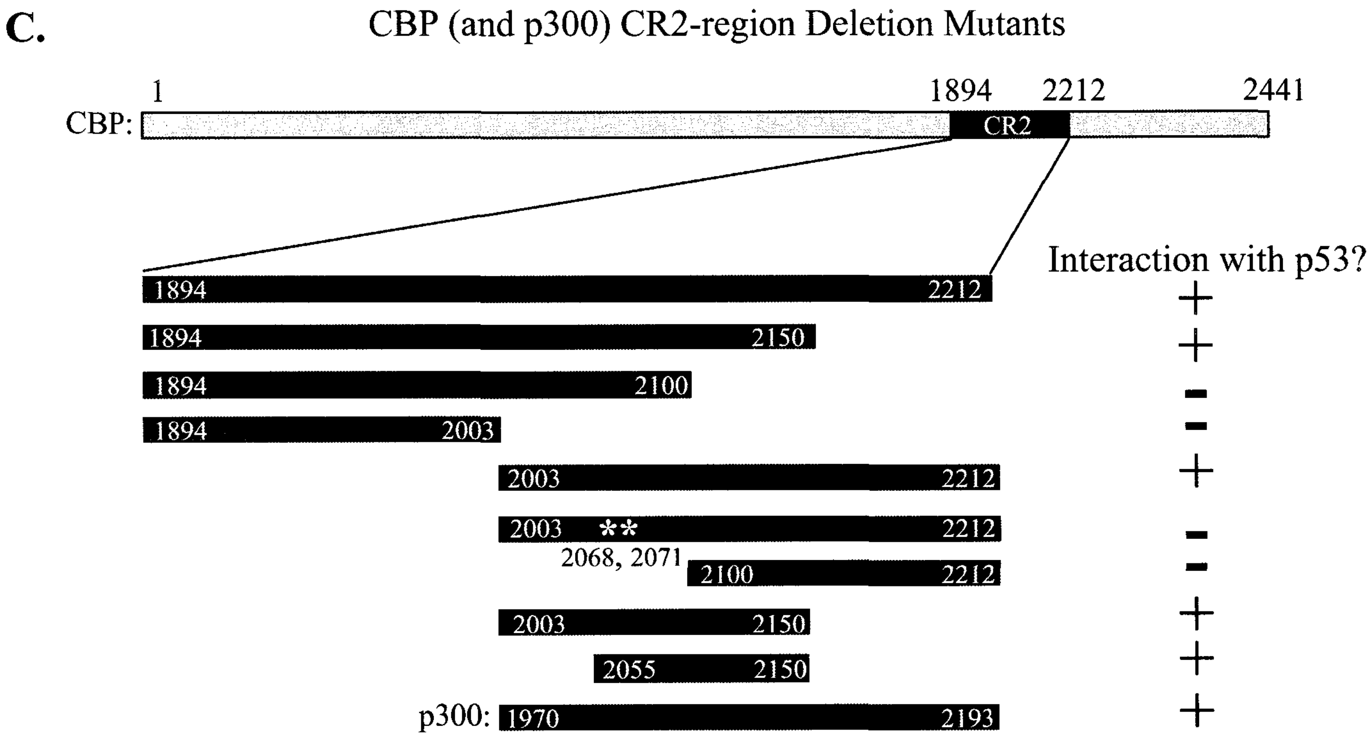


Figure 3.2. (C) Summary of the p53•CR2 interactions.

within a region that forms amphipathic α-helices (and thus, possibly, protein·protein contacts). The selected residues were changed to alanines, to minimize effects on secondary and tertiary structure. Four $CR2_{aa2003-2212}$ constructs were prepared, each carrying two point mutations as follows: F2101A/I2102A; L2068A/L2071A; L2072A/L2075A; L2140A/L2143A. Figure 3.2.B shows that only the double point mutant L2068A/ L2071A, which resides within the first of the three α-helices, had a significant effect on p53 binding (lane 5). These data provide further evidence for the specificity of the p53·CR2 interaction. A summary of the p53 interactions with the various CR2 constructs from CBP and p300 is shown in figure 3.2.C.

3.4c Fine mapping of the minimal CR2-interacting region of p53

Preliminary yeast two-hybrid studies suggested that the site of CBP interaction resides within the first 112 amino acids of p53 (see supplemental Fig. 3.7). Based on this observation, we performed GST pull-down assays using an amino-terminal fragment of p53. In vitro transcribed-translated ^{35}S-labeled full-length p53 and a ^{35}S-labeled amino-terminal truncation of p53 (aa 1-107) were tested for their ability to bind the CR2 domain of CBP. Glutathione beads were bound with the GST-$CR2_{aa1894-2221}$ or GST-C/H1-$KIX_{aa302-683}$ and then incubated with the ^{35}S-labeled in vitro translation products, and the resulting protein·protein interactions were detected by PhosphorImager analysis. Figure 3.3.A shows that both the full-length and the amino-terminal p53 fragment binds to CR2 (lanes 5 and 8). Although the binding of the amino-terminal truncation fragment to CR2 is clearly specific, the binding appears to be reduced relative to the full-length protein, possibly because the amino-terminal domain in isolation is not structurally identical to the analogous region in the full-length protein. This result is consistent with the

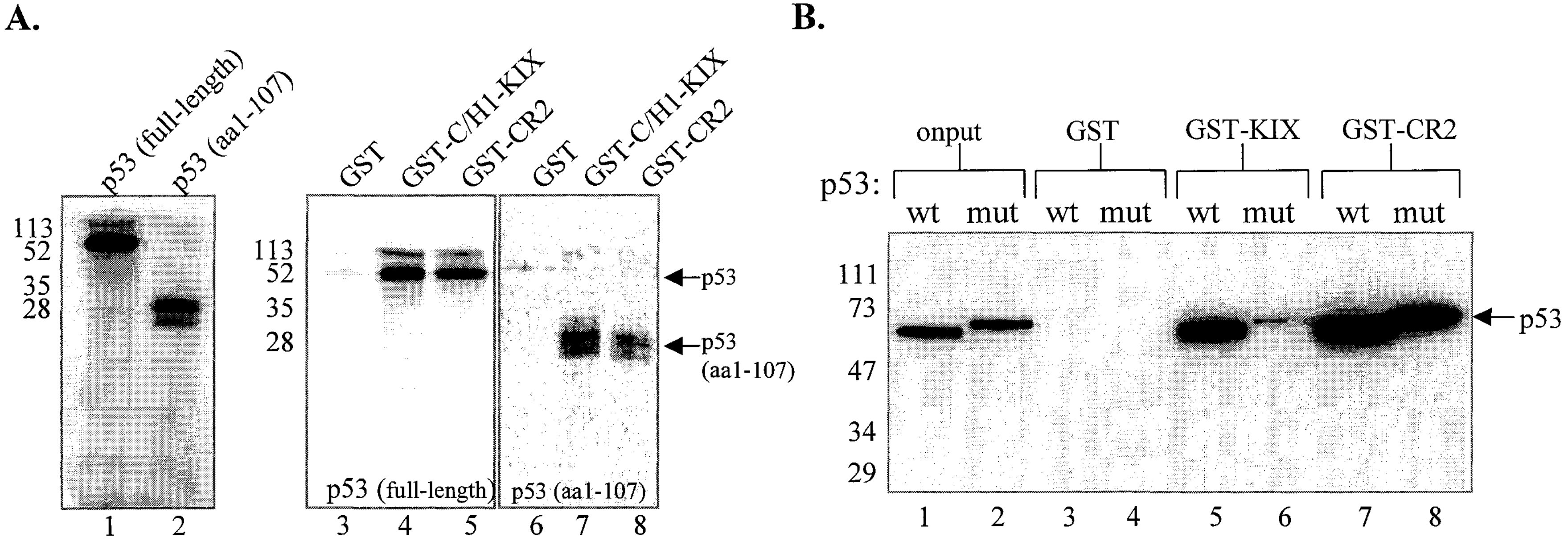


Figure 3.3. **Identification of the minimal domain of p53 responsible for CR2 binding.** (A) The amino terminus of p53 (aa 1-107) is competent for CR2 binding. The full-length p53 ^{35}S-labeled in vitro translation product (lane 1) was incubated with GST alone (lane 3), GST-C/H1-KIX$_{aa302\text{-}683}$ (lane 4), or GST-CR2$_{aa1894\text{-}2221}$ (lane 5). The p53 (aa 1-107) ^{35}S-labeled in vitro translation product (lane 2) was incubated with GST alone (lane 6), GST-C/H1-KIX$_{aa302\text{-}683}$ (lane 7), or GST-CR2$_{aa1894\text{-}2221}$ (lane 8). Bound p53 and protein molecular weight standards are indicated. The resulting protein·protein interactions were detected by PhosphorImager analysis. (B) The p53 activation domain double point mutant, L22Q/W23S, is competent for CR2 binding. Purified wild type p53 or activation domain mutant p53 (15 pmol) was incubated with GST alone (lanes 3 and 4), GST-KIXaa588-683 (lanes 5 and 6), or GST-CR2aa2055-2150 (lanes 7 and 8) (30 pmol). p53 was detected using an anti-His6 antibody. Onput wild type and mutant p53 proteins (5%) are shown (lanes 1 and 2). Bound p53 and protein molecular weight standards are indicated.

observation that amino and carboxyl-terminal interactions in p53 are important for p53 function (226).

To determine whether a previously characterized minimal activation domain of p53 may be involved in the interaction with CR2, we introduced a double point mutation (L22Q/W23S) into this region (334) and tested the ability of the purified mutant protein to bind the minimal CR2 domain (aa 2055-2150). Mutation of these residues has previously been shown to have a dramatic effect on p53 transcription function (203). Figure 3.3.B shows the results of a GST pull-down assay where we tested the binding of purified wild type and mutant p53 proteins to both CR2 and KIX. Surprisingly, the double point mutation in this minimal p53 activation domain did not have a significant effect on p53 binding to the CR2 domain (Fig. 3.3.B, lanes 7 and 8). As we have previously reported, the double point mutations did significantly reduce p53 binding to the KIX domain (Fig. 3.3.B, lanes 5 and 6) (334). These data suggests that other amino acids in the p53 tripartite activation domain likely participate in CR2 binding.

3.4d EMSA studies on the p53·CR2 interaction

As an alternate method to characterize the p53·CR2 interaction, we utilized the electrophoretic mobility shift assay (EMSA). We were interested in determining whether $CR2_{aa2055-2150}$ could form a ternary complex with p53 bound to its consensus DNA recognition element. Figure 3.4 shows that titration of the purified CR2 domain into p53-containing binding reactions decreased the mobility of the p53·DNA complex (lanes 5-7 and 12-14). The change in mobility suggested that CR2 was stably incorporated into the complex. Interestingly, we did not observe a change in the mobility of the p53·DNA complex in the presence of increasing amounts of the C/H3-containing CR1 domain (Fig.

3.4, lanes 2-4). The CR3 domain also had no effect on the migration of the p53·DNA complex, consistent with our previous observations (Fig. 3.4, lanes 8-10). As a positive control, we titrated the KIX domain of CBP into the p53·DNA binding reactions, and compared the ternary complex formation with that observed with CR2. Figure 3.4 shows that both CR2 and KIX similarly decreased the mobility of the p53·DNA complex (lanes 12-17). The specificity of the DNA binding activity of p53 was confirmed by competition assays using the p53 consensus sequence and antibody supershift assays (see supplemental Fig. 3.8). Finally, CR2, as well as the other CBP domains, did not bind DNA in the absence of p53 (see supplemental Fig. 3.9). The EMSA studies presented here were performed with unacetlylated p53, as we have observed no significant differences in the DNA binding activity, or CR2 binding activity, between the CBP/p300-acetylated and unacetylated forms of the protein (see supplemental Fig. 3.8).

3.4e Functional significance of the p53·CR2 interaction in vitro and in vivo

To test whether the p53·CR2 interaction participated in CBP/p300-mediated p53 transcriptional activation, we examined p53 transcription function in the presence of exogenous wild type p300 or a mutant form of p300 that carries a deletion of the SRC1 domain (177). We selected p300 for these studies, because p53 interacts similarly with the CR2 region of both CBP and p300, and p300 coactivator function has been well characterized in vitro (76, 96, 176, 177, 179). To measure coactivator-mediated p53 transcriptional activation, we used a DNA template containing a 567-bp fragment from the Mdm2 intragenic P2 promoter, driving synthesis of a 190-nucleotide guanine-less transcript. This Mdm2 P2 fragment carries two p53 binding sites upstream of the core promoter (157). We chose to analyze transcription in a chromatin context, because several studies have found that analysis of p300 coactivator function *in vitro* requires nucleosomal templates (76, 96, 176, 179). Chromatin assembly of the p53-responsive G-less template was performed using the recombinant *Drosophila* assembly proteins Acf1/ISWI, GST-yNAP-1, and purified *Drosophila* core histones, as previously described (145). These assembly proteins are sufficient for the ATP-dependent formation of evenly spaced nucleosomal arrays (143, 145). Figure 3.5.A shows a DNA topological analysis demonstrating the assembly of native *Drosophila* core histones onto the p53-responsive G-less template (lanes 3-9). In the presence of the assembly factors, increasing ratios (w/w) of the core histones to the DNA produced a concomitant increase in DNA supercoiling, indicating that nucleosomes were deposited onto the template. The figure shows that a histone/DNA ratio of 1.1:1.0 (w/w) fully assembled the DNA template into chromatin (lane 9); this ratio was used in subsequent in vitro transcription assays.

We performed in vitro transcription assays on this p53-responsive chromatin template using nuclear extracts from CEM cells (a mutant p53 human T lymphocyte cell line) as a source of basal transcription factors and RNA polymerase. All experiments were performed in the presence of acetyl CoA, and in the presence or absence of exogenous p53 and/or p300 or p300ΔSRC. The activator, coactivators, and nuclear extract were added following chromatin assembly. We used unacetylated p53 in this experiment, as a recent study has shown that the unacetylated form of p53 is sufficient for in vitro transcription from a chromatin assembled template (76). Figure 3.5.B shows that the addition of purified recombinant p53 alone did not activate transcription from the Mdm2 promoter (lane 3). However, addition of purified recombinant p300 together with

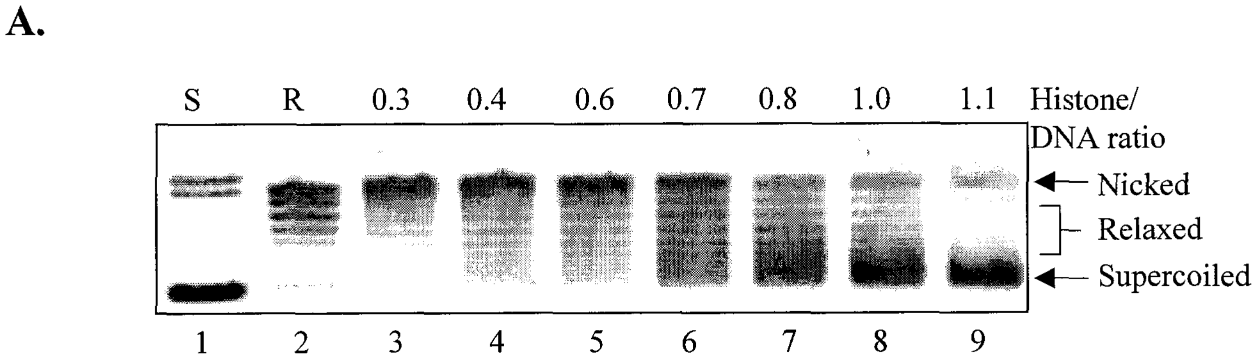


Figure 3.5. **p53-mediated transcriptional activation requires the CR2 domain of p300 in vitro and in vivo.** (A) One-dimensional DNA topological assays showing the Mdm2 P2 G-less transcription template assembled with *Drosophila* core histones in the presence of dAcf1/ISWI and GST-yNAP-1. The DNA topoisomers were resolved on an agarose gel, and the DNA stained with Sybr Gold (Molecular Probes). The supercoiled (S), relaxed (R), and nicked DNA populations, and the histone/DNA ratio, are indicated.

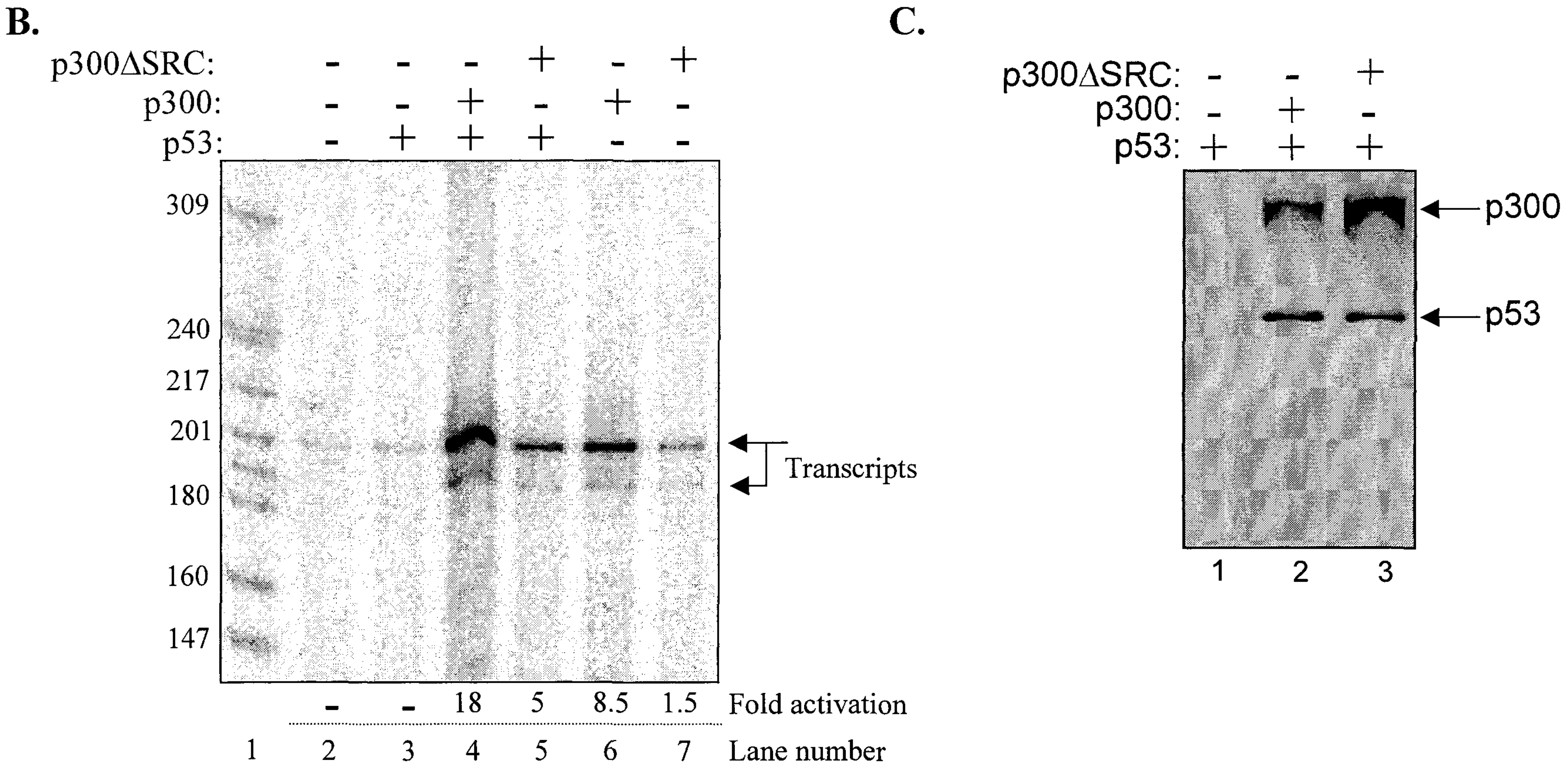


Figure 3.5. (B) Transcriptional activation on Mdm2 P2 G-less chromatin templates was analyzed in the presence of p53 (160 nM, lanes 3-5), wild-type p300 (20 nM, lanes 4, 6), and/or p300ΔSRC (20 nM, lanes 5, 7). Molecular weight size markers, recovery standard, and full length G-less transcripts are indicated. The fold activation indicated was calculated relative to transcription in the presence of exogenous p53 (lane 3). (C) Wild-type p300 and p300ΔSRC acetylation of recombinant p53. p53 (235 nM) was acetylated by p300 (lane 2), and p300ΔSRC (lane 3) (30 nM each) in the presence of [^{14}C]acetyl CoA (100 pmol, 57mCi/mmol). p53 acetylation and p300 autoacetylation are indicated.

