NOTE TO USERS

DISSERTATION

REGULATION OF HTLV-1 TRANSCRIPTION AND DEREGULATED GENE EXPRESSION BY THE VIRALLY-ENCODED PROTEIN TAX

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JILL A. LIVENGOOD ENTITLED "REGULATION OF HTLV-1 TRANSCRIPTION AND DEREGULATED GENE EXPRESSION BY THE VIRALLY-ENCODED PROTEIN TAX" BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTORATE IN CELL AND MOLECULAR BIOLOGY

Committee on Graduate Work

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ABSTRACT OF DISSERTATION

REGULATION OF HTLV-1 TRANSCRIPTION AND DEREGULATED GENE EXPRESSION BY THE VIRALLY-ENCODED PROTEIN TAX

Human T-cell Leukemia Virus Type-1 (HTLV-1) is the etiological agent of Adult T-cell Leukemia/Lymphoma (ATLL) and Tropical Spastic Paraparesis/HTLV-1 Associated Myelopathy (TSP/HAM). Oncogenesis associated with HTLV-1 is linked to the virally encoded transcription factor Tax. In combination with other cellular transcription factors, Tax activates transcription by interacting with the cellular transcription factor CREB, and the cellular coactivator CBP/p300. In addition to activating transcription, Tax has also been shown to disrupt many cellular processes that may lead to malignant transformation.

We have identified a possible mechanism for the ability of Tax to deregulate cellular function. The tumor suppressor p53 is mutated in over 60% of all cancers; however, the majority of tumor cells transformed by HTLV-1 display wild-type p53. To regulate transcription, p53 and Tax both utilize the cellular coactivator CBP/p300. We identified a domain on CBP/p300 responsible for binding p53. Tax and p53 compete for binding to this domain, possibly sequestering CBP/p300 in the cell, and limiting p53 access to these critical coactivators.

The most studied function of Tax is to activate viral transcription. However, HTLV-1 transcription is a complex process that requires many

cellular transcription factors including Sp1. Sp1 has previously been shown to bind at two distinct locations on the HTLV-1 promoter. We demonstrate that Sp1 binds with the highest affinity to the Sp1 site located between the second and third viral CREs. Sp1 is detected at the chromosomally integrated HTLV-1 promoter in living cells in both the absence and presence of Tax. Sp1 activates transcription modestly in vitro and in vivo in the absence of Tax, and a double point mutation at the preferred Sp1 binding site strongly down-regulates basal level transcription. These studies suggest a role for Sp1 in basal, and Tax-independent transcription of HTLV-1.

Understanding the transcriptional activation of HTLV-1 is important for developing treatments for individuals who are infected with the virus. There are no current treatments for HTLV-1, and the median survival rate for a person diagnosed with ATL is roughly six months. Polyamides are DNA binding drugs designed to disrupt transcription factor binding and inhibit gene expression. We designed and synthesized six Tax-directed pyrole-imidazole polyamides specifically designed to block Tax binding to DNA at the HTLV-1 promoter. We found that four of these polyamides disrupt binding of the Tax/CREB complex in vitro, and that these same molecules also inhibit Tax-mediated transcription in vitro. However, of these four Tax/CREB-specific polyamides, only one polyamide appears to be uniquely Tax specific. We show that polyamides can enter the nuclei of HTLV-1 infected T-cells, and two of the four polyamides down-regulate virion production in these cells.

In addition to down-regulating the functions of p53, Tax has also been shown to deregulate cellular transcription through its effect on the functions of the cellular NF-κB family of proteins. The majority of the studies in the past have focused on the ability of Tax to target regulatory NF-κB proteins in the cytoplasm, which leads to an increase in the levels of NF-κB in the nucleus. However, colocalization studies have shown that Tax and NF- κB reside together in transcriptionally active nuclear bodies. We have studied the mechanisms of Tax deregulation via the NF-κB proteins by focusing on the events that occur directly at the promoters of NF-κB-responsive genes in an in vitro transcription system. We found no direct ability of Tax to deregulate the function of NF-κB at the NF-κB responsive promoter.

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Chapter 1

Review of the Literature

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

The human T-cell leukemia virus type 1 (HTLV-1) was the first pathogenic human retrovirus to be isolated and characterized (38, 242, 305). It was first discovered in a T-lymphoblastoid cell line generated from a patient who was incorrectly diagnosed with a cutaneous T-cell lymphoma (224). In 1981, a retrovirus was also discovered from a cell line generated from a patient with adult T-cell leukemia/lymphoma (ATLL) (103). These two cell lines were later found to contain identical viruses, and they were named human T-cell leukemia virus type 1 (225). HTLV-1 is a Type C retrovirus belonging to the genus *Deltavirus*, and the subfamily *Orthoretrovirinae* (33). Roughly 15 to 25 million people worldwide are infected HTLV-1, and between 2% to 5% of those infected develop an HTLV-1 associated disease.