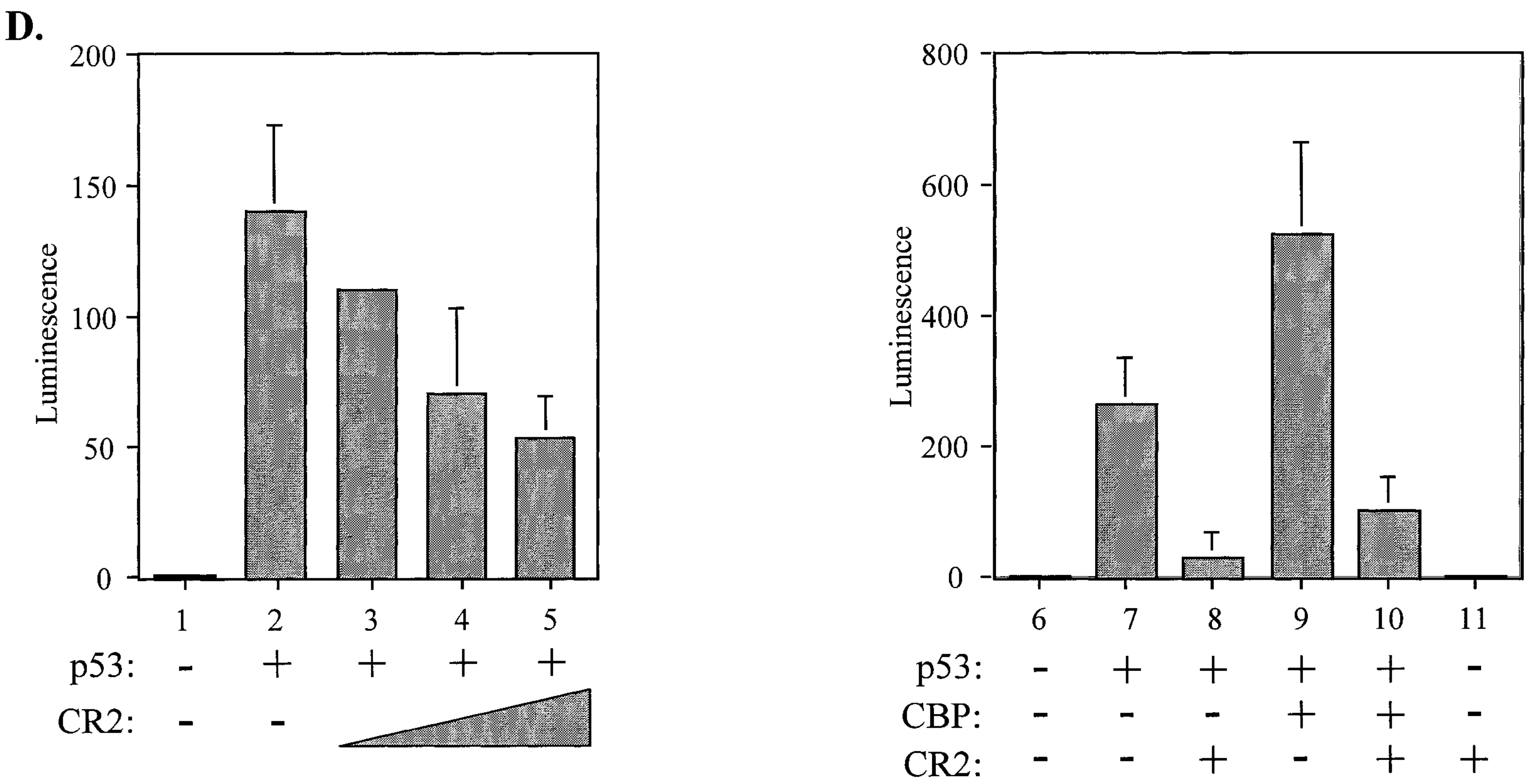


Figure 3.5. (D) The CR2 domain represses p53-activated transcription in vivo. In the *left panel*, the p53 responsive pG13-Luc reporter plasmid (400 ng) was cotransfected with an expression plasmid for p53 (200 ng, lane 2-5), and increasing amounts of an expression plasmid for CMV-CR2 (200, 400, and 800 ng respectively, lanes 3-5). In the *right panel*, the p53 responsive pG13-Luc reporter plasmid (400 ng) was cotransfected with an expression plasmid for p53 (200 ng, lanes 7-10). p53 transactivation was assayed in the presence or absence of expression plasmids for full-length CBP (400 ng, lanes 9 and10) and CMV-CR2 (800 ng, lanes 8 and 9), as indicated. As a control, cotransfection of an expression plasmid for CMV-CR2 in the absence of p53 is shown (lane 11). The values shown are the mean -fold activation (in triplicate) +/- S.D..

p53 produced a significant increase in RNA synthesis from the Mdm2 promoter (18-fold, Fig. 3.5.B, lane 4). Under these same conditions, addition of p300ΔSRC, which carries an in-frame deletion of CR2 (aa 2042 to 2157), activated transcription only 5-fold from these templates; a 3.6-fold reduction in p300 coactivator function (Fig. 3.5.B, lane 5). The absence of p53 reduced both wild type and mutant p300-stimulated transcription, indicating that optimal coactivator function required the presence of p53 (Fig. 3.5.B, lanes 6 and 7). Figure 3.5.C demonstrates that both the wild type and mutant p300 proteins similarly acetylate p53, confirming that both proteins were equivalently functional with respect to acetyltransferase activity. Furthermore, p300ΔSRC is fully functional for acetylation of free histones as well as nucleosomal core histones (177).

Finally, to determine the functional role of the p53·CR2 interaction in vivo, we examined p53 transcription activity in transient transfection assays in p53-negative Jurkat T-cells in the presence of an expression plasmid for CR2 (CMV-CR2). Because CR2 does not have intrinsic activation properties, p53 binding to free CR2 should block the p53 interaction with endogenous (or transfected) CBP/p300 and therefore have a dominant negative effect on p53 transcriptional activity. The *left panel* of figure 3.5.D shows that titration of the expression plasmid for CR2 repressed p53 transcriptional activation in a dose dependent manner (lanes 3-5). We also measured the effect of CR2 on p53 transcriptional activation in the presence of an expression plasmid for CBP. The *right panel* shows that in the presence of cotransfected full-length CBP, CR2 again repressed p53-mediated transcription. As expected, the presence of the CBP expression plasmid partially rescued the observed CR2 repression (Fig. 3.5.D, compare lanes 8 and 10). Addition of either the CR2 or the CBP expression plasmids in the absence of p53 had no effect on pG13-luc reporter activity (lane 11, see supplemental Fig. 3.10). These data support a role for the CR2 domain of CBP/p300 in p53 transcription function in vivo.

3.4f HTLV-1 Tax and p53 compete for CR2 binding in vitro

Several studies have previously reported that the human T-cell leukemia virus Tax protein represses p53 transcription function (3, 227, 253, 334). Several recent studies suggest that this transcriptional repression may occur as consequence of direct competition for binding to common regions of CBP/p300, thus compromising p53 promoter recruitment of the coactivator (10, 190, 314, 334). Recently, we reported that the HTLV-1 Tax protein binds to the CR2 domain of CBP and p300, and identified CBP aa 2003 to 2212 as the minimal region competent for interaction with Tax (280). Based on these observations, we hypothesized that the binding of Tax and p53 to CR2 might be mutually exclusive. To directly test this hypothesis, we examined whether increasing concentrations of purified recombinant p53 can displace Tax from CR2 in vitro. Glutathione beads were bound with GST-CR2$_{\text{aa2003-2212}}$ then incubated with a constant amount of Tax and increasing amounts of p53. The resulting protein·protein interactions were detected by Western blot analysis using a solution containing antibodies against both Tax and p53. Figure 3.6 shows that increasing amounts of p53 reduced Tax binding to CR2, with a concomitant increase in p53 binding (lanes 3-5). This observation was corroborated in the reciprocal experiment, where increasing concentrations of Tax similarly displaced p53 from CR2 (Fig. 3.6, lanes 7-9). This result is consistent with our observations that both Tax (280) and p53 bind to a similar, overlapping minimal domain

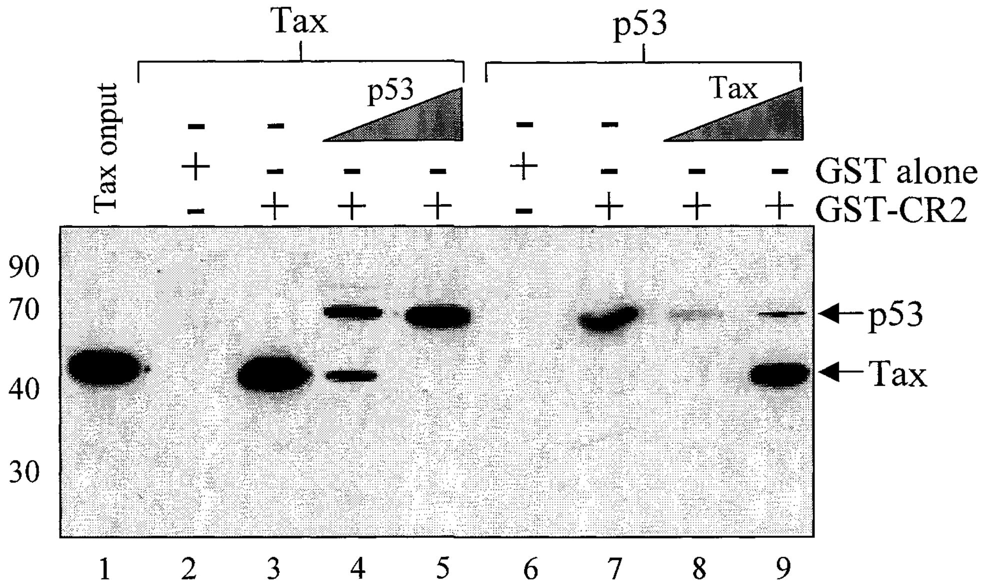


Figure 3.6. **Tax and p53 binding to CR2 is mutually exclusive.** A constant amount of purified Tax (25 pmol) was incubated with GST alone, or GST-CR2 $_{\text{aa2003-2212}}$ (25 pmol each) in the presence of increasing amounts of purified p53 (25 and 100 pmol, lanes 4 and 5). In the reciprocal experiment, a constant amount of p53 (25 pmol) was incubated with GST alone, or GST-CR2 $_{\text{aa2003-2212}}$ (25 pmol each) in the presence of the increasing amounts of purified Tax (25 and 100 pmol, lanes 8 and 9). Bound proteins were detected by Western blot analysis using both anti-Tax and anti-p53 antibodies. Bound Tax, bound p53, and protein molecular weight standards are indicated.

of CR2 (aa 2003-2212 and aa 2055-2150, respectively), and that the CR2 double point mutant L2068A/ L2071A reduces interaction with both proteins.

3.5 Discussion

In this report, we show that p53 interacts strongly with the carboxyl-terminal region 2 (CR2) of CBP, located between amino acids 2055 and 2150. We also demonstrate that p53 interacts with the corresponding CR2 region of p300, located between amino acids 1970 and 2193. The CR2 region is distinct from the C/H3 domain, the only previously identified region within the carboxyl-terminal half of CBP that has been shown to interact with p53 (67, 235). In our assays, p53 interacted more strongly with CR2 than with the region of CBP that encompasses the C/H3 domain (CR1; aa 1514-1894). We mapped the minimal CR2 region of CBP required for strong interaction with p53 to amino acids 2055-2150. This 95-amino acid minimal CR2 sequence corresponds precisely with the SRC1-interacting domain of CBP, which has been mapped to amino acids 2058-2130 (291). This domain also corresponds to the CBP region involved in binding to IRF-3 and HTLV-1 Tax (202, 280). We show that a CR2 double point mutation (L2068A/ L2071A), which specifically disrupts the first of the three α-helices that resides within this region (202), reduces interaction with p53.

The amino-terminal 107 amino acids of p53 at least partially participate in protein·protein interaction with CR2. This is consistent with our observation that CR2 binds well to full-length p53·DNA complexes, suggesting that the DNA binding and tetramerization domains are not involved in CR2 recognition. Previous studies have indicated that the p53 activation domain participates in binding to the KIX domain (334) and C/H3 domain of CBP (121, 281). We tested whether a minimal region of the p53 activation domain might interact with CR2 using a double point mutant of p53 (L22Q; W23S). Although we did not observe a significant decrease in the CR2·p53 interaction using this mutant, the activation domain of p53 is tripartite, and extends through the first 100 amino acids of the protein. Therefore, other amino acids that reside within this amino-terminal region of p53 likely participate in CR2 complex formation.

Our in vitro transcription studies clearly show that p53 interaction with the p300 CR2 domain is relevant to p53 transcription function. The addition of p300 and p53 strongly stimulated RNA synthesis from the p53-responsive Mdm2 P2 promoter assembled into chromatin. However, the p300 deletion mutant p300ΔSRC was significantly reduced in its ability to mediate coactivator function. Our observation that p300ΔSRC retained partial coactivator function in p53-mediated transcription may reflect the ability of p53 to recruit CBP/p300 to the Mdm2 promoter via interaction with other coactivator domains (such as KIX and/or C/H3) (116, 117, 121, 235, 281, 334). The in vitro transcription result was corroborated using transient transfection assays, confirming a functional role for the CR2 domain in mediating p53 transcription function in vivo.

Previous studies have shown that the HTLV-1 Tax protein inhibits many of the tumor suppressor functions of p53 (3, 45, 93, 223, 227, 253). Several recent studies suggest that this may occur through competition for CBP/p300 (10, 190, 314, 334). We have recently shown that, like p53, Tax also recognizes the CR2 region of CBP/p300 (280), raising the possibility that both Tax and p53 bind mutually exclusively to this region. Using a competition binding assay, we directly show that Tax specifically disrupts the p53·CR2 interaction, providing further evidence for coactivator competition between these two proteins. It appears that both proteins recognize the same surface structure of CR2, as p53 and Tax are unable to bind the CR2 domain that harbors the double point mutation (L2068A/ L2071A) (shown in Fig. 3.2.B, and 280). Together, these data provide further evidence for a model of Tax repression of p53 transcription function mediated through direct competition, at multiple sites, for CBP/p300. This coactivator competition between Tax and p53 may contribute to the molecular mechanism of HTLV-1-associated malignant transformation.

3.6 Acknowledgments. We thank J.T. Kadonaga for gifts of reagents including Acf1, ISWI, *Drosophila* embryos, and yNAP-1. We also thank W. Lee Kraus for the p300 constructs, as well as Andy Vendel and Melissa Gonzales for their initial work on this project. This work was supported by a Public Health Service grant CA-55035 from the NCI, National Institutes of Health (to J.K.N).

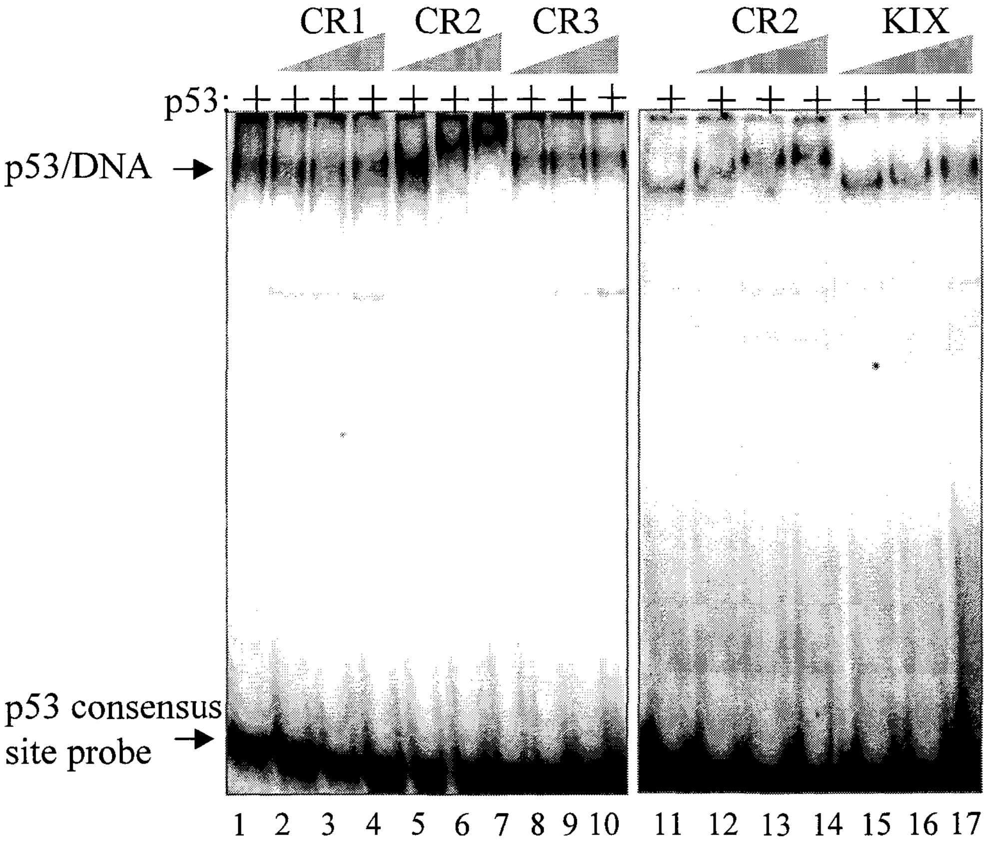


Figure 3.4. **Characterization of CR2 binding to the p53·DNA complex.** Purified, recombinant p53 (0.15 μM) was incubated with a p53 consensus site probe together with increasing amounts of GST-CR1$_{aa1514-1894}$, GST-CR2 $_{aa2055-2150}$, GST-CR3 $_{aa2212-2441}$, or GST-KIX $_{aa588-683}$ protein (0.20, 0.44, and 0.65 μM). Protein·DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The position of the p53·DNA complex with the p53 consensus site probe is indicated.

Supplemental Figures for Chapter 3

The following figures were cited as “data not shown” in the text.

DNA-Binding Domain	Activation Domain	10mM AT	20mM AT
DB alone	AD alone	+	-
DB alone	AD-p53	+	-
DB-CR2	AD alone	+	-
DB-CR2	AD-p53	+	+
DB-CR2	AD-p53 (aa 73 to 393)	+	+
DB-CR2	AD-p53 (aa 73 to 201)	+	+
DB-CR2	AD-p53 (aa 73 to 112)	+	+

Figure 3.7. **p53 binds to the CR2 domain in vivo**. Growth phenotypes of the designated DNA-binding (DB) and activation domain (AD) constructs were assayed by streaking cells on plates lacking histidine and containing 10 or 20 mM aminotriazole (AT). Plates were analyzed following 5 days of incubation at 30°C.

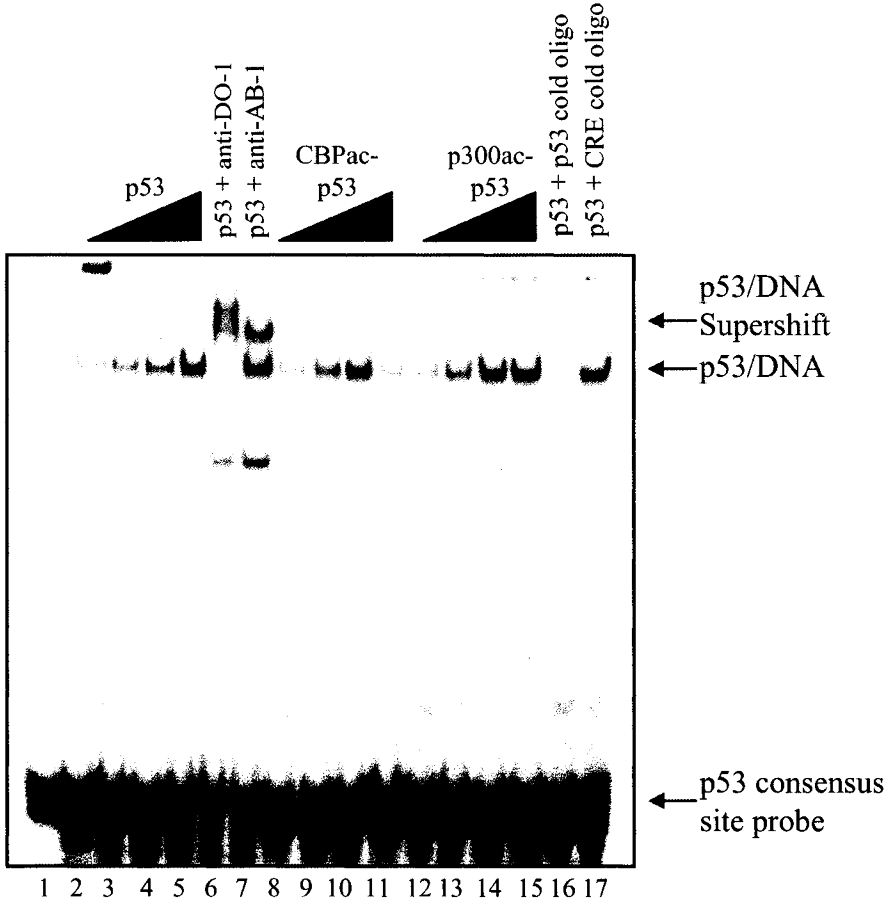


Figure 3.8. **Characterization of CBP-acetylated p53 versus p300-acetylated p53 binding to the p53 consensus probe.** Increasing amounts of CBP-acetylated p53 (60, 120, 240, and 480 ng) (lanes 8 to 11), p300-acetylated p53 (60, 120, 240, and 480 ng)(lanes 12 to 15), and/or purified, recombinant p53 (60, 120, 240, and 480 ng) (lanes 2 to 5) was incubated with a p53 consensus site probe. The specificity of the p53·DNA complex was confirmed by competition assay, adding a p53 cold competitor DNA (lane 16) compared with the non-specific CRE cold competitor DNA (lane 17) (each at 100-fold excess). The specificity of the p53·DNA complex was also confirmed by addition of p53-specific antibodies, anti-DO-1 (lane 6) and anti-AB-1 (lane 7). Protein·DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The position of the p53·DNA complex with the p53 consensus site probe is indicated.

Figure 3.9. **The CBP domains do not bind to the p53 consensus probe.** GST-CR1aa1514-1894, GST-CR2 aa2055-2150, GST-CR3 aa2212-2441, or GST-KIX aa588-683 protein (200 ng each) were incubated with the p53 consensus probe (lanes 3, 4, 5, and 6, respectively). Purified, recombinant p53 (100 ng) (lane 2) was incubated with the p53 consensus probe as a positive control. Protein·DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The position of the p53·DNA complex with the p53 consensus site probe is indicated.

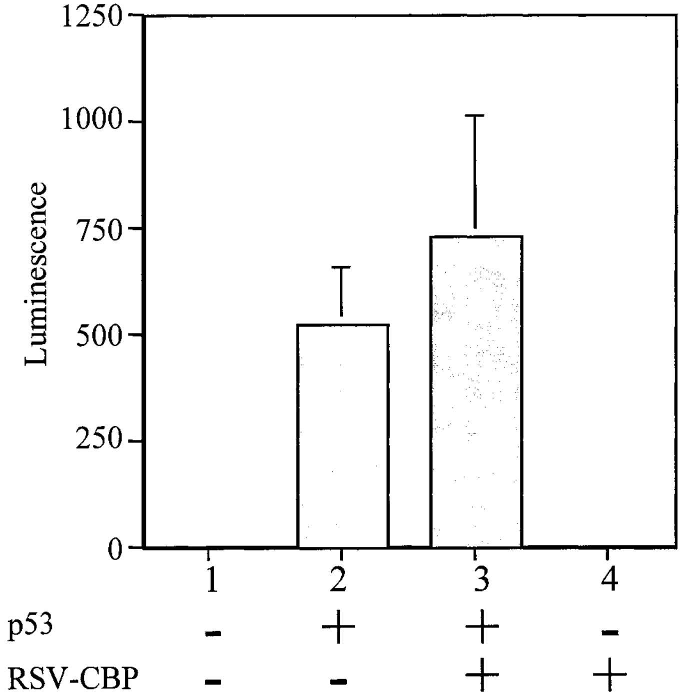


Figure 3.10. **Full-length CBP does not activate transcription from the pG13-Luc promoter in vivo.** The p53 responsive pG13-Luc reporter plasmid (400 ng) was cotransfected with an expression plasmid for full-length CBP (400 ng, lanes 3 and 4), and an expression plasmid for p53 (200 ng, lanes 2 and 3). As a control, cotransfection of an expression plasmid for full-length CBP in the absence of p53 is shown (lane 4). The values shown are the mean -fold activation (in triplicate) +/- S.D..

Chapter 4

Baculovirus Expressed P/CAF Does Not Mediate Tax Transactivation From Nucleosomal-Assembled HTLV-1 DNA In Vitro

The following study was undertaken to analyze the role of the p300/CBP-associating factor (P/CAF) coactivator in mediating Tax transactivation from HTLV-1 chromatin assembled templates. Upon the commencement of this study, the addition of exogenous, baculovirus expressed P/CAF had not been studied in vitro in the context of chromatin assembled DNA templates. It was hypothesized that like the cellular coactivator p300, the addition of exogenous, recombinant P/CAF would stimulate Tax-activated transcription from chromatin assembled HTLV-1 templates in vitro. Two previous studies found that P/CAF addition in vivo stimulated Tax transactivation similar to the enhancement found with p300 addition; thus, we hypothesized that P/CAF would enhance Tax transactivation in vitro.

4.1 Abstract

The human T-cell leukemia virus type 1 (HTLV-1) encodes the viral oncogenic transcription factor, Tax, to generate high levels of expression of the viral genome. Tax activation of HTLV-1 transcription requires the cellular transcription factor CREB to further enhance HTLV-1 expression. The recombinant chromatin assembly was used to examine the effects of P/CAF on Tax/CREB transactivation in vitro. HTLV-1 chromatin templates were assembled using either recombinant *Xenopus* core histones or *Drosophila* core histones. Interestingly, Tax/CREB activation from the HTLV-1 chromatin assembled templates remained unchanged in the presence of exogenous P/CAF. The stimulation of Tax/CREB mediated transcription by p300 was also unaffected by the presence of P/CAF. These data indicate that baculovirus-expressed P/CAF is not sufficient to enhance Tax transactivation in vitro from HTLV-1 chromatin assembled templates.

4.2 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus that causes adult T-cell leukemia (256, 344) and a neurodegenerative disorder known as tropical spastic paraparesis (reviewed in 211, 344, 363). Tax is a 40 kDa regulatory oncoprotein that stimulates HTLV-1 viral gene expression and is critical in HTLV-1 transformation (112, 113). Tax associates with these GC-rich minor groove DNA sequences located in the HTLV-1 promoter (169, 194, 209), while CREB also forms protein-DNA complexes (83, 365). The formation of the Tax/CREB/viral CRE nucleoprotein complex then is able to recruit the multifunctional cellular coactivator CBP/300 to the promoter (101, 182), resulting in strong transcriptional activation of the viral genome. Interestingly, this quaternary complex is able to directly interact with another coactivator, p300/CBP-associated factor (P/CAF), to form a multiple-enhancer containing complex (124).

P/CAF is an 832 amino acid cellular coactivator that was first identified in 1996 through its sequence conservation with yeast GCN5 protein. P/CAF is a member of the GNAT (GCN5 related N-acetyltransferase) superfamily and contains a P/CAF specific domain, located in the first 352 amino acids, linked to its GCN5-related domain (354). P/CAF has been characterized to bind to the CR1 domain of CBP/p300 at amino acids 1801 to 1851, and specifically competes with the adenoviral oncoprotein, E1A, for binding to CBP/p300 (261, 354). P/CAF is able to associate with a significant number of transcription factors, such as Tax (153), adenovirus E1A (261), MyoD (259), and HIV-I Tat (25). P/CAF also contains intrinsic histone acetyltransferase (HAT) activity that preferentially acetylates histone H3 (lysine 14) and to a lesser degree, histone H4 (lysine 8) (127, 279). Chromatin remodeling through HAT activity could allow for specific protein-protein or protein-DNA contacts that might otherwise not occur due to an inability to access the DNA and thus inhibiting transcription (reviewed in 27, 294). The P/CAF-acetylation sites on H3 and H4 overlap with the preferential acetylation sites by CBP/p300 for histones H3 and H4; however, CBP/p300 is also able to acetylate histones H2A and H2B (279). In addition, P/CAF has been shown to acetylate transcription factors such as MyoD (278), HMG-17 (126), and p53 (204). Interestingly, P/CAF is able to stimulate transcription, independent of its HAT activity (153, 261). Presently, only one study has examined whether or not baculovirus-expressed P/CAF is able to enhance transcriptional activation. Wallberg et al. (342) found that P/CAF is unable to promote transcription on its own, however, P/CAF in the presence of p300 act cooperatively to enhance transcription of the Notch signaling pathway from chromatin-assembled templates.

To date, only two papers have examined the interaction between Tax and P/CAF. Tax has been characterized to bind to amino acids 465 to 529 of P/CAF (153), while the carboxy-terminus of P/CAF might also aid in the stability of the Tax-P/CAF interaction. In vivo studies using the transient cotransfection assay suggests that exogenous P/CAF enhanced Tax transactivation in a HAT-independent fashion, while deletions of either the amino-terminus (ending at residue 465) or the carboxy-terminus (beginning at residue 529) completely abrogated the enhanced transcriptional activation (153). Other transient cotransfection assays examining the effects of exogenous coactivators found that P/CAF in addition to p300, did not exhibit synergistic effects on Tax transactivation. The carboxy-terminal transactivation domain of Tax is responsible for forming the protein-protein contacts with P/CAF. More specifically, the Tax M47 (L319R, I320S) double point mutant, which is defective for transactivation from the HTLV-1 LTR, is unable to interact with P/CAF and form a functional activator complex (124).

In this study, we examined the mechanistic role of P/CAF in Tax/CREB transcriptional activation in a chromatin context. We used a biochemically defined chromatin system as previously described in Georges et al. (96) in which recombinant histones lacking posttranslational modifications or histone variants were assembled onto the HTLV-1 promoter. We found that, as expected, nucleosome assembly strongly repressed basal transcription and that Tax and CREB were able to relieve this repression. Unexpectedly, we found that the addition of exogenous P/CAF did not further increase

Tax/CREB-mediated activation. As expected, we also found that the addition of exogenous p300 did increase Tax/CREB transcriptional activation; however, the addition of exogenous P/CAF to p300-activated transcription did not further enhance this activation. Together, these results suggest that while recombinant baculovirus expressed P/CAF is active in its ability to acetylate free core histones, it is unable to enhance Tax/CREB-mediated transcription in a chromatin context.

4.3 Materials and Methods

4.3a Expression and purification of recombinant proteins. CREB (83) and Tax-His_6 (366) were expressed and purified as previously described (101). CREB was serine-133 phosphorylated by protein kinase A (PKA) as previously described (101). GST-Tax (full length), GST-$Tax_{aa286-337}$ (63), GST, and GST-KIX $_{aa588-683}$ were purified using glutathione-agarose chromatography as previously described (280). Purified proteins were stored at -70°C in TM buffer (50mM Tris-HCl [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA [pH8.0], 1mM dithiotrhreiotol, 0.1% [vol/vol] Tween-20, 20% [vol/vol] glycerol).

Recombinant *Xenopus* histone octamers were expressed in *Escherichia coli* and purified to homogeneity as previously described (96, 207). *Drosophila* core histones and were purified as previously described (39). *Drosophila* NAP-1 (dNAP-1) (His_6-tagged) was expressed from recombinant baculovirus (142) and purified first by nickel chelate chromatography followed by +Source 15Q-column chromatography as previously described (96, 144). We co-expressed FLAG-tagged ISWI and Acf1 from baculovirus, and purified the complex by anti-FLAG affinity batch binding and elution as previously described (145). Baculovirus stocks of human P/CAF and three deletion mutant forms of human P/CAF, P/CAF ΔN (aa Δ1-465), P/CAF ΔC (aa Δ529-832), and P/CAF ΔHAT (aa Δ445-512), were a generous gift from Dr. Yoshihiro Nakatani (Harvard University). Wild-type P/CAF and the three deletion mutant forms of P/CAF were expressed from recombinant baculoviruses and purified using anti-FLAG resin as previously described (354). His_6-tagged p300 protein was expressed from recombinant baculovirus and purified using nickel-chelate chromatography as previously described (177).

4.3b GST pulldown assay. All GST pull-down experiments were performed as previously described (334). Anti-FLAG M2 antibody was used in the GST pull-down experiment.

4.3c Chromatin assembly and topological assay. Nucleosomes were assembled on the 4TxRE G-less plasmid as previously described (96, 144). Following the addition of the DNA, ATP (3 mM), creatine phosphokinase (1 μg/ml), and phosphocreatine (30 mM) were added in a 70 μl reaction containing 10 mM HEPES (K^+), [pH 7.6], 50 mM KCl, 5 mM $MgCl_2$, and 5% (v/v) glycerol. Briefly, histone octamers were preassembled with dNAP-1 (8:1 dNAP-1/core histones) on ice for 30 minutes. Assembly reactions were incubated overnight at 27°C. DNA topological assays were performed as previously described (96). The samples were analyzed on a 1% agarose gel, and the degree of supercoiling was visualized by Sybr Gold (Molecular Probes) staining.

4.3d Recombinant plasmids and in vitro transcription assays. The 4TxRE G-less cassette carries four copies of the third 21-bp TxRE located upstream of the HTLV-1 core promoter (7). Preinitiation complexes were formed on 100ng of DNA template in TM buffer (supplemented with 10 μM acetyl CoA [Sigma]) by the addition of the indicated amounts of CREB and/or Tax and 12 μg CEM nuclear extract (73) in a final reaction volume of 30 μl. The reactions were incubated for 60 minutes at 30°C. RNA synthesis was initiated by the addition of 250 μM of ATP, 250 μM of CTP, 12 μM UTP, and 0.8 μM [α-^{32}P] UTP (3,000 Ci/mmol) and incubated for an additional 35 minutes at 30°C. Transcription reactions were processed and analyzed as previously described (194). Molecular weight markers (radiolabeled *Hpa* II digested pBR322) were used to estimate the size of the RNA products. For the chromatin-assembled in vitro transcription assay, the supercoiled 4TxRE G-less plasmid was assembled into chromatin using dNAP-1, dAcf1 and recombinant *Xenopus* histones (or *Drosophila* histones where indicated) at a 0.6:1 histone:DNA ratio. Following chromatin assembly, preinitiation complexes were formed on the equivalent of 150 ng of the plasmid DNA in the absence or presence of the indicated amounts of CREB, Tax, p300, and/or P/CAF. All reactions contained 100 μM acetyl CoA (USB) and the remaining steps were followed as described above.

4.3e In vitro acetylation assay. The acetyltransferase activity of P/CAF and the P/CAF mutants was assayed by incubation of recombinant *Xenopus* histone octamers (500 ng) with P/CAF (250 ng), P/CAFΔHAT (250 ng), P/CAFΔN (250 ng), or P/CAFΔC (250 ng) and ^{14}C-acetyl CoA (100 pmol; 57mCi/mmol, Amersham) in a solution containing 50 mM Tris, pH 8, 10% glycerol, 10 mM sodium butyrate, 1 mM DTT and 1 mM PMSF. Samples were incubated at 30°C for 60 minutes, proteins were separated by 18% SDS-PAGE, fixed, and acetylation was visualized by PhosphorImager analysis.

4.3f Electrophoretic mobility shift assay. The singly-end labeled viral CRE consensus site double stranded oligonucleotide probe (3 fmol) was incubated with purified Tax (25 ng), phosphorylated CREB (1.25 ng) and increasing amounts of P/CAF (3 ng, 15 ng, and

30 ng) for 45 minutes on ice as previously described (101). GST-KIX$_{aa588-683}$ protein (5ng and 50 ng) was incubated with the respective amounts of Tax and phosphorylated CREB as a positive control. Protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel.

4.4 Results

4.4a Tax binds to amino acids 465 to 529 of P/CAF

Two previous studies have shown that Tax physically interacts with P/CAF both in vitro and in vivo, mediated through amino acids 465 to 529 of P/CAF (124, 153). We obtained wild-type P/CAF baculovirus as well as three deletion mutant forms of baculovirus P/CAF, P/CAF ΔN (amino acids Δ1-465), P/CAF ΔC (amino acids Δ529-832), and P/CAF ΔHAT (amino acids Δ445-512) (Y. Nakatani, Harvard University). A schematic of the P/CAF proteins used in this study are represented in figure 4.1.A and the FLAG-affinity purified P/CAF proteins are shown in figure 4.1.B. An in vitro acetylation assay was used to confirm the histone acetyltransferase (HAT) activity of each respective P/CAF protein, as the HAT domain resides within amino acids 352 to 658 (261). The acetyltransferase assay was performed as described in Kraus et al. (177) using recombinant *Xenopus* histone octamers in the presence and absence of the various forms of P/CAF. As shown in figure 4.1.C, both wild-type P/CAF and P/CAF ΔN were able to acetylate histones H3 and H4 (lanes 2 and 3, respectively), while p300 was used as a positive control for examining acetylation of all four core histones (lane 1). The P/CAF acetylated histones H3 and H4 run slower in the acetylation electrophoresis gel because of the different number of acetylated lysine residues on P/CAF-acetylated H3 and H4

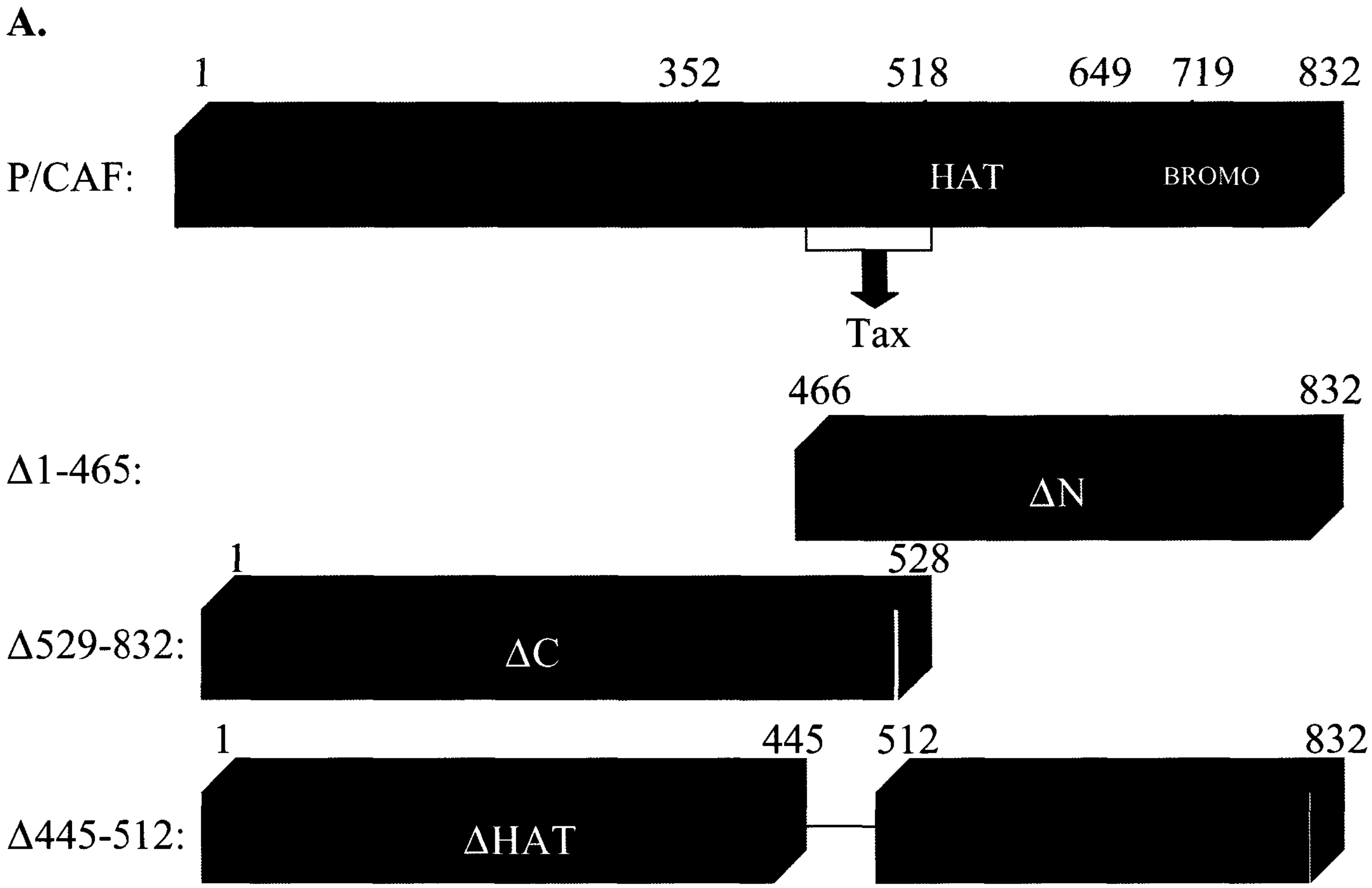


Figure 4.1. **Tax interacts with amino acids 465 to 529 of P/CAF in vitro**. (A) Schematic representation of full-length P/CAF and the P/CAF mutants used in this study.