1.1a Virology and genetic structure

HTLV-1 viral RNA is packaged with virally encoded envelope proteins that can bind the T-cell receptor to trigger viral uptake. For efficient transmission of HTLV-1, cell-cell contact is required (213), as only one out of a million cell-free virions are infectious (61). Recently, it was suggested that the ubiquitous vertebrate glucose transporter Glut-1 is the cellular receptor for HTLV-1 (183). However, the virus may require more than one cellular receptor for efficient infection. HTLV-1 has been shown to be transferred from an infected cell to an uninfected cell through an "immunological synapse" at which small domains of proteins mediate adhesion, antigen recognition, and secretion of cytokines (108). Mitotic transmission of the HTLV-1 provirus is also a preferred course of transmission, as there are little observed differences in DNA sequences among HTLV-1 isolates, indicating that replication occurs via the highly accurate cellular DNA synthesis machinery (109, 257).

Once the virus infects a host cell, the capsid proteins are released, which allows for the genome to be copied from single stranded RNA to double stranded DNA by its reverse transcriptase (as shown in figure 1.1). The viral polymerase uses a cellular tRNA primer to initiate replication from the 3' end of the viral genome. Intrinsic RNase H activity carried by the polymerase enables it to destroy the viral RNA after it is copied to DNA. Once the synthesis of the double stranded DNA is complete, the genome is imported into the nucleus of the cell and is randomly integrated into the host cell genome by the viral integrase enzyme. Efficient transcription of the viral

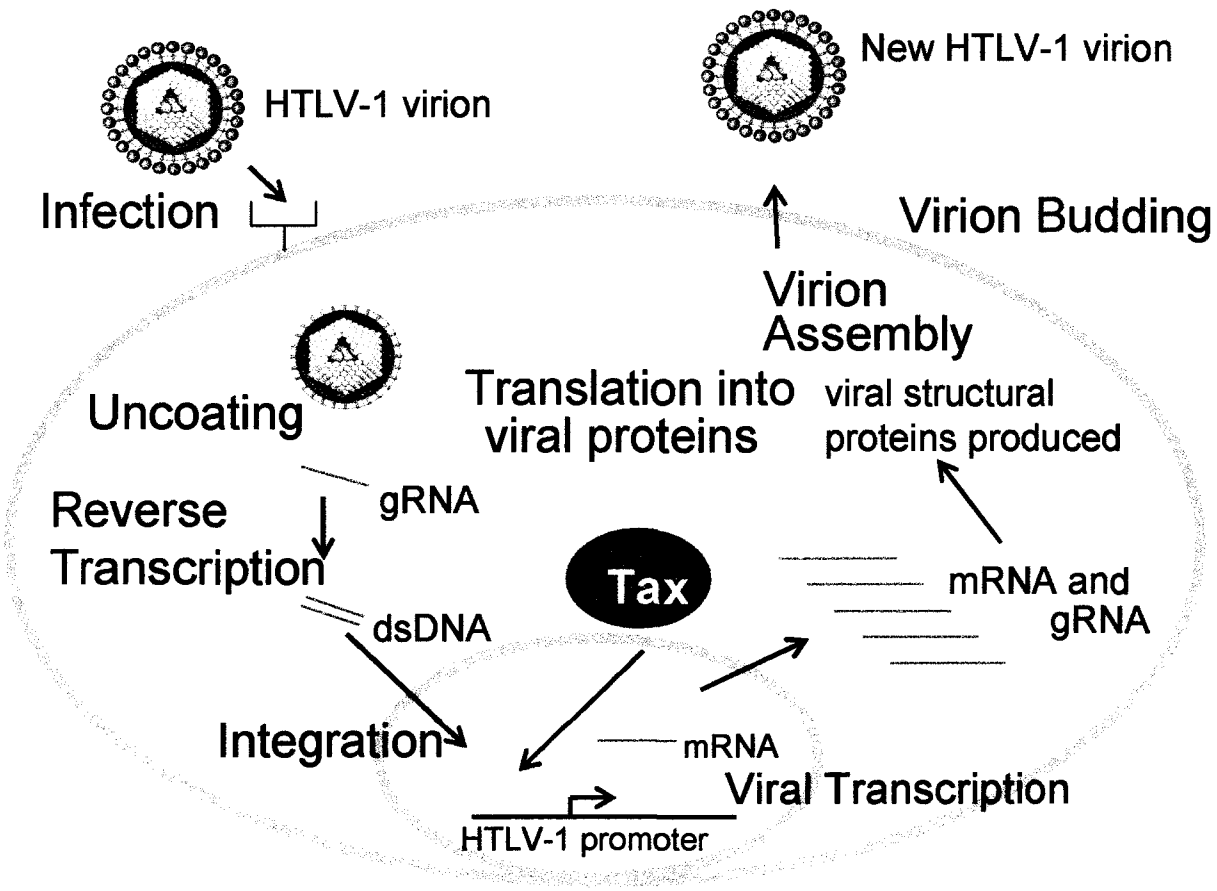


Figure 1.1 **The HTLV-1life cycle**. The virus binds a receptor on the host cell membrane and is taken into the cell. The virion capsid proteins are uncoated and the RNA genome is reverse transcribed into double stranded DNA. The viral genome is taken into the nucleus and is integrated into the host cell genome randomly. The viral regulatory proteins are synthesized, and are transported back into the nucleus to regulate viral gene expression. The structural proteins are synthesized, and the newly assembled virions bud from the cell membrane.

genome involves the viral transcription factor Tax and the host cell RNA polymerase II machinery. Since HTLV-1 carries an RNA genome, transcription from the HTLV-1 long terminal repeat (LTR) produces single stranded RNA copies of the viral genome to be packaged, and viral mRNAs to be translated into viral proteins. First, the mRNAs are spliced to give rise to the regulatory proteins Tax and Rex. Later, unspliced and singly-spliced RNA is produced as templates for synthesis of viral structural proteins (244, 285). Finally, new virions assemble at the host cell membrane (91).