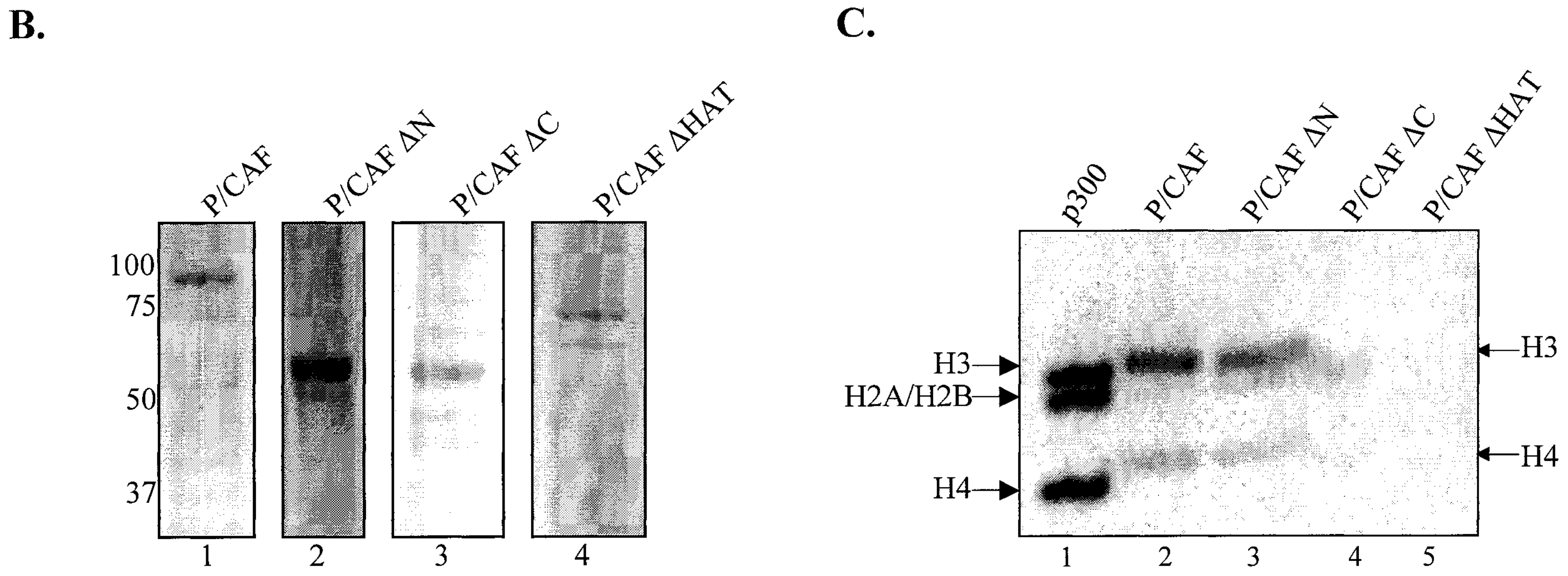


Figure 4.1. (B) Wild-type P/CAF and P/CAFΔN contain HAT activity. Purified P/CAF and P/CAF deletion mutants expressed from baculovirus were analyzed by SDS-10% PAGE and stained with Coomassie. Protein molecular size standards are indicated in kilodaltons. (C) In vitro acetylation assay of recombinant histones (500 ng) by P/CAF and by the P/CAF deletion mutants (250 ng each). P/CAF (lane 2) and P/CAF ΔN (lane 3) acetylate recombinant *Xenopus* histone octamers, whereby histones H3 and H4 are indicated. As a positive control, p300 (250 ng) HAT activity was also assayed. Acetylation reactions were run on a long SDS-18% PAGE with a 10% stacker.

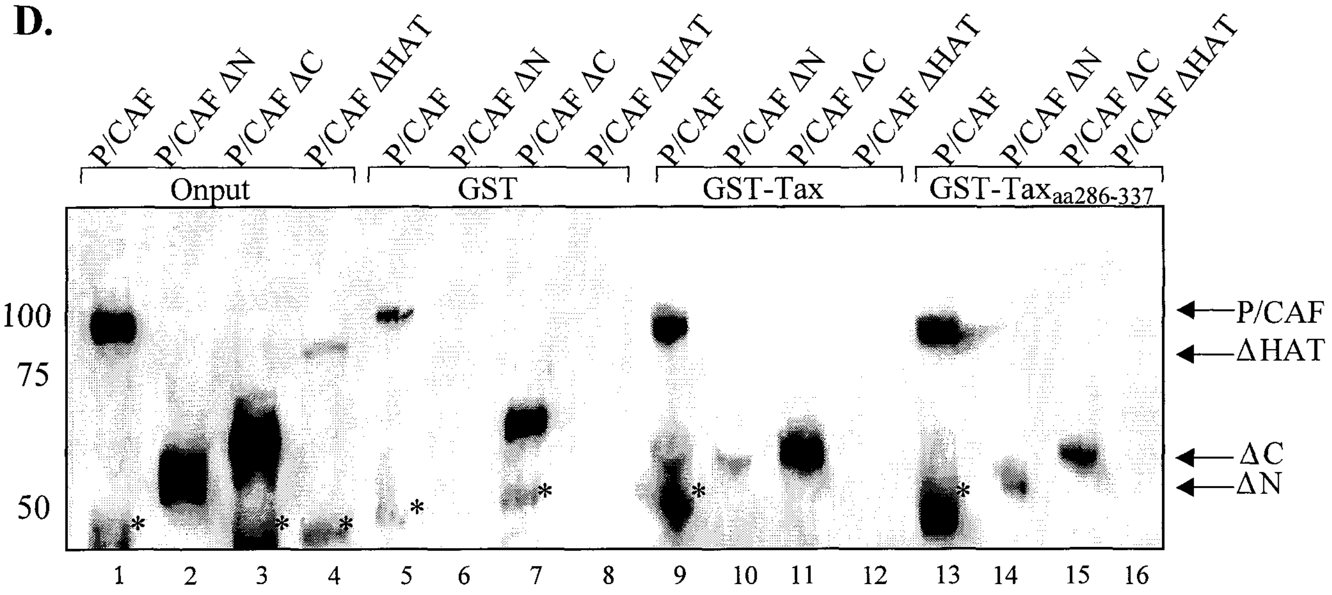


Figure 4.1. (D) Purified P/CAF (50 pmol) and the designated P/CAF deletion mutants (50 pmol each) were incubated with GST alone or GST-Tax (50 pmol). The bound proteins were separated on a SDS-10% PAGE, transferred to nitrocellulose, and detected using an anti-FLAG M2 antibody. The onputs are shown in lanes 1, 2, 3, and 4 (10% each). The positions of bound P/CAF and protein molecular weight standards are indicated (in kilodaltons). Asterisks denote degradation products detected using the anti-FLAG M2 antibody.

compared with p300-acetylated H3 and H4. Because two of the P/CAF mutants differed in their amino acid deletion coordinates from those used in the Jiang et al. (153) study, we analyzed their ability to bind Tax in a GST pulldown assay. GST-Tax and a carboxyl-terminal region of Tax bound to GST (GST-Tax $_{\text{aa287-336}}$) were bound to glutathione-agarose beads, and used in a GST pulldown assay with either wild-type P/CAF or the deletion mutants of P/CAF. As expected, wild-type P/CAF bound well to both GST-Tax and the carboxy-terminal region of GST-Tax (Fig. 4.1.D, lanes 9 and 13). Also shown in figure 4.1.D, the P/CAF ΔN deletion mutant (lacking the first 465 amino acids of the protein) bound weakly to GST-Tax (lanes 10 and 14). The P/CAF ΔC mutant (lacking the carboxyl-terminal region of the protein) bound equally well to both the negative control, GST, and to GST-Tax; thus, it is difficult to draw conclusions using this carboxyl-terminal deletion of P/CAF due to non-specific binding (Fig. 4.1.D, lanes 7 and 11). Surprisingly, the P/CAF ΔHAT deletion mutant did not interact with GST-Tax (Fig. 4.1.D, lanes 12 and 16); however, the onput signal is relatively weak (lane 4) suggesting that lack of binding could be attributed to poor protein expression that would be difficult to visualize even if it was present due to the low signal. While we do not have a positive control for functional binding to the P/CAF ΔHAT deletion mutant, we have shown that the purified protein lacks its histone acetyltransferase activity, as expected (Fig. 4.1.C, lane 5). The results comparing wild-type P/CAF with P/CAF ΔN, combined with the study performed by Jiang et al. (153), suggests that Tax indeed binds to amino acids 465 to 529 of P/CAF.

4.4b EMSA studies on the Tax-P/CAF interaction

As an alternate method to characterize the Tax-P/CAF interaction, we utilized the electrophoretic mobility shift assay (EMSA). We were interested in determining whether full-length P/CAF could form a quaternary complex with Tax and Ser133-phosphorylated CREB bound to the viral CRE DNA recognition element. Figure 4.2 shows that titration of the purified P/CAF into Tax/pCREB-containing binding reactions had no effect on their mobility (lane 3, and lanes 6 to 8). As a positive control, we titrated the KIX domain of CBP into the Tax/pCREB/viral CRE DNA binding reactions to examine successful quaternary complex formation (Fig. 4.2, lanes 4 and 5).

4.4c P/CAF does not stimulate Tax transactivation on unassembled templates

We were then interested in examining the effect of P/CAF on Tax activated transcription from unassembled templates. We first examined P/CAF activity in an in vitro transcription assay, using a plasmid carrying a Tax-responsive promoter driving synthesis of a two tandem G-less cassettes. The Tax-responsive promoter, which drives the G-less cassette, carries four viral CRE elements cloned immediately upstream of the core HTLV-1 promoter (7). A schematic representation of the promoter construct is shown at the top of figure 4.3.A. We performed in vitro transcription assays using CEM (an HTLV-1-negative human T-lymphocyte cell line) nuclear extracts. Figure 4.3.A shows the results of the in vitro transcription experiment. As expected, the addition of wild type Tax and CREB produced an increase in transcription from the 4TxRE/G-less template (6.8-fold) (Fig. 4.3.A, lane 2). The addition of increasing amounts of wild-type P/CAF did not affect the levels of Tax/CREB transcriptional activation on naked templates (Fig. 4.3.A, lanes 3 to 5). This result is not altogether unexpected as previous

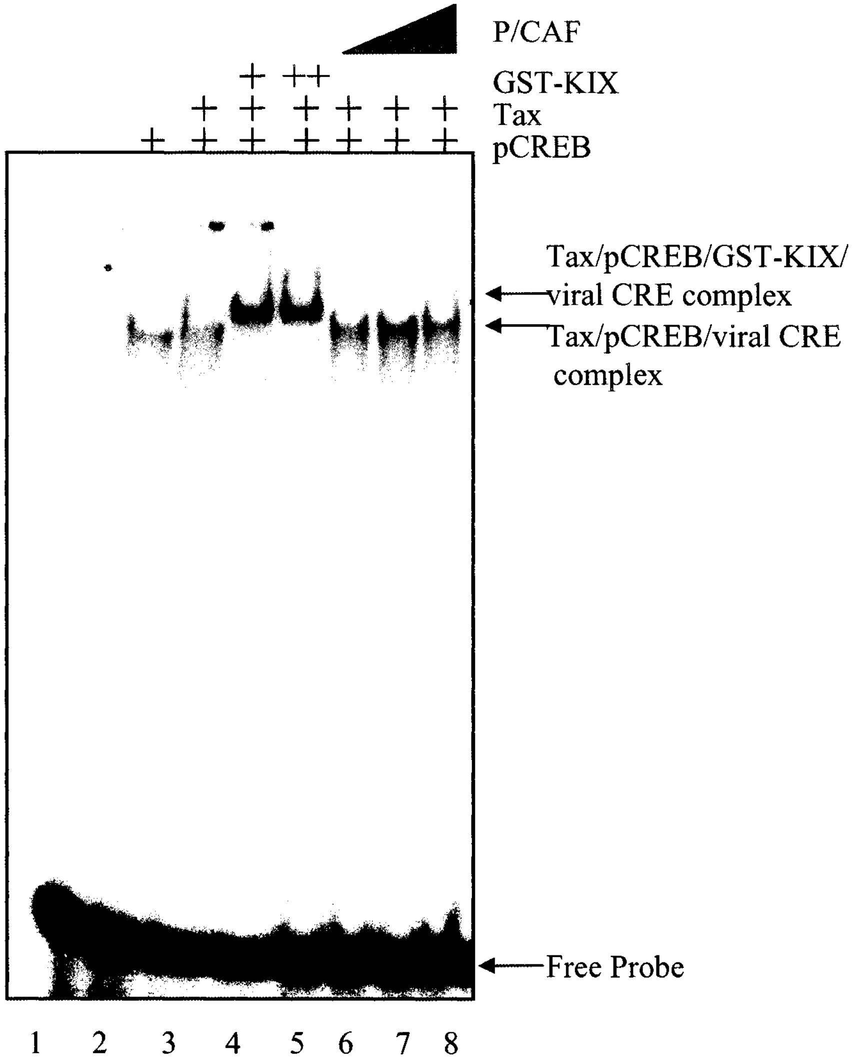


Figure 4.2. **Tax does not recruit P/CAF to Ser133-phosphorylated CREB on a viral CRE.** Purified, recombinant Tax (25 ng, lanes 3 to 8) was incubated with 3 fmol of viral CRE (lanes 1 to 8) together with Ser133-phosphorylated CREB (1.25 ng, lanes 2 to 9), and increasing amounts of P/CAF (3 ng, 15 ng, 30 ng, lanes 6 to 8), and/or GST-KIX $_{aa588-683}$ protein (5 ng and 50 ng, lanes 4 and 5). Protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The position of the ternary (Tax/ pCREB/ DNA) and quaternary (Tax/ pCREB/ GST-KIX $_{aa588-683}$/ DNA) complexes and the free probe are indicated.

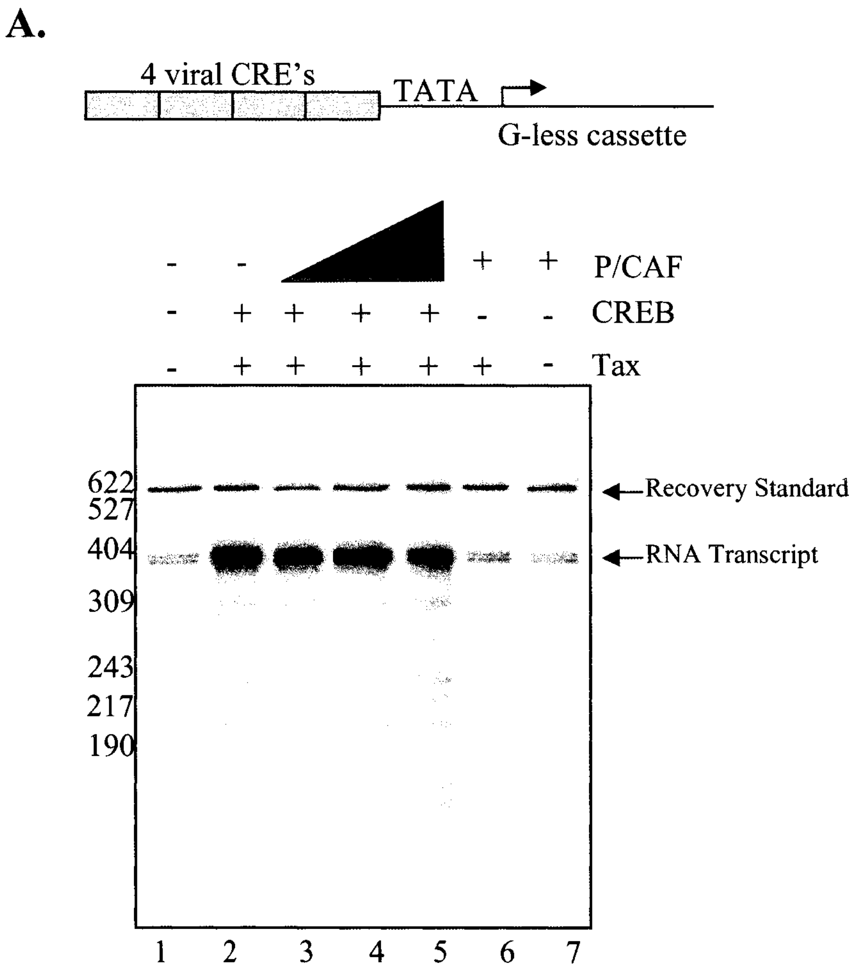


Figure 4.3. **P/CAF does not enhance Tax activation from unassembled DNA templates.** (A) Tax transactivation on unassembled DNA templates. The in vitro transcription assay was performed on a 4TxRE G-less cassette template that carries four tandem copies of the third (promoter proximal) viral CRE driving expression of a 380-nt RNA (shown schematically above panel 4.3.A). Transcription reaction mixtures contained the 4TxRE G-less template (150 ng), CEM nuclear extract (12 μg), purified, recombinant Tax (50 ng) (lanes 2 to 7), CREB (50 ng) (lanes 2 to 7), and increasing amounts of P/CAF (75, 150, and 300 ng) (lanes 3 to 5). All reactions were performed in the presence of acetyl-CoA. The full-length 380-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated. This experiment is representative of three independent experiments.

B.

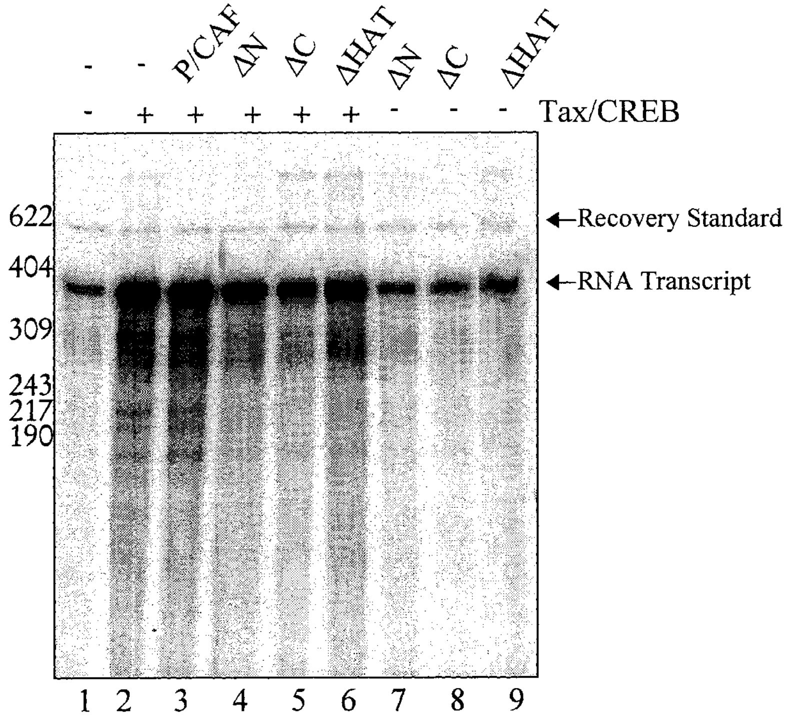


Figure 4.3. (B) The P/CAF mutants do not activate Tax transactivation from unassembled DNA templates. Transcriptional activation on 4TxRE G-less unassembled templates was analyzed in the presence of Tax (50 ng) (lanes 2 to 6), CREB (50 ng) (lanes 2 to 6), P/CAF (150 ng) (lane 3), P/CAF ΔN (150 ng) (lane 4 and 7), P/CAF ΔC (150 ng) (lane 5 and 8), and/or P/CAF ΔHAT (150 ng) (lane 6 and 9). All reactions were performed in the presence of acetyl-CoA. The full-length 380-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated. This experiment is representative of two independent experiments.

studies have shown that the addition of exogenous full-length coactivator, p300, does not activate transcription in vitro in the absence of chromatin (96, 176, 179). We also examined the P/CAF deletion mutants for their effect on Tax/CREB transactivation on unassembled templates. As shown in figure 4.3.B, the addition of exogenous P/CAF or the P/CAF mutants had no affect on wild-type Tax/CREB mediated transcriptional activation (lanes 2 to 6). Also shown in figure 4.3.B, the addition of the P/CAF mutants had no affect on basal-mediated transcription (lanes 7 to 9).

4.4d P/CAF does not stimulate Tax transactivation in a chromatin context

Nucleosome assembly has been shown to strongly repress transcription from various promoters (96, 177). The addition of transcription factors and coactivators, such as p300, has been shown to counter this repression leading to increased transcriptional activation. A study performed by Jiang et al. (153) found that P/CAF activated Tax transactivation using either exogenously expressed wild-type P/CAF or a P/CAF mutant lacking HAT activity, suggesting that P/CAF activates Tax transcription in a HAT-independent manner in vivo. It was of interest to then ask if exogenous P/CAF acts in a HAT-independent manner to enhance Tax/CREB-mediated transcription from an HTLV-1 promoter in a chromatin context. Recombinant *Xenopus* core histones are an ideal source of histones because they are expressed and purified from *E.coli* and are, thus, unmodified and lack histone variants (207, 348). Chromatin assembly of the HTLV-1/G-less template was performed using the purified recombinant *Drosophila* assembly proteins Acf1/ISWI and dNAP-1(nucleosome assembly protein-1), an H2A-H2B histone chaperone (145). The ACF complex is involved in chromatin assembly as well as in nucleosome remodeling (143). These assembly proteins are sufficient for the ATP-dependent formation of evenly spaced nucleosomal arrays (143, 145). A DNA topological assay is used to determine the proper histone to DNA ratio used in a chromatin assembly that yields full assembly of nucleosomes onto the DNA. This analysis was used to determine the efficiency of nucleosome deposition on the 4TxRE/G-less template. The assembly of HTLV-1 transcription templates is optimized using increasing ratios of histones to DNA (w/w), in which the assembly of the nucleosomes can be visualized by an increase in DNA supercoiling. As shown in figure 4.4.A, increasing ratios of histone octamers to DNA, lead to a concomitant increase in the supercoiling of the DNA, with complete assembly at a ratio of 1.4:1 (lanes 2 to 8). The increase in supercoiling, which is indicative of nucleosomes deposited onto the DNA, can also be visualized using the in vitro transcription assay. Following chromatin assembly, an in vitro transcription assay is performed to examine the effect that increased nucleosome deposition has on HTLV-1 basal transcription. As shown in figure 4.4.B, the increasing concentrations of histone octamers present on the 4TxRE/G-less plasmid lead to complete repression of basal activated transcription at the ratio of 0.6:1.0. Thus, the histone to DNA ratio of 0.6:1 was used to examine what role P/CAF plays in Tax transactivation from chromatin assembled templates. We performed the in vitro transcription assay as described above, using nuclear extracts from CEM cells (HTLV-1 negative T-lymphocyte cell line) as a source of basal transcription factors and RNA polymerase. All experiments were performed in the presence of acetyl CoA, and in the presence or absence of exogenous Tax and CREB and/or P/CAF. Figure 4.4.C shows that the addition of Tax and CREB together strongly stimulated transcription ~73-fold above basal transcription levels (lane 2). The addition of increasing amounts of P/CAF,

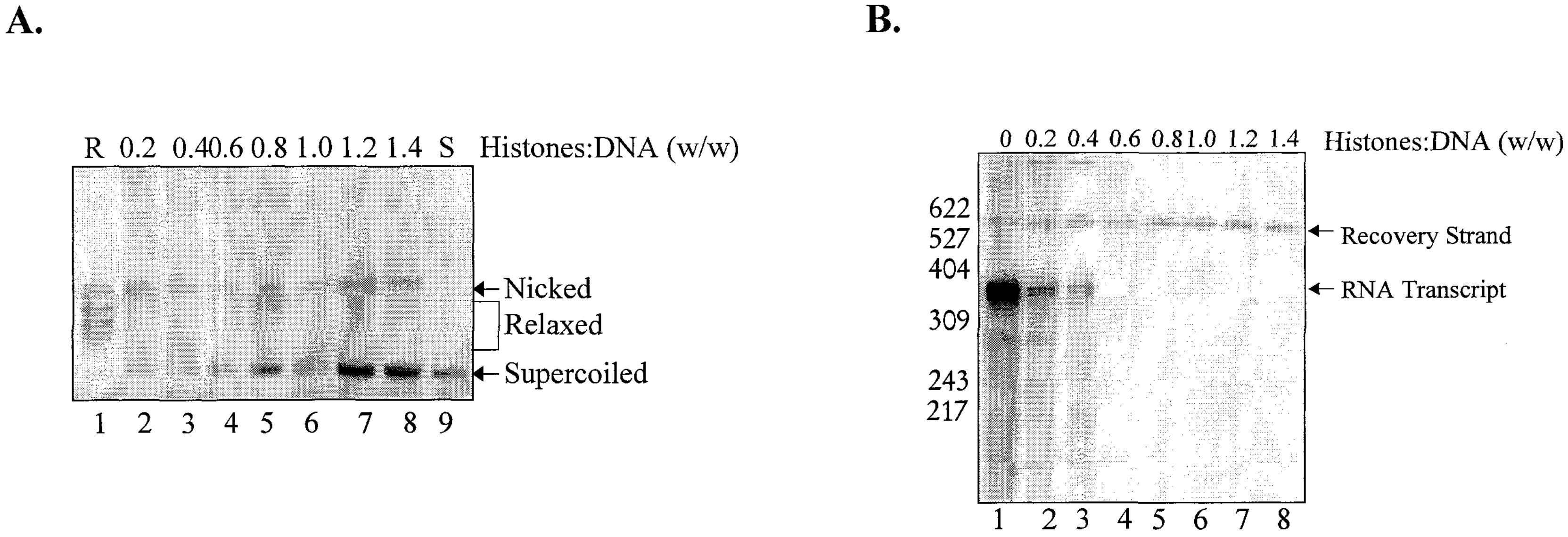


Figure 4.4. **P/CAF does not stimulate Tax transactivation from HTLV-1 assembled templates.** (A) Analysis of the purified proteins and their transcriptional activity in the chromatin assembly system. One-dimensional DNA topological assays showing the 4TxRE G-less transcription template assembled with *Xenopus* core histones in the presence of dAcf1/ISWI and dNAP-1. The DNA topoisomers were resolved on an agarose gel, and the DNA stained with Sybr Gold (Molecular Probes). The supercoiled (S), relaxed (R), and nicked DNA populations, and the histone/DNA ratio, are indicated. (B) Increasing amounts of core histones were deposited on the 4TxRE/G-less template and basal transcription was assayed. Basal transcription from an unassembled template was assayed as a positive control (lane 1). The full-length 380-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated.

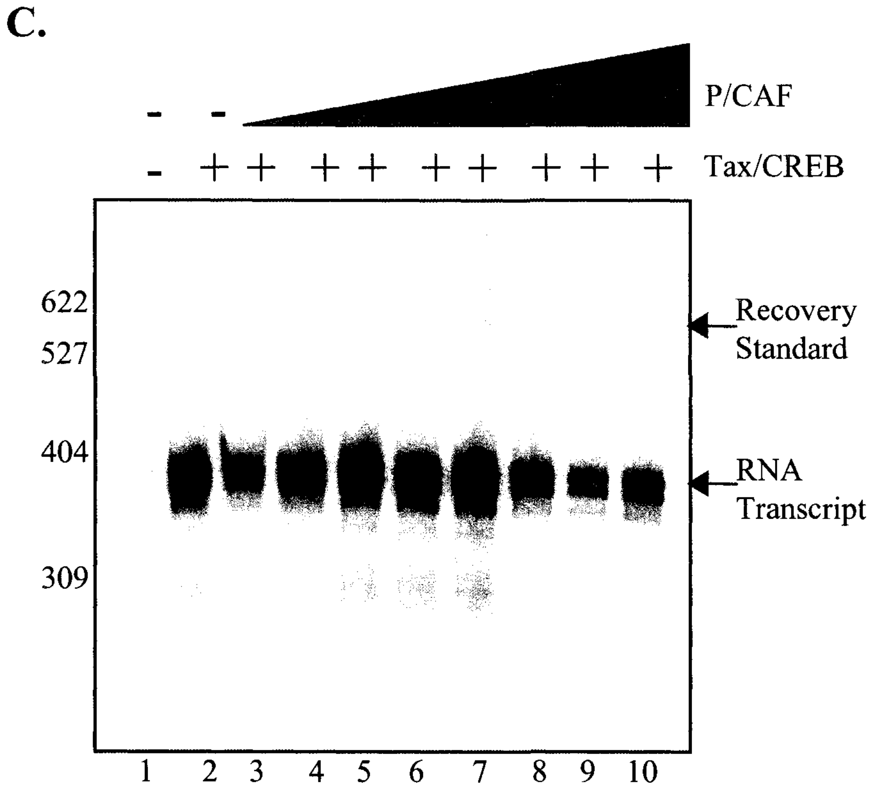
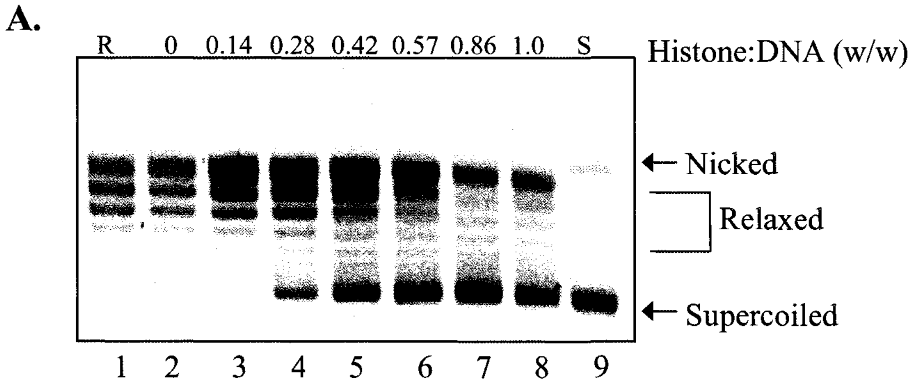


Figure 4.4. (C) Transcriptional repression and activation on 4TxRE G-less chromatin templates (using *Xenopus* core histones) was analyzed in the presence of Tax (100 ng) (lanes 2 to 10), CREB (80 ng) (lanes 2 to 10), and increasing amounts of P/CAF (1 ng to 200 ng) (lanes 3 to 10). The full-length 380-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated.

ranging from 1 ng to 200 ng, did not stimulate Tax/CREB mediated transcription in a chromatin context (Fig. 4.4.C, lanes 3 to 10). This result examining Tax transactivation from chromatin-assembled templates in vitro was somewhat surprising as a previous in vivo study suggests that exogenous P/CAF enhanced Tax transactivation (153).

Based upon these preliminary results, we examined P/CAF stimulation of Tax/CREB mediated transcription using native *Drosophila* core histone chromatin assembled 4TxRE/G-less templates. Native *Drosophila* core histones were assayed because they may contain posttranslational modifications on the amino-terminal histone tails that are not present on the recombinant *Xenopus* core histones. Figure 4.5.A shows a DNA topological assay demonstrating the assembly of native *Drosophila* core histones onto the HTLV-1/G-less template (lanes 2 to 8). This figure shows that a histone/DNA ratio of 1.0:1.0 (w/w) fully assembled the DNA template into chromatin (lane 8). We performed in vitro transcription assays on this Tax/CREB-responsive HTLV-1 chromatin template as described above. As shown in figure 4.5.B, we did not detect P/CAF stimulation of transcription in the presence of Tax/CREB (compare lane 3 with lanes 5 to 7). To ensure that the chromatin assembly system was functional, we added p300 to the Tax/CREB transcription reaction and found noticeable stimulatory effects upon addition (Fig. 4.5.B, lane 4). We also examined the additive effect of p300 and P/CAF on Tax/CREB transactivation to determine whether or not these coactivators can work synergistically in a chromatin context in vitro. As shown in figure 4.5.B and figure 4.5.C, p300 and P/CAF did not act synergistically on Tax/CREB mediated transcription from either native or unmodified chromatin assembled templates (lanes 8 and 6, respectively). This result is not altogether surprising as in vivo studies performed by Jiang et al. (153)



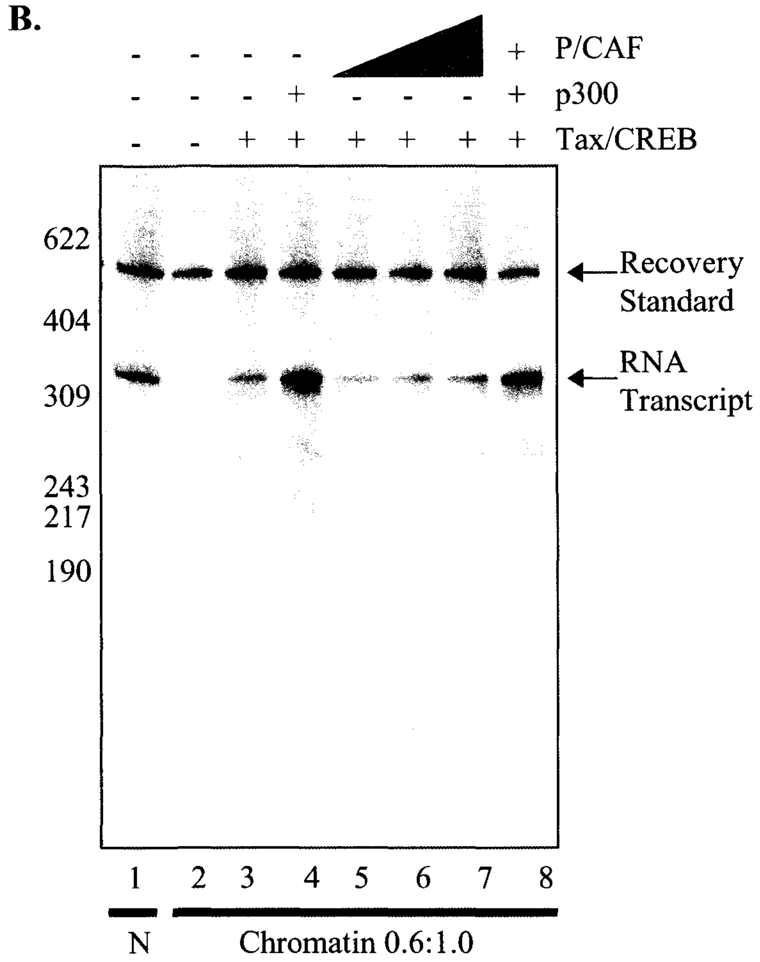


Figure 4.5. **P/CAF does not synergize with p300 to enhance transcriptional activity from chromatin templates assembled with native *Drosophila* core histones.** (A) Chromatin Assembly using *Drosophila* core histone proteins. One-dimensional DNA topological assays showing the 4TxRE G-less transcription template assembled with *Drosophila* core histones in the presence of dAcfl/ISWI and dNAP-1. The DNA topoisomers were resolved on an agarose gel, and the DNA stained with Sybr Gold (Molecular Probes). The supercoiled (S), relaxed (R), and nicked DNA populations, and the histone/DNA ratio, are indicated. (B) The 4TxRE G-less template was assembled into chromatin using *Drosophila* core histones in the presence of dAcfl/ISWI and dNAP-1. Transcriptional repression and activation was analyzed in the presence of Tax (100 ng) (lanes 3 to 8), CREB (75 ng) (lanes 3 to 8), p300 (100 ng) (lanes 4 and 8), and/or increasing amounts of P/CAF (41.5 ng, 83 ng, 166 ng) (lanes 5, 6, and 7). N designates unassembled transcription. The full-length 380-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated.

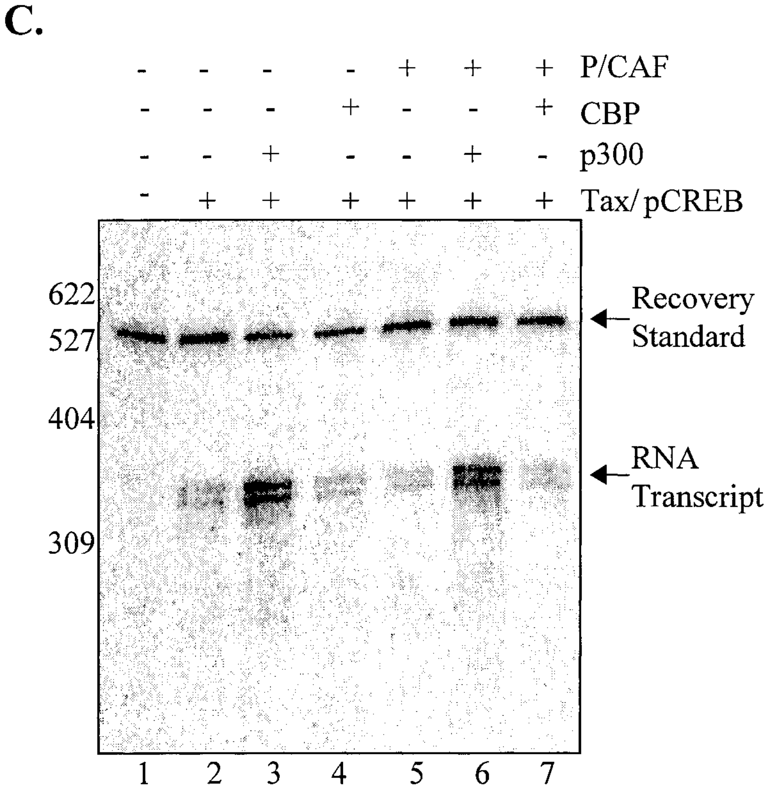


Figure 4.5. (C) P/CAF does not synergize with p300 or CBP to activate Tax transcription from chromatin templates assembled using recombinant *Xenopus* core histones. The 4TxRE G-less template was assembled into chromatin using *Drosophila* core histones in the presence of dAcf1/ISWI and dNAP-1. Transcriptional repression and activation on 4TxRE G-less chromatin templates was analyzed in the presence of Tax (100 ng) (lanes 2 to 7), CREB (100 ng) (lanes 2 to 7), p300 (150 ng) (lanes 3 and 6), CBP (150 ng) (lanes 4 and 7), and P/CAF (150 ng) (lanes 5 to 7). The full-length 380-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated. This experiment is representative of three independent experiments.

suggest that P/CAF, in addition to p300, did not exhibit synergistic effects on Tax transactivation. We also examined the additive effects of CBP and P/CAF in the chromatin assembled transcription assay. Figure 4.5.C suggests that full-length CBP does not stimulate Tax transactivation from recombinant, unmodified chromatin (lane 4). Furthermore, the addition of P/CAF to the CBP-Tax-pCREB reaction does not affect this result (Fig. 4.5.C, lane 7). This result is consistent with those examining the p300-P/CAF effect on recombinant chromatin assembled templates.

4.4e Tax/CREB does not stimulate P/CAF HAT activity

Research by Georges et al. (96) demonstrated that the presence of Tax/CREB stimulates p300 HAT activity on chromatin templates. Thus, we asked whether or not the addition of Tax/CREB could enhance acetylation of histones H3 and H4 by P/CAF when assembled into chromatin. 4xTRE/G-less chromatin templates were assembled at the predetermined ratios and examined for their P/CAF-dependent acetylation of the histones using ^{14}C-labeled acetyl-CoA. Figure 4.6 shows that the addition of Tax/CREB does not enhance acetylation of histones H3 or H4 by P/CAF, either as assembled into chromatin or as free histones (lanes 1 to 4). P/CAF autoacetylation also appears unaffected by the presence of Tax/CREB (Fig. 4.6, lanes 1 to 4). This result, together with the in vitro transcription assays from chromatin assembled templates, suggests that full-length P/CAF purified from baculovirus does not function in and of itself in Tax/CREB-mediated activation from the HTLV-1 promoter.