HTLV-1 carries two copies of its nine kilobase single stranded RNA genome. Like other retroviruses, the genome carries genes for Gag (group specific antigen), Pol (polymerase), and Env (envelope). The Gag gene is initially translated into a large polyprotein that is cleaved to form three proteins; matrix (19-kDa), capsid (24-kDa), and nucleocapsid (15-kDa) (reviewed in 154). The Gag open reading frame (ORF) overlaps with the 5' end of the protease ORF. The protease protein is responsible for proteolytic cleavage of the polyprotein after translation, and for processing of the mature gag products (91). The Pol region encodes an RNA-dependent DNA polymerase (reverse transcriptase) and an integrase that is responsible for integration of the viral genome into the host cell genome (33, 298). The Env ORF encodes a large polyprotein that is later cleaved to form two mature products; surface glycoprotein and transmembrane protein. These proteins are detected on the surface of HTLV-1 infected cells (91).

In addition, there is also a unique region at the 3' end of the genome termed pX, which contains four open reading frames. ORFIII and IV encode the regulatory proteins Rex (27 kDa) and Tax (40 kDa) (199). Tax is involved in activating HTLV-1 transcription and will be discussed later in the chapter. Tax has also been shown to be the transforming agent of HTLV-1 through its ability to immortalize helper T-cells and induce the growth of tumors in mouse cells expressing Tax (5, 90, 95). Rex has a role in controlling viral gene expression by regulating the splicing of the HTLV-1 mRNA (64). The remaining ORFs in the pX region encode the HTLV-1 accessory proteins p12, p27, p13, and p30. These proteins are important for viral infectivity and for retaining high viral loads in the cell, (6), but are not necessary for HTLV-1 replication in cultured cells (228).

The HTLV-1 genome contains a 3' and 5' long terminal repeat (LTR), which consists of a U3, R, and U5 region (outlined in figure 1.2). The U3 region is the transcriptional control region that contains a TATA box and three imperfectly conserved 21 base pair enhancer elements that are required for high levels of transcription. These repeats consist of an eight base-pair imperfect cyclic-AMP response element (CRE) flanked by GC-rich base pairs and are essential for transcriptional activation by the viral protein Tax (30, 72, 80, 120, 164, 192, 216, 232, 242). Both the 5' and 3' ends of the genome contain a U3 region. However, only the U3 element in the 5' LTR is responsible for driving transcription of the viral mRNA and HTLV-1 RNA genome. The U3

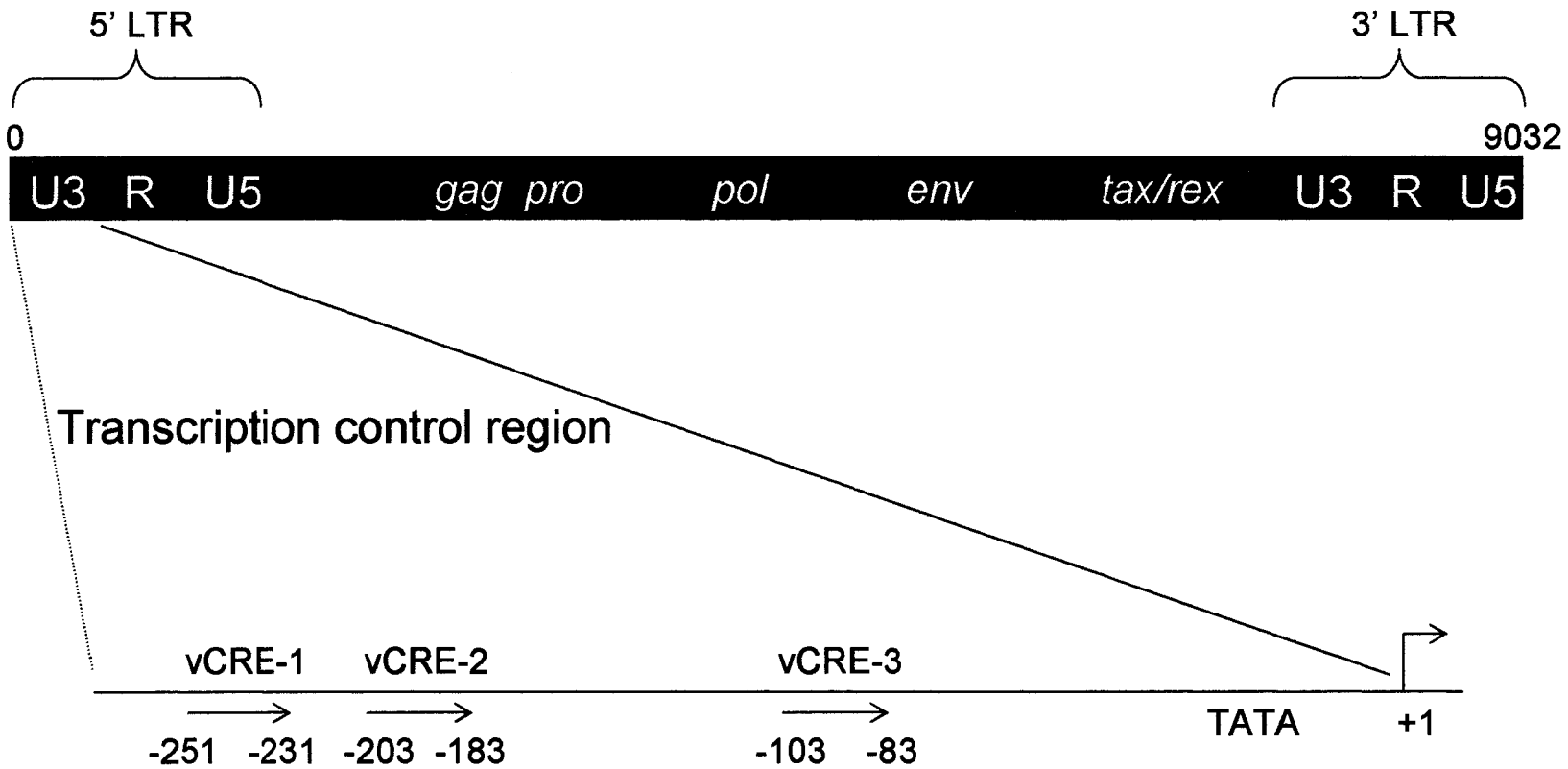


Figure 1.2 **Genomic structure of the human T-cell leukemia virus type 1.** The common viral genes *gag, pro, pol, env, tax*, and *rex* are indicated. The organization of the U3 region, and viral CREs, are indicated.

region also contains binding sites for other cellular proteins. These proteins will be described in further detail later in chapter 3.

1.1b Disease

In cultured cells, HTLV-1 is able to infect many cell types including B-lymphocytes, dendritic cells, fibroblasts, rat cells, and mouse cells. However, the provirus is mainly found in CD4+ and CD8+ T lymphocytes in vivo, and has only been shown to transform T-cells (reviewed in 64). Infection requires cell to cell contact, and occurs by vertical transmission from mother to child via breast milk, or through infected maternal T-lymphocytes passed from mother to child during pregnancy (91). HTLV-1 is also transmitted through sexual contact, or contact with any infected blood product (139, 212, 213).

In a small number of cases (2-6%), HTLV-1 has been shown to cause adult T-cell leukemia/lymphoma (ATLL), and this generally occurs at least 20 to 30 years following the initial infection (103, 225). ATLL patients are seropositive for HTLV-1 antigens, and HTLV-1 viral particles can be observed budding from leukemic cells taken from these patients (103, 131, 132, 191, 224, 229, 243, 292, 306). There are several stages to ATLL including the carrier state, preleukemic state, chronic/smouldering state, lymphoma development, and acute ATLL (reviewed in 91). 55% of persons diagnosed with ATLL have the most aggressive form (acute ATLL), which is characteristic of a dominant malignant HTLV-1 clone (254, 255). These patients have a high white blood cell count, with greater than 5% abnormal T-

lymphocytes containing lobulated nuclei, eosinophilia, enlarged lymph nodes, spleenomegaly, and neutrophilia (208). Patients also exhibit hypercalcemia, and lytic bone lesions in the skull and long bones due to infiltration of leukemic cells (25). Patients with ATLL are also immunocompromised and are prone to opportunistic infections. After diagnosis with acute ATLL, the median survival rate is approximately 6 months (117, 256). Although the viral protein Tax is strongly linked to the development of ATLL, the precise molecular mechanism leading to disease remains to be elucidated.

Another disease caused by HTLV-1 infection is Tropical Spastic Paraparesis/HTLV-1 Associated Myelopathy (TSP/HAM) (79, 115, 215). TSP/HAM was found independently in a group of West Indian patients (and was termed TSP), and also in patients in Japan (termed HAM) (215). The neurological disease known as TSP/HAM is characterized by demyelination of the nerves of the spinal cord resulting in paralysis of the lower extremeties. The lifetime risk for developing this after infection with HTLV-1 is below 1%, but the time of onset is much shorter than ATLL and can develop within six months after infection. Patients with TSP/HAM exhibit neurological difficulties including weakness, hyperreflexia, Babinski sign, incontinence, and mild peripheral sensory loss (214). High levels of cytotoxic T-lymphocytes are seen in patients with TSP/HAM, and this may be due to infiltration of HTLV-1 infected T-cells in to the central nervous system (92, 116, 147). These patients also display abnormal T-lymphocytes, but they are different from those found in the blood of ATLL patients (214). TSP/HAM is three times

more likely to occur in females, while ATLL is found more often in males (298). This disease is much different than ATLL, and the factors that lead to the progression of TSP/HAM also remain to be defined.