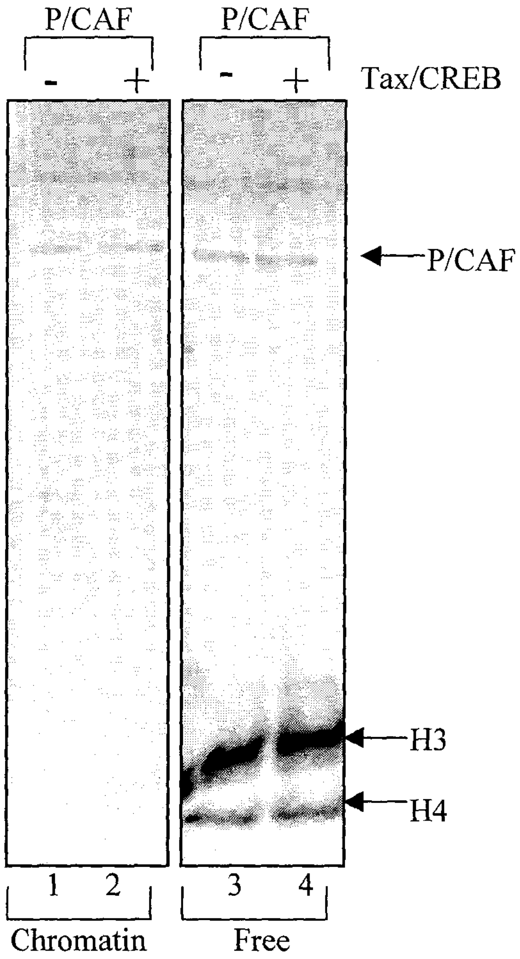


Figure 4.6. **In vitro acetylation assay on chromatin templates assembled with recombinant *Xenopus* core histones.** 4TxRE G-less templates (150 ng) were assembled into chromatin in the presence and absence of Tax and CREB using *Xenopus* core histones (500 ng). Acetylation reactions for both free and assembled core histones were assayed in the presence of ^{14}C-labeled acetyl-CoA, P/CAF (500 ng) (lanes 1 to 4), and/or Tax (333 ng) (lanes 2 and 4) and CREB (333 ng) (lanes 2 and 4). Histones H3, H2A/H2B, and H4 are indicated. This experiment is representative of three independent experiments.

4.5 Discussion

In this study, we show that recombinant, full-length P/CAF is not sufficient for mediating Tax/CREB transcriptional activity from the integrated HTLV-1 promoter. Chromatin templates were generated using either recombinant *Xenopus* core histones as an unmodified source of nucleosomes or native *Drosophila* core histones as nucleosomes containing posttranslational modifications. We show that chromatin assembled with either native or recombinant histones are efficiently assembled onto the HTLV-1 template DNA, through repression of basal transcription. In addition, Tax/CREB transcriptional activation was evident from either chromatin-assembled template; however, P/CAF was unable to enhance transcriptional activity from either template. We found that p300 and P/CAF do not enhance Tax-activated transcription in a cooperative manner, as previous studies have shown Tax transactivation is potentiated by the addition of either p300 or P/CAF (96, 206). We further show that the recombinantly purified P/CAF from baculovirus is active in an acetyltransferase assay using free core histones, but that this acetyltransferase activity is not sufficient for acetylating histones H3 and H4 from chromatin-assembled templates. This result is consistent with those described in Ogryzko et al. (236) in which only the P/CAF complex purified from nuclear extracts was able to acetylate core histones in a nucleosomal context. Consistent with a study done by Jiang et al. (153), we also found that P/CAF interacts with the carboxyl-terminal region of Tax through amino acids 465 to 529 of P/CAF.

The major difference in the requirement for HAT activity between p300 and P/CAF could be one explanation for the lack of effect on HTLV-1 transcription from chromatin-assembled templates. A study performed by Jiang et al. (153) suggests that

P/CAF lacking HAT activity was able to activate HTLV-1 transcription in vivo exactly as wild-type P/CAF. This result differs from that found when using another HAT, p300. While P/CAF stimulates Tax transactivation in a HAT-independent mode, p300 primarily functions as a HAT in HTLV-1 transcription (96, 153). Additionally, P/CAF is able to acetylate only lysine residue 14 of histone H3 and lysine residue 8 of H4 (279). These acetylated residues overlap with those acetylated by p300; moreover, p300 is able to acetylate all four core histones, while acetylating multiple lysine residues on histones H2B, H3, and H4 (279). The need for different HAT containing complexes in the presence of different transcription factors would support the observation that we do not visualize a P/CAF effect on HTLV-1 transcription in vitro. Thus, the HAT activity of p300 is most likely sufficient to mediate Tax transactivation and dual HAT activity is potentially unnecessary to remodel chromatin from the HTLV-1 promoter as evidenced by the lack of synergistic effects when P/CAF is added to p300/Tax/CREB mediated transcription in vitro and in vivo (Fig. 4.5.B; 153). Promoter dependence is another factor dictating the requirement for the P/CAF and/or p300 HAT domain for transcriptional activity. Whereas the HAT domain of P/CAF is necessary for myogenic transcription and differentiation (259, 278), for Fetal Kruppel-like factor DNA binding and transcriptional activation (307), and for retinoic acid receptor dependent transcription (30, 175), the p300 HAT activity is dispensable.

An alternate explanation for the lack of P/CAF effect on Tax-mediated transcription is that there is adequate amounts of endogenous P/CAF present in the CEM nuclear extract. This endogenous P/CAF could be contributing to the Tax/CREB effect seen above basal transcription. Jiang et al. (153) found that P/CAF was present in similar amounts when comparing HTLV-1 negative nuclear extracts (CEM and Jurkat) to HTLV-1 positive nuclear extracts (C81 and MT2). Research by Harrod et al. (124) suggests that p300 is able to form a multimeric complex with P/CAF, Tax, and CREB at the viral promoter using a DNA pulldown assay. This result would support the hypothesis that p300 forms a multimeric complex with Tax, CREB, and P/CAF using the endogenous P/CAF present in the nuclear extract. Alternatively, P/CAF was not found at the HTLV-1 promoter in vivo using the chromatin immunoprecipitation assay (191); however, this result could be attributed to a masked epitope rather than an actual lack of P/CAF at the promoter.

Interestingly, P/CAF purified in its native form is complexed with 20 associated polypeptides (236, 337). This 400 kDa, native P/CAF complex includes subunits for the human forms of the yeast transcriptional cofactors, ySPT3, yADA2, and yADA3 (337). In addition, this P/CAF histone acetyltransferase complex contains histone-like TBP-associated factors (TAFs) as well as polypeptides containing significant sequence homology to other TAFs, such as $hTAF_{II}15$, $hTAF_{II}20$, $hTAF_{II}30$, and $hTAF_{II}31$ (337). This P/CAF complex was able to acetylate core histones in a nucleosomal context, while recombinantly purified P/CAF was only able to acetylate free core histones (236). This result suggests that there are other key factors present in the P/CAF complex that are responsible and necessary for mediating acetylation of chromatin-assembled DNA. Thus, it is possible that the recombinant purified P/CAF will not enhance activation from chromatin-assembled HTLV-1 templates in an in vitro transcription assay because P/CAF is lacking a necessary subunit. Studies done by Vassilev et al. (337) would also support the transient transfection assays used to examine the effects of exogenous P/CAF on Tax transactivation. The native P/CAF complex would be present in cell lines used in these transient transfection assays; thus, P/CAF is able to enhance Tax/CREB mediated transcription in vivo as opposed to the in vitro results suggesting exogenous baculovirus-expressed P/CAF has no effect on Tax-activated transcription (153).

Lastly, p300 and P/CAF interact with different forms of RNA polymerase II. p300 has been shown to directly interact with the initiation-competent, nonphosphorylated form of RNA polymerase II, while P/CAF directly interacts with the elongation-competent, hyperphosphorylated form of it (53). Thus, p300, rather than P/CAF, is potentially responsible for recruiting RNA polymerase II and the general transcription machinery to the promoter for activated transcription. Time course experiments examining the dynamics of estrogen receptor mediated transcription complex assembly found that p300, CBP, and P/CAF were recruited to the promoter in a step-wise fashion with p300 specifically involved in transcription initiation (289). These results suggest that P/CAF may have a distinct role in the estrogen receptor mediated transcription; thus, it is also possible that P/CAF is not recruited to other promoters, such as the HTLV-1 promoter, until after transcription initiation has taken place.

Based upon the above mentioned observations, it is not altogether surprising that recombinantly purified P/CAF does not have any effect on Tax/CREB mediated transcription. One could test the hypothesis that the native P/CAF acetyltransferase complex would be active for HTLV-1 chromatin-assembled templates by purifying this P/CAF complex from FLAG-epitope tagged P/CAF-expressing HeLa cells. One could then add this native complex to an HTLV-1 chromatin-assembled in vitro transcription assay and determine if one sees an effect on Tax transactivation. Additionally, one could compare this P/CAF-depleted nuclear extract with HeLa nuclear extract containing a full-complement of P/CAF to examine what contribution, if any, endogenous P/CAF has on Tax/CREB mediated transcription in a chromatin-context. One could also examine the effects of recombinant purified P/CAF on another chromatin-assembled template, such as a p53-responsive template, to determine if this form would activate another transcription factor that it is known to interact with. p53 directly interacts with P/CAF and is also acetylated by P/CAF at lysine 320 (204). p53-responsive chromatin-assembled templates have been shown to be enhanced by the addition of p300 (76, 205); thus, this template would make an attractive control to test P/CAF activated transcription.

4.6 Acknowledgements

We thank Y. Nakatani (Harvard University) for the baculovirus stocks of human P/CAF and three deletion mutant forms of human P/CAF, P/CAF ΔN (aa Δ1-465), P/CAF ΔC (aa Δ529-832), and P/CAF ΔHAT (aa Δ445-512).

Chapter 5

Tax Is Unable To Activate Transcription From Nucleosomal-Assembled HIV-1 DNA In Vitro

The human T-cell leukemia virus type-1 (HTLV-1) virally encoded oncoprotein Tax is known to activate transcription from the human immunodeficiency virus type-1 (HIV-1) LTR in vivo, through activation of the NF-κB pathway. Thus, we hypothesized that Tax would activate transcription from nucleosomal-assembled HIV-1 DNA in vitro. We were also interested in determining if the HIV-1 virally encoded protein Tat in combination with Tax would activate transcription from nucleosomal-assembled HIV-1 DNA templates. Transcriptional activation from the HIV-1 promoter in vitro has been well characterized; thus, it would make an effective biochemical system to examine the role of Tax transactivation from the HIV-1 LTR in vitro.

5.1 Abstract

The human immunodeficiency virus type 1 (HIV-1) genome is naturally packaged into chromatin and is able to integrate into multiple sites within a host-cell genome. The p65 subunit of NF-κB in combination with other enhancer and promoter binding proteins such as LEF-1, Ets-1, and Sp1 generates strong transcriptional activation of the chromatin assembled HIV-1 DNA. HIV-1 transcription is also regulated by the virally encoded protein Tat. In this study, we examined the role of the human T-cell leukemia virus type 1 virally encoded protein Tax in transcriptional activation of the HIV-1 LTR. Tax is known to activate transcription from the HIV-1 LTR in vivo, through activation of the NF-κB pathway. Interestingly, we found that Tax was sufficient to mediate transcription from unassembled HIV-1 DNA; however, Tax alone was insufficient to derepress transcription from nucleosomal-assembled HIV-1 DNA.

5.2 Introduction

Human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus that integrates its genome into the host chromatin. Viral gene expression of the integrated HIV-1 genome is regulated by the 5' long terminal repeat (LTR) of the proviral genome. HIV-1 enters CD4+ T lymphocytes, where increased viral replication leads to progressive immunodeficiency in infected individuals. HIV-1 encodes a regulatory activator protein, Tat, which is important for RNA polymerase II transcription. Tat is an 86 amino acid polypeptide that binds to an RNA hairpin called TAR (trans-activating response element) found at the 5' termini of viral transcripts (reviewed in 82). Tat enhances the processivity of RNA polymerase II and increases efficient elongation through its interaction with cyclin T, cyclin T1, and CDK9 of the pTEFb complex (128, 129, 345, 353). This interaction leads to phosphorylation of to the carboxyl-terminal domain of RNA polymerase II, generating enhanced elongation of the viral promoter and, thus, Tat transactivation (210, 212, 368).

DNase I hypersensitive sites, indicating regions of accessible chromatin, have been identified in the integrated HIV-1 genome (338, 339). Five major hypersensitive sites were identified in a chronically infected cell line that generates a low level of transcription, two of which are found in the U3 region of the 5' LTR (338). Nucleosome positions in the 5' LTR have also been well characterized. There are two nucleosomes positioned in the 5' LTR: nuc-0 spans nucleotides 40 to 200 associated with the enhancer elements while nuc-1 spans nucleotides 465 to 610 associated with the RNA start site (whereby nucleotide position 0 is the beginning of the U3 region) as shown in figure 5.1 (339). Upon transcriptional activation of the HIV-1 promoter, nuc-1 becomes the only nucleosome that is repositioned or rearranged which allows access to the RNA start site.

In vitro chromatin assembly of the HIV-1 promoter has been well characterized. The binding sites for transcription factors in the 5' LTR of HIV-1 are defined as four functional domains: the negative regulatory element (NRE), the distal and proximal enhancer region, the core promoter and the trans-activating response (TAR) element (reviewed in 156, 331). In particular, the distal and proximal enhancer region contains DNA-binding sites for such transcription factors as NF-κB, LEF-1, and Ets-1 (331). The core promoter consists of the TATA box as well as Sp1 binding sites (331). The TAR element is responsible for forming a stem-loop RNA structure that interacts with Tat, yielding increased transcriptional activity of the HIV-1 promoter in vivo (81, 272). A

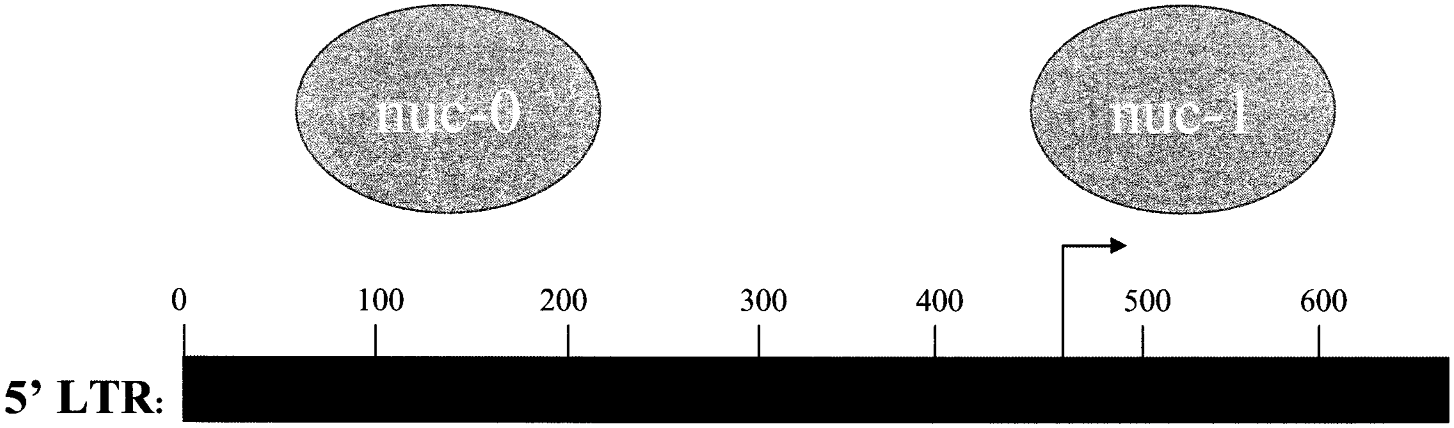


Figure 5.1. **Nucleosome position sites on the HIV-1 5' LTR**. Nuc-0 spans nucleotides 40 to 200 associated with the enhancer elements while nuc-1 spans nucleotides 465 to 610 associated with the RNA start site (whereby nucleotide position 0 is the beginning of the U3 region).

study done by Sheridan et al. (293) suggested that LEF-1 and Ets-1 had no stimulatory effect on HIV-1 transcription from unassembled templates; however, the combination of LEF-1, Ets-1, TFE-3 (an E-box binding protein), and Sp1 yielded a 200- to 450-fold derepression of the HIV-1 promoter in a chromatin-dependent manner. Further studies were done by Pazin et al. (249) to characterize the role of two tandem binding sites for NF-κB in the HIV-1 promoter and the importance of different subunits of NF-κB in mediating transcription. Pazin et al. (249) found that the p65 subunit of NF-κB, but not the p50 subunit of NF-κB, enhanced transcription of chromatin-assembled HIV-1 DNA. Additionally, researchers found that p65 in the presence of Sp1 and other enhancer binding factors (i.e., LEF-1) enhance transcription synergistically (249). The addition of Sp1 alone to the HIV-1 chromatin assembled template was not able to activate transcription (249). Both of these studies point to the importance of trans-activating factors in mediating transcription from nucleosomal-assembled HIV-1 DNA as well as their importance in the recruitment of RNA polymerase II general transcription factors.

Initial studies have alluded to the importance of histone acetyltransferases for activation of the integrated HIV-1 provirus. Tat forms a ternary complex with CBP/p300 and P/CAF, both histone acetyltransferases, which is required for Tat transactivation (25, 136, 215). Acetylation of Tat by coactivators, CBP/p300 and P/CAF, has also proven to be an important step in the regulation of HIV-1 transcription from chromatin-assembled templates (69, 168, 243). Tat is acetylated by p300 at lysine residue 50 in its TAR binding domain, and by P/CAF at lysine residue 28 in its activation domain (168). These two distinct acetylation sites within Tat have different consequences on its regulation. p300 acetylation of Tat dissociates Tat from TAR and P/CAF acetylation of Tat regulates binding of Tat to the pTEFb complex. Histone deacetylase inhibitors have also been shown to strongly induce HIV-1 enhancer activity from nucleosomal DNA templates in vitro (292). Additionally, these inhibitors are known to increase virus production at the transcription level from latently infected T cells in vivo (332). These studies underline the importance of histone acetylation for nucleosome remodeling and recruitment of the RNA polymerase II general transcription machinery to the HIV-1 promoter.

The human T-cell leukemia virus type 1 (HTLV-1) is another complex retrovirus that integrates into the host cell genome, although the site of HTLV-1 integration is not known. The molecular basis of HTLV-1-associated diseases is strongly linked to the expression of the virally-encoded Tax protein (195). Tax is similar to Tat in that it is a potent transcriptional activator that stimulates HTLV-1 viral gene expression leading to high-level virion production in the infected T cell. Several studies have suggested that Tax is able to mediate transactivation from the HIV-1 promoter. More specifically, both NF-κB binding sites within the 5' LTR of HIV-1 are critical for Tax-mediated HIV-1 gene expression, as transactivation with only one NF-κB binding site severely reduced this expression (32, 298, 369). An initial study by Smith and Greene (303) suggested that different regions of Tax are responsible for mediating activation from either the HIV-1 promoter or the HTLV-1 promoter. Smith and Greene (303) found that the double point mutant at amino acids 137 and 138 (G137A/L138S) activated the HTLV-1 promoter, but was unable to activate transcription from the HIV-1 LTR. Semmes and Jeang (288) then expanded on these results by analyzing single amino acid changes in Tax and their ability to mediate transcription from the HIV-1 LTR versus the HTLV-1 LTR. Semmes and Jeang (288) found that point mutations at amino acids 113, 160, and 258 completely eliminated Tax transactivation from the HIV-1 promoter. Researchers concluded that this 160 to 258 amino acid region of Tax was specific for mediating activation from the NF-κB containing promoter, as activation from the HTLV-1 LTR using these mutants appeared as wild-type.

In this study, we analyzed the ability of Tax, alone or in combination with Tat, to enhance transcription from nucleosomal-assembled HIV-1 DNA in vitro. Transcriptional activation from the HIV-1 promoter has been well characterized; thus, it would make an effective biochemical system to confirm the in vivo transient cotransfection results using Tax and the HIV-1 promoter. Tax has previously been shown to interact with both the p50 and p65 subunits of NF-κB (311) (J. Livengood, personal communication). Thus, this interaction may be important in mediating transcription from the HIV-1 promoter. We find that Tax is able to activate transcription from the unassembled HIV-1 promoter. However, we were unable to detect Tax transcriptional activation from chromatin-assembled HIV-1 templates. These studies outline the initial steps that were taken to analyze the role of Tax transactivation from the HIV-1 LTR. Future directions for this project could include adding Tax during different time points during chromatin assembly, or include coactivators during the assembly process. Other directions for this project could include analyzing transcription from a promoter containing multiple copies of NF-κB binding sites.