Although there has been extensive progress in the molecular biology of ATLL, the prognosis of patients with ATLL remains poor. Treatments for ATLL include combination chemotherapy regimen, but have not been shown to be useful in prolonging the lives of those with ATLL (reviewed in 19). ATLL cells are refractory to apoptosis, which may render them resistant to chemotherapy (204, 275). In one study median survival was increased from six to ten months in patients given zidovudine (AZT) and alpha interferon (19). Treatments with interferon alpha, beta, and gamma have been shown to be uneffective, but alpha interferon treatment for patients with TSP/HAM may be useful (91). Lamuvidine, an anti-retroviral agent, decreased the HTLV-1 proviral load of five TSP/HAM patients in one study (19). A successful bone marrow transplantation of a patient with ATLL was reported making it a promising future treatment (26). Since acquisition of HTLV-1 is preventable, the most obvious form of treatment is through education and prevention programs. However, at this time there is no effective treatment for patients diagnosed with, TSP/HAM, or ATLL.

1.1c Epidemiology

HTLV-1 infection is endemic to regions of southwestern Japan, Africa, northeastern South America, and the Caribbean basin (276). In addition, high

seroprevalence rates in Melanesia, Papua New Guinea, the Solomon Islands, and among Australian aborigines has been reported (55). Approximately 15 to 25 million people worldwide are infected with HTLV-1, while only 2-5% of the people infected with HTLV-1 develop an HTLV-1 associated condition (144). Out of a population of roughly 121 million, approximately 1 million people in Japan are seropositive for HTLV-1 (102). Most of these infections occur perinatally, but many are also acquired through blood transfusions (236). The number of people infected with HTLV-1 seems to be increasing in Western Europe and the United States. This increase is primarily seen in intravenous drug users (IVDA) and homosexuals. For example, in New York City approximately 9% of IVDA are infected with HTLV-1 (230). In Trinidad, 15% of the homosexuals are seropositive for HTLV-1, compared to 2.4% of the general population (18). HTLV-1 is not a new phenomenon, as proviral DNA was isolated from a 1500 year old Andean mummy (169).

1.2 Tax

1.2a Tax function; HTLV-1 transcription

The Tax protein is synthesized from a doubly-spliced RNA transcript from the pX region of ORF IV, and is imported into to the nucleus after translation. Tax is a 353 amino acid viral phosphoprotein that is a potent transcriptional activator of the viral promoter (see schematic, figure 1.3) (38, 62, 71, 260). Its nuclear localization signal is found at the amino terminus (85, 258), and