5.3 Materials and Methods

5.3a Expression and purification of recombinant proteins. The plasmid for GST-Tat was obtained from the AIDS Reagent and Reference Program (NIH). GST-Tat was purified as previously described using glutathione agarose chromatography (280). The plasmid for His_6-tagged Tat was a gift from A.Vendel (Colorado State University), and was purified using nickel-affinity chromatography as previously described (280). Tax was expressed from the $pTaxH_6$ expression plasmid (366), and purified as previously described (101). The plasmids for the NFκB subunits p65 and p50 were a generous gift from Dimitri Thanos (Columbia University) and were purified under denaturing conditions using nickel-affinity chromatography as previously described (320). p65 and p50 subunits were renatured slowly by dialysis against buffer B (500 mM NaCl, 20 mM HEPES-KOH (pH 7.9), 0.1% NP-40, 0.2 mM EDTA, 1 mM dithiothreiotol, 0.5 mM phenylmethylsulfonyl fluoride) containing 6, 4, 2, 1, 0.5 and 0 M UREA, followed by dialysis in TM buffer (50mM Tris-HCl [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA [pH8.0], 1mM dithiothreiotol, 0.1% [vol/vol] Tween-20, 20% [vol/vol] glycerol). Sp1 was purchased from Promega. Purified proteins were stored at -70°C in TM buffer.

Drosophila core histones were purified as previously described (39). *Drosophila* NAP-1 (dNAP-1) (His_6-tagged) was expressed from recombinant baculovirus (142) and purified first by nickel chelate chromatography followed by +Source 15Q-column chromatography as previously described (96, 144). We co-expressed FLAG-tagged ISWI and Acf1 from baculovirus, and purified the complex by anti-FLAG affinity batch binding and elution as previously described (145).

5.3b Recombinant plasmids and in vitro transcription assays. The HIV+TAR/G-less cassette carries the transcription elements located upstream of the HIV-1 core promoter, generating a 400-nt RNA transcript (212, 367). Preinitiation complexes were formed on 150 ng of DNA template (or 100 ng where indicated) in TM buffer (supplemented with

10 μM acetyl CoA [Sigma]) by the addition of the indicated amount of Tax and 12 μg CEM nuclear extract (73) in a final reaction volume of 30 μl. The reactions were incubated for 60 minutes at 30°C. RNA synthesis was initiated by the addition of 250 μM ATP, 250 μM CTP, 12 μM UTP, and 0.8 μM [α-^{32}P] UTP (3,000 Ci/mmol) and incubated for an additional 35 minutes at 30°C. Transcription reactions were processed and analyzed as previously described (194). Molecular weight markers (radiolabeled *Hpa* II digested pBR322) were used to estimate the size of the RNA products. For chromatin-assembled in vitro transcription assays, the supercoiled HIV+TAR/G-less plasmid was assembled into chromatin using dNAP-1, dAcf1 and *Drosophila* histones at the indicated histone:DNA ratio. Following chromatin assembly, preinitiation complexes were formed on the equivalent of 150 ng of the plasmid DNA in the absence or presence of the indicated amounts of Tax, p65 homodimer, p50/p65 heterodimer, Sp1, and/or GST-Tat. All reactions contained 100 μM acetyl CoA (USB) and the remaining steps were followed as described above.

5.3c Chromatin assembly and topological assay. Nucleosomes were assembled on the HIV+TAR/G-less plasmid as previously described (96, 144). Following the addition of the DNA, ATP (3 mM), creatine phosphokinase (1 μg/ml), and phosphocreatine (30 mM) were added in a 70 μl reaction containing 10 mM HEPES (K^+), [pH 7.6], 50 mM KCl, 5 mM $MgCl_2$, and 5% (v/v) glycerol. Briefly, histone octamers were preassembled with dNAP-1 (8:1 dNAP-1/core histones) on ice for 30 minutes. Assembly reactions were incubated for 4 hours at 27°C. DNA topological assays were performed as previously described (96). The samples were analyzed on a 1% agarose gel, and the degree of supercoiling was visualized by Sybr Gold (Molecular Probes) staining.

5.4 Results

5.4a Tax transactivation on unassembled HIV-1 DNA templates

We were interested in examining the potential for Tax activation, as well as Tat activation, from unassembled HIV-1 DNA templates in vitro. We first examined Tax transactivation in an in vitro transcription assay, using a plasmid carrying the transcription elements of the 5'LTR of the HIV-1 promoter driving synthesis of a G-less cassette (212, 367). A schematic representation of the promoter construct is shown in figure 5.2. We performed in vitro transcription assays using CEM (an HTLV-1- and HIV-1-negative human T-lymphocyte cell line) nuclear extracts. Figures 5.3.A and 5.3.B show the results of the in vitro transcription experiments on naked HIV-1 templates. The addition of Tax produced an increase in transcription from the HIV+TAR/G-less template when using either 100 ng or 150 ng of template DNA (18- and 20-fold, respectively) (Fig. 5.3.A, lanes 2 and 4). We also examined the effect of increasing amounts of Tat on transcriptional activation from the HIV+TAR template. The addition of Tat produced an increase in transcription from the HIV+TAR/G-less template (5-fold) (Fig. 5.3.B, lanes 1 and 2). This result is consistent with that found in Zhou and Sharp (367) in which a 5-fold effect over basal transcription was seen in the presence of Tat. Tax in combination with Tat did not enhance the level of transcription (Fig. 5.3.B, lane 7). This result was somewhat unexpected as previous in vivo results suggest that Tax in the presence of Tat exhibit synergistic effects from the HIV-1 promoter (52).

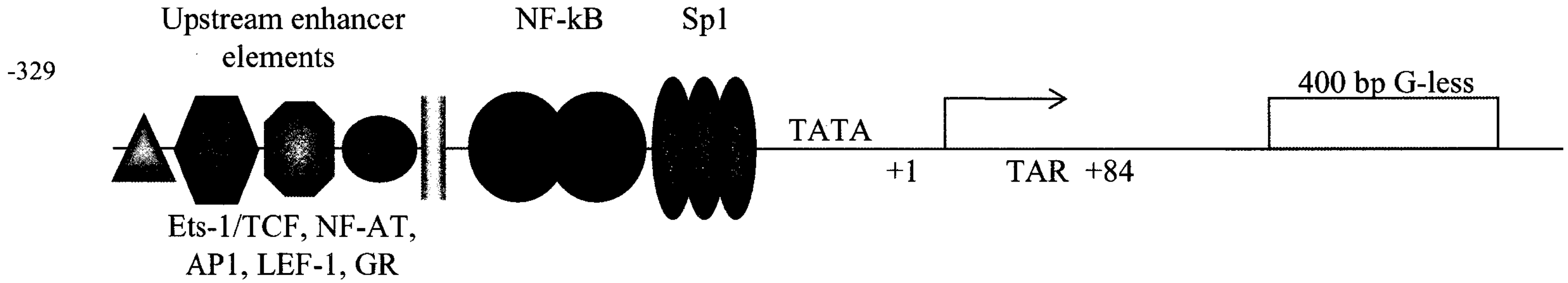


Figure 5.2. **Schematic representation of the HIV-1 5' LTR/ G-less promoter**. Transcription factor binding sites are indicated.

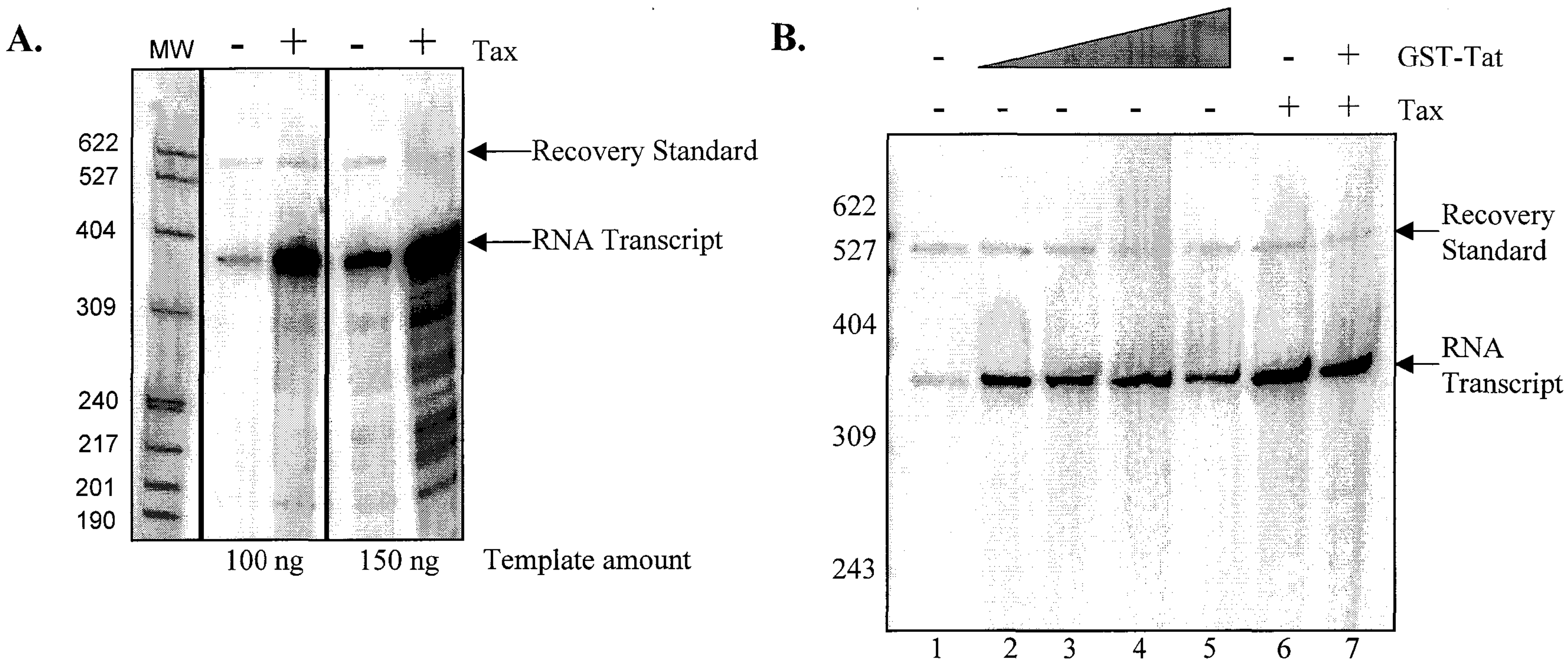


Figure 5.3. **Tax is a potent activator of HIV-1 transcription from unassembled templates in vitro**. (A) Tax transactivation on unassembled DNA templates. The in vitro transcription assay was performed on a HIV+TAR/G-less cassette (27, 51) template that carries the upstream negative regulatory element, proximal and distal elements, core promoter, and TAR region driving expression of a 400-nt RNA (shown schematically in figure 5.2). Transcription reaction mixtures contained the HIV+TAR/G-less template (100 ng or 150 ng, where indicated), CEM nuclear extract (12 μg), and purified recombinant Tax (100 ng) (lanes 2 and 4). All reactions were performed in the presence of acetyl-CoA. The full-length 400-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated. This experiment is representative of three independent experiments. (B) Transcriptional activation on HIV+TAR/G-less unassembled templates was analyzed as described above, in the presence of Tat (100 ng) (lanes 2 to 5 and lane 7) and in the presence of Tax (100 ng) (lanes 6 and 7). All reactions were performed in the presence of acetyl-CoA. The full-length 400-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated. This experiment is representative of two independent experiments.

5.4b Tax does not stimulate transcription from the HIV-1 promoter in a chromatin context

The HIV-1 genome is naturally integrated into chromatin following retroviral infection, and the structure of the integrated HIV-1 provirus is well characterized. Thus, it is of interest to determine if Tax is able to activate viral transcription from HIV-1 nucleosomal DNA. Nucleosomes have been shown to strongly repress transcription from the HIV-1 promoter (70, 249, 292, 293). The addition of transcription factors, such as NF-κB, Sp1, and Ets-1; coactivators, such as p300 and P/CAF, and inhibitors of histone deacetylases, have been shown to counter this repression leading to increased transcriptional activation. It was of interest to then ask if exogenous Tax is able to counter this repression or if Tax acts in a cooperative manner with other transcription factors, such as NF-κB, to mediate transcription from an HIV-1 promoter in a chromatin context. Chromatin assembly of the HIV+TAR/G-less template was performed using *Drosophila* core histones and the recombinant *Drosophila* assembly proteins Acf1/ISWI and dNAP-1(nucleosome assembly protein-1), an H2A-H2B histone chaperone (145). The ACF complex is involved in chromatin assembly as well as in nucleosome remodeling. These assembly proteins are sufficient for the ATP-dependent formation of evenly spaced nucleosomal arrays (143, 145). A DNA topological assay is used to determine the proper histone to DNA ratio used in a chromatin assembly that yields full assembly of nucleosomes onto the DNA. This analysis was used to determine the efficiency of nucleosome deposition on the HIV+TAR/G-less template. The assembly of HIV-1 transcription templates is optimized using increasing ratios of histones to DNA (w/w), in which the assembly of the nucleosomes can be visualized by an increase in

DNA supercoiling. As shown in the top panel of figure 5.4.A, increasing ratios of histone octamers to DNA, lead to a concomitant increase in the supercoiling of the DNA, with complete assembly at a ratio of 1.0:1.0 (lanes 2 to 8). We also examined the assembly of HIV-1 templates in the presence of the NF-κB p50/p65 heterodimer to determine if the presence of transcription factor(s) during chromatin assembly affected the deposition of nucleosomes onto the DNA. As shown in the bottom panel of figure 5.4.A, the addition of 150 ng of the NF-κB p50/p65 heterodimer to the assembly reaction did not appear to change the ratio for complete assembly of the HIV+TAR/G-less template (lanes 2 to 8).

The increase in supercoiling, which is indicative of nucleosomes deposited onto the DNA, can also be visualized using the in vitro transcription assay. Following chromatin assembly, an in vitro transcription assay is performed to examine the effect that increased nucleosome deposition has on HIV-1 basal transcription. As shown in figure 5.4.B, the increasing concentrations of histone octamers present on the HIV+TAR/G-less plasmid lead to complete repression of basal activated transcription at the ratio of 0.6:1.0 (lane 4). Also shown in figure 5.4.B, the repression was not consistent from one histone:DNA ratio to the next (compare lanes 4 and 5). The fact that we were never consistently able to see repression at the histone:DNA ratio of 0.6:1.0 suggests that there is a problem with the chromatin assembly reaction. Nonetheless, we performed the in vitro transcription assay as described above at the indicated histone:DNA ratios, using nuclear extracts from CEM cells as a source of basal transcription factors and RNA polymerase. As shown in figure 5.4.C, exogenous Tax was unable to derepress the HIV-1 chromatin-assembled template (lane 3, 7, and 11). Also shown in figure 5.4.C,

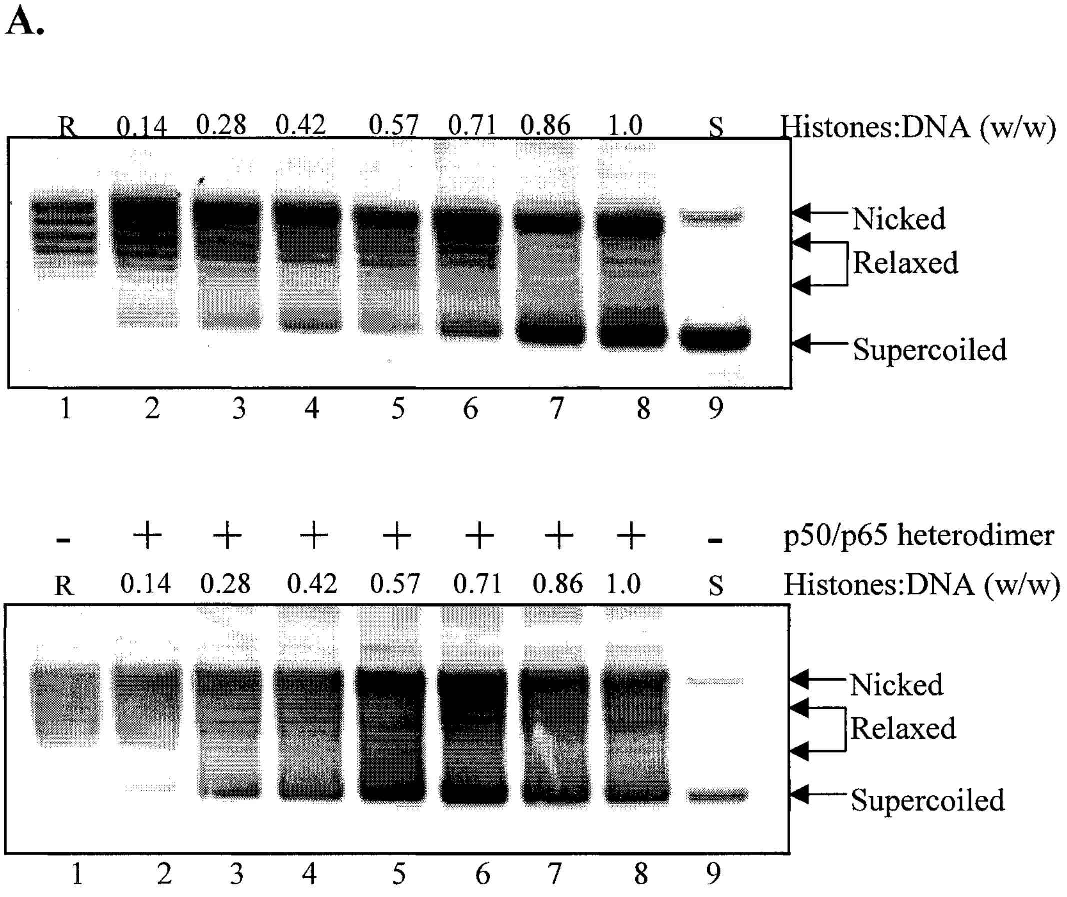


Figure 5.4. **Exogenous Tax does not stimulate transcription from the HIV-1 DNA template.** (A) Chromatin assembly of the HIV+TAR/G-less template in the presence and absence of p50/p65 heterodimer. *Top panel.* One-dimensional DNA topological assays showing the HIV+TAR/G-less transcription template assembled with *Drosophila* core histones in the presence of dAcf1/ISWI and dNAP-1. *Bottom panel.* One-dimensional DNA topological assays showing the HIV+TAR/G-less transcription template assembled in the presence of p50/p65 heterodimer (150 ng) with *Drosophila* core histones in the presence of dAcf1/ISWI and dNAP-1. The DNA topoisomers were resolved on an agarose gel, and the DNA stained with Sybr Gold (Molecular Probes). The supercoiled (S), relaxed (R), and nicked DNA populations, and the histone/DNA ratio, are indicated.