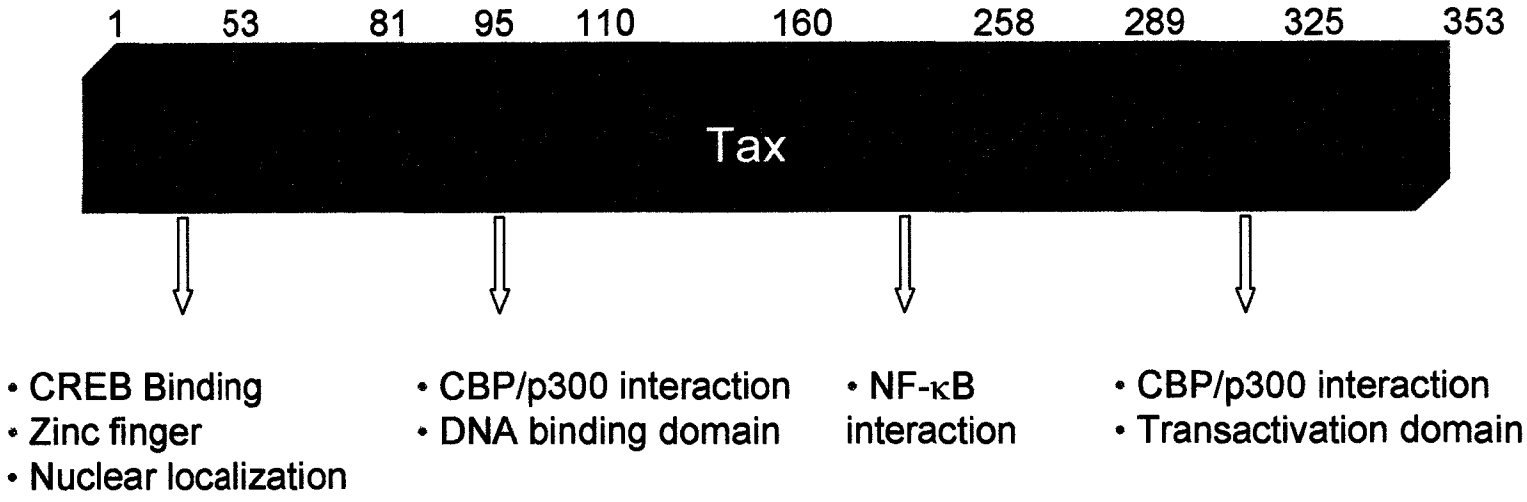


Figure 1.3 **Functional domains of the viral oncoprotein Tax.**

contains a high amount of cysteine and histidine residues that are hypothesized to form a zinc-finger-like motif (246). Tax interacts with the cellular protein CREB (cyclic-AMP response element binding protein) through these amino terminal 59 amino acids of Tax (91). The central domain of Tax, from amino acids 124 to 204, contains a dimerization domain (43, 125). Dimerization is important to HTLV-1 transcription, as point mutations in this domain result in a transcriptionally inactive protein (125, 271). To activate transcription, Tax and CREB bind to the viral CREs in the HTLV-1 promoter. The DNA binding domain of Tax lies between residues 80 and 110 (141). Tax makes DNA contacts in the minor groove at GC-rich nucleotides that flank the off-consensus viral CRE (140, 141, 164, 181). The carboxy-terminus of Tax (amino acides 284 to 325) contains the transactivation domain (69). A double point mutation at residues 319 and 320 (M47: L319R/L320S) abrogates Tax activation of transcription in vivo (259), and residues 315 to 325 are also indispensable for activation of the HTLV-1 LTR in vivo (246).

Early studies of HTLV-1 found that the U3 region of the LTR carried Tax-responsive elements (71, 217, 232, 260). These consist of three 21 base pair repeats (called viral CREs) positioned at -100, -200, and -250 with respect to the start site of transcription (128, 232, 242). At least two of the three viral CREs are thought to be required for efficient Tax transactivation (29, 71, 120, 200, 231). The viral CREs bind members of the ATF/CREB family, but the transcription factor CREB appears to be the protein most

utilized (1, 2, 20, 30, 66, 304, 309). The ATF/CREB family of transcription factors contains a basic leucine zipper (bZIP) DNA binding domain, and uses a transactivation domain to mediate activated transcription (reviewed in 157, 248).

To activate HTLV-1 transcription, Tax binds to the bZIP domain of CREB to increase its DNA binding affinity (reviewed in 165). This ternary complex of Tax-CREB-DNA is responsible for recruiting the cellular coactivator CBP/p300 to the promoter as outlined in figure 1.5 (80, 101, 149, 153, 302). It is hypothesized that this stable quaternary complex then recruits the general RNA polymerase II general transcription machinery to activate high levels of HTLV-1 transcription (80, 153). It has been shown that Tax can directly interact with members of the Pol II transcriptional machinery including TFIIA (44), hTAFII28 (34), and TBP (35).

1.2b Tax activation and repression of cellular genes

The main role of the viral transcription factor Tax is to activate transcription from the HTLV-1 promoter. However, like many other viral proteins, it has also been shown to affect transcription from cellular promoters. Deregulation of cellular gene expression is believed to be the mechanism by which Tax transforms cells (figure 1.4). For example, Tax is able to form protein-protein interactions with serum responsive factor (SRF) to activate the early response c-Fos gene (7, 67, 264). Tax can also activate the platelet-derived growth factor (PDGF) B promoter (274), interleukin-1, and

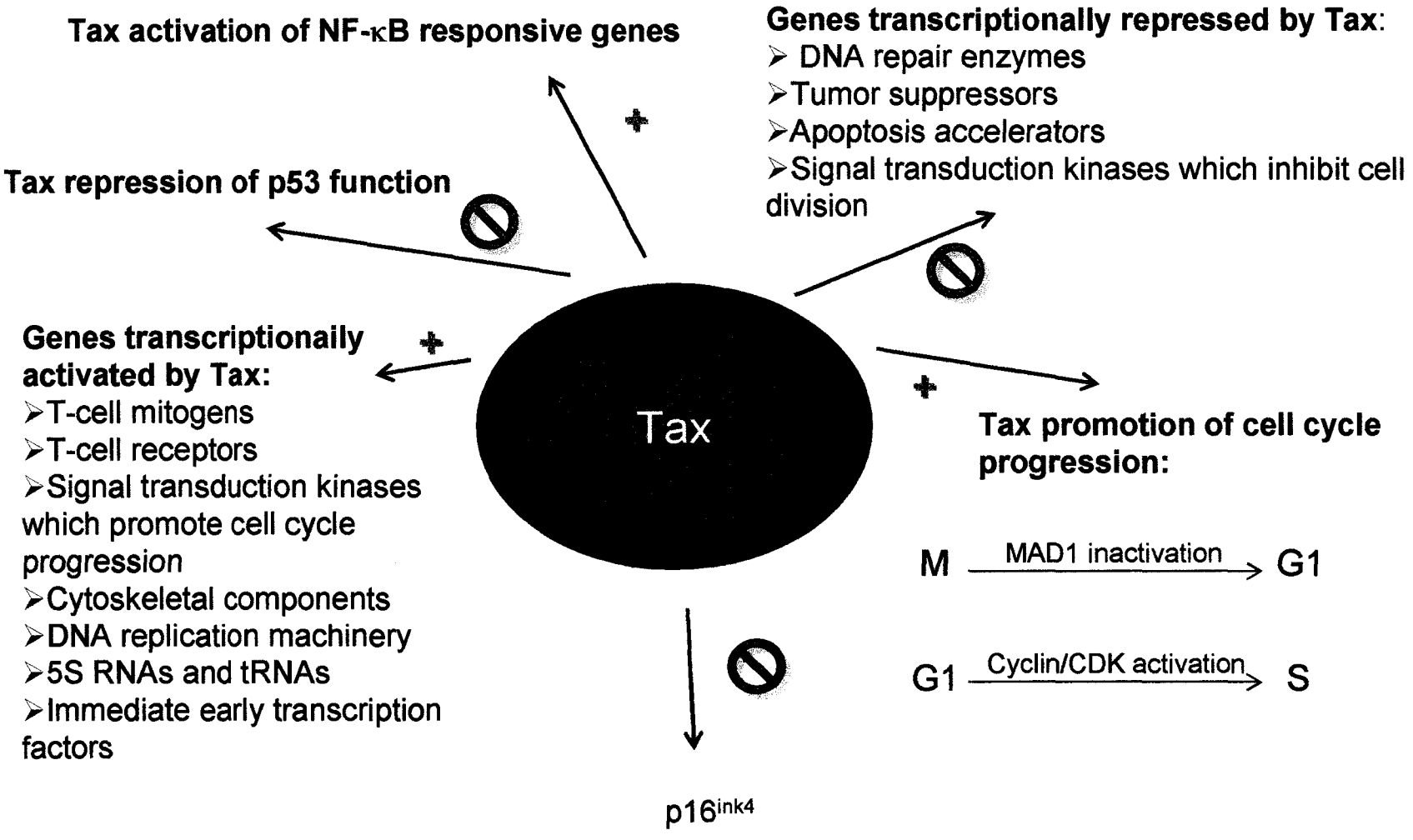


Figure 1.4 **Overview of the cellular processes deregulated by the HTLV-1 Tax protein**. This figure is modified from Lenzmeier, B. A., and J. K. Nyborg. 1999. Molecular mechanisms of viral transcription and cellular deregulation associated with the HTLV-I Tax protein. Gene Ther. Mol. Biol **3**:327-345.

interleukin-2 receptor alpha (15, 49). Tax upregulates the NF-κB pathway (reviewed below) resulting in constitutive activation of genes responsive to NF-κB.

In addition to the pathways mentioned above, Tax has been shown to target members of the cellular basic-helix-loop-helix (bHLH) family of transcription factors. These factors bind to DNA elements called E-boxes in the promoter of cellular genes to activate transcription. Tax was shown to repress promoters containing E-boxes that bind bHLH proteins (245, 278). Promoters repressed through this mechanism include β-polymerase, p53, Bax, p18, and lck (31, 122, 160, 267, 279). Tax can also repress transcriptional activity of c-jun, p53, and c-myb through competition for the coactivator CBP/p300 (reviewed below) (45, 280, 282, 295).

1.2c Tax and CBP

CBP and its paralog p300 are large (2441 amino acids), highly conserved cellular pleiotropic coactivator proteins involved in a number of different cellular processes. They have been implicated in processes such as hematopoetic development, embryogenesis, differentiation, and cell cycle control (reviewed in 81, 82). The importance of CBP/p300 in the cell is profound as evidenced not only by its ability to recruit transcriptional machinery by serving as a scaffold to bridge transcription factors to the general transcription machinery on promoter DNA, but also by its ability to act as an acetyltransferase (16, 188).

CBP was originally identified by its ability to bind Serine-133 phosphorylated CREB (cyclic AMP response element binding protein) (41, 150). Its name is somewhat misleading, as CBP is known to bind over 30 cellular transcription factors including p53, and viral transcription factors including Tax (80, 149, 239, 280). An important activity of CBP/p300 is its intrinsic acetyltransferase activity, and ability to recruit other acetyltranferases. CBP and p300 are known to acetylate all four core histones either free or in nucleosomes, and this hyperacetylation of histones has historically been associated with actively transcribed DNA (210). In addition to their ability to acetylate histones, CBP and p300 have also been shown to acetylate other transcription factors including p53 and GATA-1 (28, 97).

Since Tax has been shown to bind to multiple domains of CBP, one model of Tax repression of cellular genes is through competition with cellular transcription factors for CBP/p300 in the nucleus (reviewed in 281). Although CBP is ubiquitously expressed, the level of CBP in the nucleus is limiting (220, 251, 300). Many cellular factors interact with CBP (reviewed in 81, 118, 252). Tax binding to the KIX and CR2 domains of CBP/p300 competes with cellular proteins that bind to these regions, and this could lead to disruption of transcription of many cellular genes.