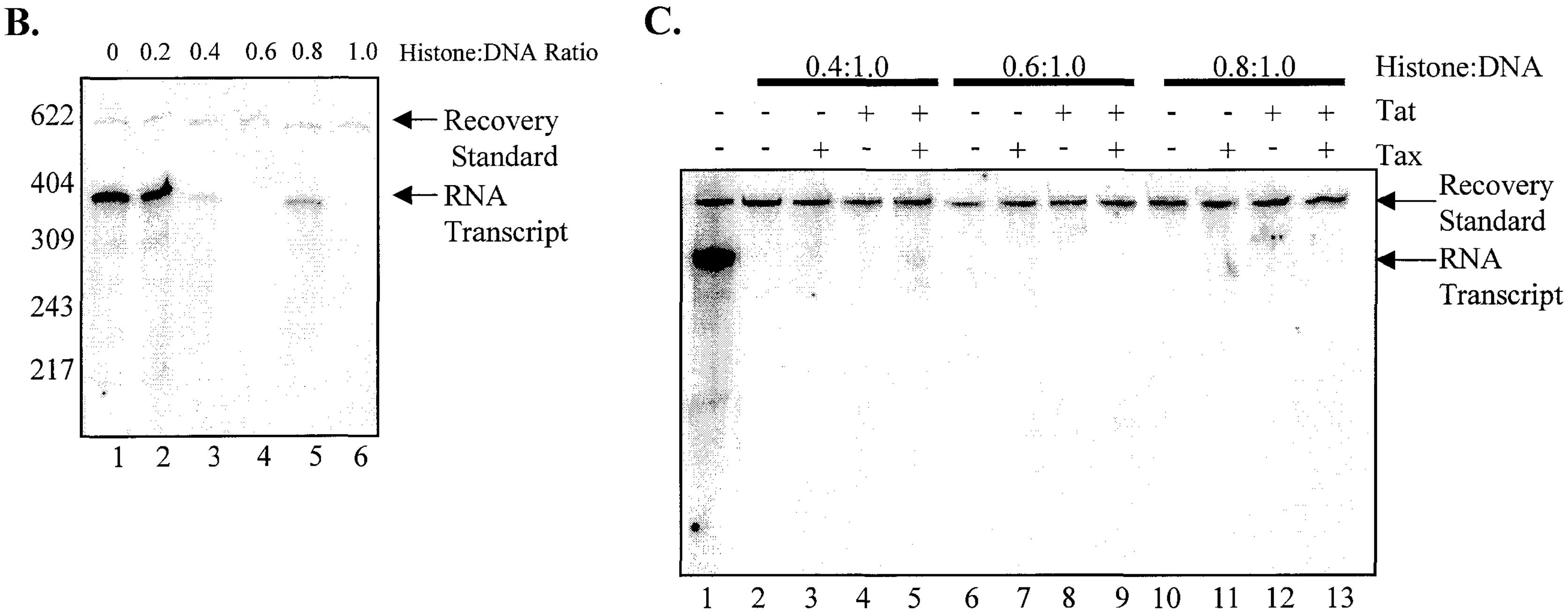


Figure 5.4. (B) Increasing amounts of core histones were deposited on the HIV+TAR/G-less template and basal transcription was assayed. The histone:DNA ratios (w/w) assayed are indicated. Basal transcription from an unassembled template was assayed as a positive control (lane 1). The full-length 400-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated. (C) Transcriptional activation on HIV+TAR/G-less chromatin templates was analyzed in the presence of Tax (75 ng, lanes 3, 5, 7, 9, 11, and 13) and His$_6$-tagged Tat (100 ng, lanes 4, 5, 8, 9, 12, and 13) at the indicated histone:DNA ratios. Basal transcription from an unassembled template was assayed as a positive control (lane 1). The full-length 400-nt RNA transcript and labeled DNA recovery standard are indicated.

exogenous Tat was unable to derepress the HIV-1 chromatin-assembled template (lane 4, 8, and 12).

Because exogenous Tax was unable to counter the repressive effects of nucleosomes on the HIV+TAR/G-less template, we added other transcription factors to the reaction that have previously been shown to counter the repressive effects of nucleosome deposition. All subsequent experiments were performed in the presence of acetyl CoA and in the presence or absence of exogenous Tat, p65 homodimer, p50/p65 heterodimer, and/or Sp1. The addition of Sp1 and p65 to unassembled HIV-1 DNA templates yielded a 2-fold stimulation in transcription levels (Fig. 5.5.A, lanes 7 and 8). Figure 5.5.A shows that preincubating the p65 homodimer, Sp1, and 5 μM trichostatin A (TSA) with the HIV+TAR/G-less template (incubated on ice 20 minutes prior to addition to the chromatin assembly reaction) strongly stimulated transcription 77-fold above basal transcription levels (lane 5). This p65/Sp1 stimulation effect was not observed in the absence of exogenous TSA in the 20 minute preincubation step with the HIV+TAR/G-less template (Fig. 5.5.A, lane 4). The addition of p65 heterodimer and Sp1 also enhanced transcription in the absence of TSA when added post-assembly to the transcription reaction (127-fold, Fig. 5.5.A, lane 6). Thus, the combination of Sp1 and p65 stimulated transcription synergistically, consistent with studies done by Pazin et al. (249) (Fig. 5.5.A, compare lanes 3 and 5). However, we did not see the same potent activation of the HIV-1 chromatin templates in our study using the p65 and Sp1 as compared with the activation found in Pazin et al. (249). These differences in relative amounts of transcription (2000-fold in Pazin et al. (249) versus 77-fold in the present study) could be attributed to different sources of Sp1 and p65. The p65 used in the Pazin

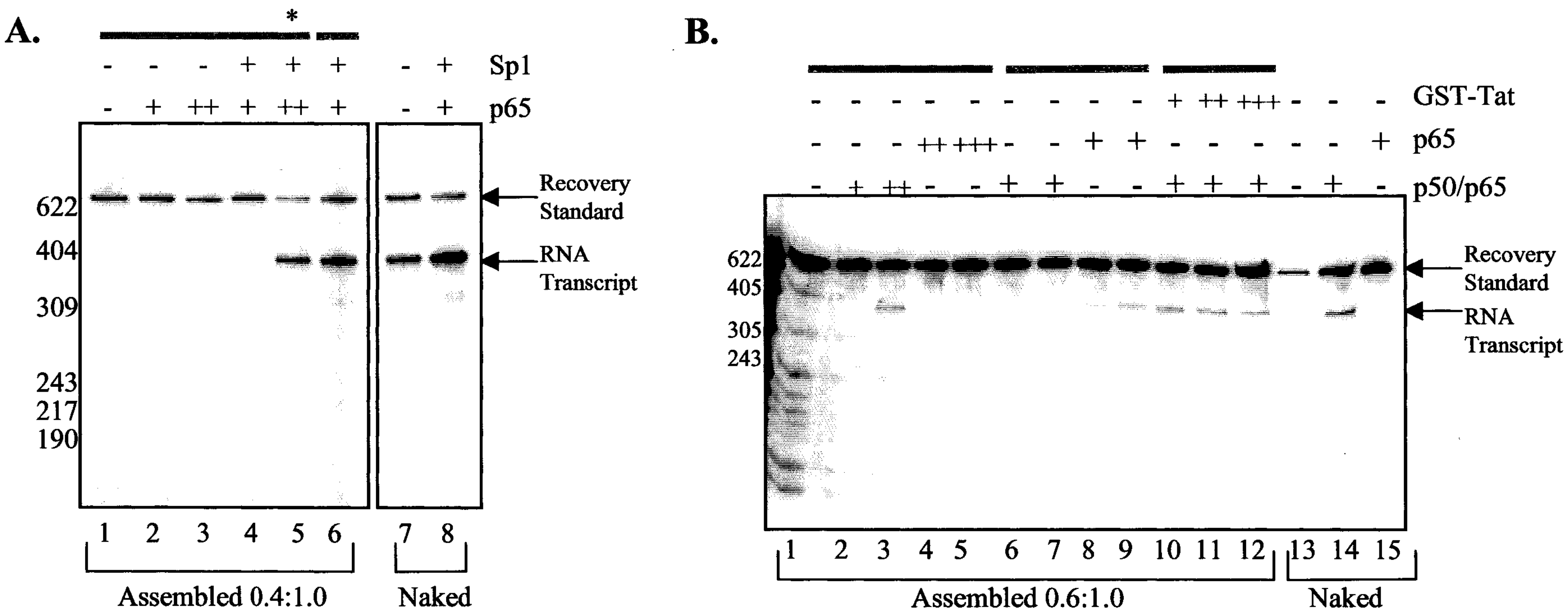


Figure 5.5. **The NF-κB p65 subunit in combination with Sp1, but not Tax, is a potent activator of HIV-1 chromatin-assembled transcription templates.** (A) Transcriptional activation on HIV+TAR/G-less chromatin templates was analyzed in the presence of p65 homodimer (+ and ++: 100 ng and 200 ng) (lanes 2 to 6, 8) and Sp1 (129 ng) (lanes 4 to 6, 8) at a histone:DNA ratio of 0.4:1.0. The p65 and Sp1 purified proteins that were preincubated with the HIV-1 DNA on ice for 20 minutes prior to addition to chromatin assembly are indicated. Naked DNA templates were preincubated with the indicated purified proteins and not subjected to chromatin assembly (lanes 7 and 8). The * represents the addition of 5 μM TSA. The full-length 400-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated. The blue line represents samples in which the transcription factors were preincubated with the DNA and the red line represents transcription factors added post-assembly. (B) Transcriptional activation on HIV+TAR/G-less chromatin templates was analyzed in the presence of p65 homodimer (+, ++, and +++: 37.5, 75, and 150 ng) (lanes 4, 5, 8, 9, and 15), p50/p65 heterodimer (+ and ++: 37.5 and 75 ng) (lanes 2, 3, 6, 7, 10 to 12, and 14), and GST-Tat (lanes 10 to 12) at a histone:DNA ratio of 0.6:1.0. The purified proteins that were preincubated with the HIV-1 DNA on ice for 20 minutes prior to addition to chromatin assembly are indicated. Naked DNA templates were preincubated with the indicated purified proteins and not subjected to chromatin assembly (lanes 13 to 15). The full-length 400-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers are indicated.

et al. (249) study was expressed and purified from HeLa cells infected with recombinant vaccinia virus. Thus, the p65 expressed in mammalian cells may contain posttranslational modifications that are not present in the bacterially expressed form. Additionally, Sp1 was purified from either Jurkat or HeLa nuclei as compared with the present studies done using commercially available Sp1.

The addition of GST-Tat to the transcription reactions was also pursued as a means to generate activated transcription from the HIV+TAR/G-less template. We compared p65 homodimer to p50/p65 heterodimer in the presence of GST-Tat to determine which transcription factor combination would be most active in our chromatin assembly transcription system. Figure 5.5.B is representative of the amount of transactivation detected above basal levels. This chromatin assembled transcription was performed as described above; however, we used HeLa nuclear extract as a source of RNA polymerase II general transcription machinery. Unfortunately, we were unable to consistently see derepression in the presence of p65 homodimer or p50/65 heterodimer in the absence of exogenous Sp1. Sp1 was not pursued further as a means for enhancing transcriptional activation (and no longer added to the transcription reactions) as Promega no longer produces it and, thus, quantities were extremely limited. As shown in figure 5.5.B, 75 ng of p50/p65 heterodimer was able to enhance transcription approximately 112-fold above basal levels while 37.5 ng of p50/p65 heterodimer had no detectable effect on transcription (compare lanes 2 and 3). Thus, when GST-Tat is added to the reactions in figure 5.5.B (lanes 10 to 12), it is unclear as to whether or not the Tat effect is due to a true effect of Tat on viral transcription or that the levels are the same as those found in the presence of p50/p65 heterodimer alone (lane 2). The Tat effect was not reproducible as the p50/p65 and p65 transcriptional activation of the HIV-1 enhancer was also inconsistent. We also compared the preincubation of the enhancer factors with the HIV+TAR/G-less template prior to addition to the assembly reaction versus the addition of enhancer factors post assembly; however, we could find no consistent pattern to definitively say that preincubation aids in the transcriptional activation. Thus, the addition of Tax to the chromatin-assembled transcription reaction was not pursued further, as we did not visualize consistent NF-κB-mediated transcription.

5.5 Discussion

In this study, we examined the role of Tax in transcriptional activation from HIV-1 chromatin assembled templates in vitro. We found that Tax alone was sufficient to regulate HIV-1 transcription from unassembled templates. We also found that the addition of Tax alone to nucleosomal HIV-1 templates was not sufficient to derepress transcription. Several alternate means to relieve the repressive effects of nucleosomes were examined to then study the role of Tax in the regulation of chromatin-assembled HIV-1 templates. We preincubated the p50 and p65 subunits of NF-κB, as well as with Sp1, with the HIV+TAR/G-less template prior to addition to the chromatin assembly reactions in order to generate transcription from the repressed basal state. We found that the addition of exogenous Tat to the NF-κB/Sp1 derepressed chromatin did not enhance transcription. Also, we did not consistently see repression using the same conditions from one day to the next; thus, it was hard to draw conclusive results because the repression was somewhat sporadic. Thus, conditions that generated activated transcription from chromatin assembled templates on one day would yield either no activation or yield incomplete repression on the following day. We also tried varying the histone:DNA ratio for chromatin assembly; however, the conditions that yielded complete repression on one day at times yielded incomplete repression on further attempts. There appears to be some variable either in the chromatin assembly reaction or in the transcription reaction that causes inconsistent results. Additionally, reagents that were found to be crucial for activating transcription in the chromatin context, such as Sp1, were limiting. Thus, we were unable to examine the addition of exogenous Tax to the NF-κB/Sp1 derepressed chromatin templates.

Inconsistent repression from the HIV+TAR/G-less template could be attributed to improper nucleosome positioning on the DNA. Previous studies using an HIV-1 promoter have used *Drosophila* S-190 extracts supplemented with native core histones for chromatin assembly (249, 292, 325). Different sources of assembly factors and core histones as well as other endogenous proteins present in the *Drosophila* S-190 extracts could possibly create differences in nucleosome positioning (39). We did not perform micrococcal nuclease digestion assays with the HIV+TAR/G-less template assembled into chromatin in the presence of native *Drosophila* core histones, so it is difficult to know if we were getting consistent, proper positioning of the nucleosomes. The DNA topological assay using the HIV-1 template and the above described chromatin assembly system appeared to yield assembly of nucleosomes onto the DNA; however, the reactions never appeared to be fully assembled in the topological assay (see Fig. 5.4.A and Fig. 5.4.B). If nuc-1 that is positioned over the RNA start site is not disrupted (339), it would be difficult to visualize true activation from the HIV-1 promoter. Also, the nuclease hypersensitive sites present when NF-κB and Sp1 are added to the assembly reaction are not altered when Tat is added to the reaction (347). This result suggests that NF-κB and Sp1 must be bound to the enhancer region first in order to see derepression of the promoter, to then exert their transactivation function. Only one study has suggested that Tat alone is able to activate transcription from HIV-1 nucleosomal assembled templates; however, the signal was relatively weak and remained virtually unchanged in the presence of the p300 histone acetyltransferase domain (70).

Once conditions have been established in order to consistently visualize transcriptional activation from the nucleosomal HIV-1 DNA, we could test the addition of coactivators, such as CBP/p300 and P/CAF, to the reaction. Treatment of chronically HIV-1-infected T cells with histone deacetylase inhibitors, such as sodium butyrate, trapoxin, and trichostatin A, generate a significant increase in HIV-1 expression, suggesting the importance of histone acetylation in HIV-1 transcriptional regulation (31, 108, 332, 333). In vitro experiments using chromatin-assembled HIV-1 DNA also suggests that trichostatin A specifically induces HIV-1 expression (292). These in vivo and in vitro results point to the importance of histone acetylation, and potentially, acetylation of Tat, as a means of HIV-1 transcriptional regulation. Thus, it would be of interest to then examine the role of exogenous p300 and P/CAF on enhancer activated transcription from nucleosomal DNA.

Other future directions for this project could include the addition of Tax at different time points during the assembly and the transcription reactions. It is possible that we did not find the right time frame for Tax addition to see the maximum possible enhancement of transcription by Tax. Additionally, we could combine Tax with the coactivators, CBP/p300 and/or P/CAF, to determine if their presence has any additive effects on transactivation. It would also be of interest to examine another promoter that contains multiple copies of the single NF-κB binding site, which is found in the HIV-1 LTR. This would negate the need for multiple enhancer binding factors, such as Sp1, LEF-1, and Ets-1, in the reaction and simplify the chromatin assembly system.

5.6 Acknowledgements

We thank D. Thanos (Columbia University) for the p65 and p50 NF-κB expression plasmids and A. Vendel for the GST-Tat expression plasmid.

Chapter 6

Future Considerations

The importance of multiple binding sites for Tax within CBP/p300 has yet to be determined. Independent interaction sites for Tax may be important as Tax is bound as a dimer; however, the domains on CBP/p300 that are accessible when CBP/p300 is folded in its native conformation are currently unknown. The study described in chapter 2 proposes that the CR2 domain is important for Tax transactivation both in vitro and in vivo. Interestingly, another study done by Nyborg and coworkers (101, 193) found that the KIX domain is also important for Tax transactivation both in vitro and in vivo. Both the KIX domain and the CR2 repress Tax transactivation by roughly 50% when added exogenously during a transient cotransfection assay. Thus, it would be interesting to add both the exogenous KIX domain and CR2 domain simultaneously to see if Tax transactivation was completely repressed.

We have also found that different domains of Tax are responsible for interacting with the independent domains of CBP/p300. The results described in chapter 2 propose that Tax interaction with the CR2 domain is mediated through its carboxyl-terminal transactivation domain, while Harrod et al. (125) established that the protease-sensitive region in the amino terminus of Tax was responsible for the interaction with KIX. The V89A Tax mutant (defective for an interaction with KIX) and the M47 Tax mutant (a double point mutant in its transactivation domain) were investigated for their ability to immortalize primary T lymphocytes in culture. The V89A Tax point mutant was competent for immortalization of human primary T lymphocytes ex vivo (266). The M47 Tax mutant was also functional for immortalization of CD4+ and CD8+ T lymphocytes also in culture (265). Perhaps these Tax mutants retained their ability to immortalize T lymphocytes because they were still able to recruit CBP/p300 through their alternate binding sites. Thus, it would be interesting to examine immortalization of T lymphocytes using a Tax mutant that was defective for both KIX binding and CR2 binding to determine if the immortalization process was dependent or independent of the Tax-CBP/p300 interaction.

Initial studies suggest that Tax binding to the KIX domain versus the CR2 domain may have important consequences on quaternary complex formation and Tax binding to the viral CRE DNA. Using a glutathione-S-transferase pulldown assay, we have found that the Tax interaction with KIX is notably enhanced in the presence of CREB and the viral CRE DNA (J. Mick, personal communication). On the other hand, the Tax interaction with CR2 is wholly independent of CREB and the viral CRE DNA (K. Scoggin, unpublished observations). While we have been unsuccessful in forming a quaternary complex with CR2-Tax-CREB-viral CRE DNA, as visualized by the electrophoretic mobility shift assay (EMSA), a study done by Harrod et al. (125) found that the M47 Tax transactivation mutant exhibited a roughly 50% reduction in p300 binding in an EMSA suggesting that Tax binding to CR2 does aid in the recruitment of p300 to the Tax-CREB-viral CRE DNA complex. These results suggest that there are important differences in the role of KIX binding versus CR2 binding to Tax both free in solution and in the context of the cell.

Tax interaction with the CR2 domain of CBP/p300 may also be important for Tax transactivation from non-HTLV-1 promoters. Tax has previously been shown to activate transcription from the HIV-1 promoter, mediated through activation of the NF-κB pathway (32, 288). We hypothesize that Tax binds to the p50 and p65 subunits of NF-κB and then recruits CBP/p300 through the CR2 region to mediate transactivation. To explore this hypothesis, we could assemble a promoter containing multiple NF-κB binding sites into chromatin, and then add exogenous Tax, CBP/p300 and/or the NF-κB subunits to see if transactivation was affected in a positive manner. If Tax transactivation from this NF-κB-chromatin assembled promoter was stimulated by the presence of exogenous p300, we could then ask if the CR2 domain in isolation acts as a dominant-negative in transcriptional activation. Additionally, the role of Tax and CBP/p300 could be examined from other cellular promoters such as c-Fos or tumor necrosis factor-α. A recent study by Tzagarakis-Foster et al. (326) suggests that estrogen receptor α and β as well as estradiol inhibits Tax transactivation in vivo from the tumor necrosis factor-α promoter; however, it was unclear as to how estradiol exerted this repression. One could hypothesize that estrogen receptor α and/or β competes with Tax for binding to the CR2 domain of CBP/p300; thus, acting as a dominant-negative repressor of Tax transactivation.

Lastly, while much research has focused on the importance of CREB in mediating Tax transactivation, other members of the CREB/ATF family may contribute to Tax transcriptional activation. Recent studies with ATF-4 (or CREB-2) and Tax have found that Tax enhances the binding of ATF-4 to the viral CREs and the addition of ATF-4 in vivo enhances Tax transactivation (91, 92). Thus, it would be of interest to examine ATF-4/Tax-mediated transcription in the presence and absence of exogenous p300 from HTLV-1 chromatin-assembled templates in vitro. One could then ask whether or not CR2 and/or KIX acts as a dominant negative regulator of transactivation suggesting that recruitment of CBP/p300 is mediated through either CR2 and/or KIX. The results described herein propose that Tax interaction with CBP/p300 is potentially mediated through independent mechanisms; thus, future research will shed more light as to the precise role of CR2-mediated versus KIX-mediated recruitment of CBP/p300 by Tax to HTLV-1 and non-HTLV-1 promoters.

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