1.3 Tax and p53

1.3a p53 function

p53 is often referred to as the "guardian of the genome." Its profound and varied functions in response to genotoxic stress implicate this protein as a major tumor suppressor in the cell (reviewed in 142). Its function as a transcriptional activator helps keep the cell's genome intact and functional. p53 is found to have mutations in over 60% of all cancers. The large majority of these debilitating mutations most frequently map to its DNA binding domain (107, 167). p53 has been shown to bind to free DNA, and at insertion or deletion mismatches. This binding induces the cell to repair the DNA (287). p53 functions as a transcription factor via is its sequence-specific DNA binding ability. This protein is known to bind to promoters to activate genes that help control cell cycle progression and apoptosis. p53 is a 393 amino acid protein that resides at very low levels in the cytoplasm when it is not activated. Once activated by certain genotoxic stresses like ionizing radiation, hypoxia, nucleotide depletion, and serum starvation, p53 is translocated to the nucleus to activate gene expression of cell cycle regulatory proteins. Like most transcriptional activators, p53 has a modular structure with separable domains. Its four main functional domains include an amino terminal acidic domain (amino acids 1-43), a central core DNA binding domain (amino acids 100-301), a tetramerization domain (amino acids 324-355), and a carboxy-terminal regulatory domain (amino acids 363-393). This

highly basic C-terminal domain is thought to regulate the DNA binding ability of p53 through modifications such as phosphorylation and acetylation (reviewed in 107).

p53 is involved in many important cellular processes including differentiation, cell cycle progression, apoptosis, senescence, and inhibition of angiogenesis. Its ability to promote cell cycle arrest is tied to its function as a DNA binding transcriptional activator. Genes that p53 regulates include but are not limited to p21, MDM2, GADD45, 14-3-3, Bax, and Fas/Apo1 (261). For example, upregulation of p21 by p53 allows p21 to bind to cyclin dependent kinases (which regulate cell cycle progression) and inhibit their activity. This interaction allows for an increase in the amount of hypophosphorylated Rb forcing it to remain associated with E2F, and promoting cell cycle arrest.

In vitro studies have shown that deletion of the C-terminal domain causes p53 to bind to DNA with similar affinity as wild-type p53 with a covalently modified C-terminus. Unmodified, this domain is thought to be in a conformation that sterically inhibits the DNA binding domain. Upon modification (acetylation or phosphorylation), this domain is thought to change conformation to allow for DNA binding. Several studies have shown that acetylation of the C-terminal domain stimulates the high affinity binding of p53 to DNA, and also upregulates its transcriptional activity (96). Acetylation of p53 at Lys 382 and 373 by p300/CBP (96), and Lys 320 by P/CAF has also been demonstrated (174). Many kinases have also been shown to

phosphorylate p53, including ATM, CDK, CKI, CKII, PKC, MAPK, JNK, Raf, and DNAPK. This phosphorylation has been demonstrated after genotoxic stresses that activate p53.

In addition to binding DNA, p53 has been shown to bind to several other proteins including TBP, TAF 40, TAF 60, mdm2, CBP/p300, and BRCA1. Binding of p53 by several oncoproteins like SV40 T antigen, adenovirus E1A, and human papillomavirus 16 have also been shown to silence the activities of p53. This binding results in cell cycle progression.

1.3b Tax inhibition of p53 and cell cycle

Tax is known to disrupt p53 function; however, there is no direct evidence for an interaction between p53 and Tax. Although p53 is often mutated in many human cancers, p53 mutations in HTLV-1 infected cells are relatively rare (36, 73, 168, 227). Since cellular transformation has been linked with the inhibition of p53 (63), functional inactivation of p53 by Tax may be necessary for the induction of transformation. Although p53 is generally wild-type in HTLV-1 infected cells, the transcription function of p53 in these cells is blocked (4, 36, 73, 223), and this disruption has been shown to be mediated by Tax (4, 197, 223, 235, 280). Tax and p53 compete for binding to the cellular coactivator CBP/p300 (175, 280), and this may be a significant event in the progression to leukemogenesis.

Tax has also been implicated in disruption of the cell cycle and DNA repair pathways. The length of the G1 phase of Tax expressing cells is

decreased while the S phase is unaffected (161). In HTLV-1 infected T-cells, various members of the cyclin/CDK family are deregulated. For example, $p27^{KIP1}$ is limiting, cyclin E-CDK2 is constitutively active, and cyclin D2 levels are elevated (3, 37, 193, 234). Tax has been shown to bind and inactivate $p16^{INK4A}$, and transrepress $p18^{INK4C}$ activity (98, 176, 267, 268). Despite functional inactivation of p53, the level of $p21^{WAF1/CIP1}$ is increased in HTLV-1 infected cells (3, 36). The promotion of the G1/S transition by Tax may prevent cells from pausing to repair DNA. This would be consistent with the lobulated nuclei found in HTLV-1 infected T-cells (224). Alterations in DNA repair have also been associated with HTLV-1 and Tax expressing cells, as these cells display an enhanced mutation frequency of the cellular genome (162, 190). Base excision and nucleotide excision repair have both been reported to be inhibited by Tax (122, 135). In addition, Tax has been shown to disrupt the mitotic spindle checkpoint by binding hsMAD1, a mitotic spindle checkpoint protein (123).

1.4 LTR binding proteins; Sp1

The 5' LTR of the HTLV-1 provirus is responsible for viral gene expression. The U3 region of the HTLV-1 5' LTR contains the viral CREs that recruit the Tax/CREB complex and are responsible for high levels of viral transcription (outlined in figure 1.5). However, the viral CREs are not the only

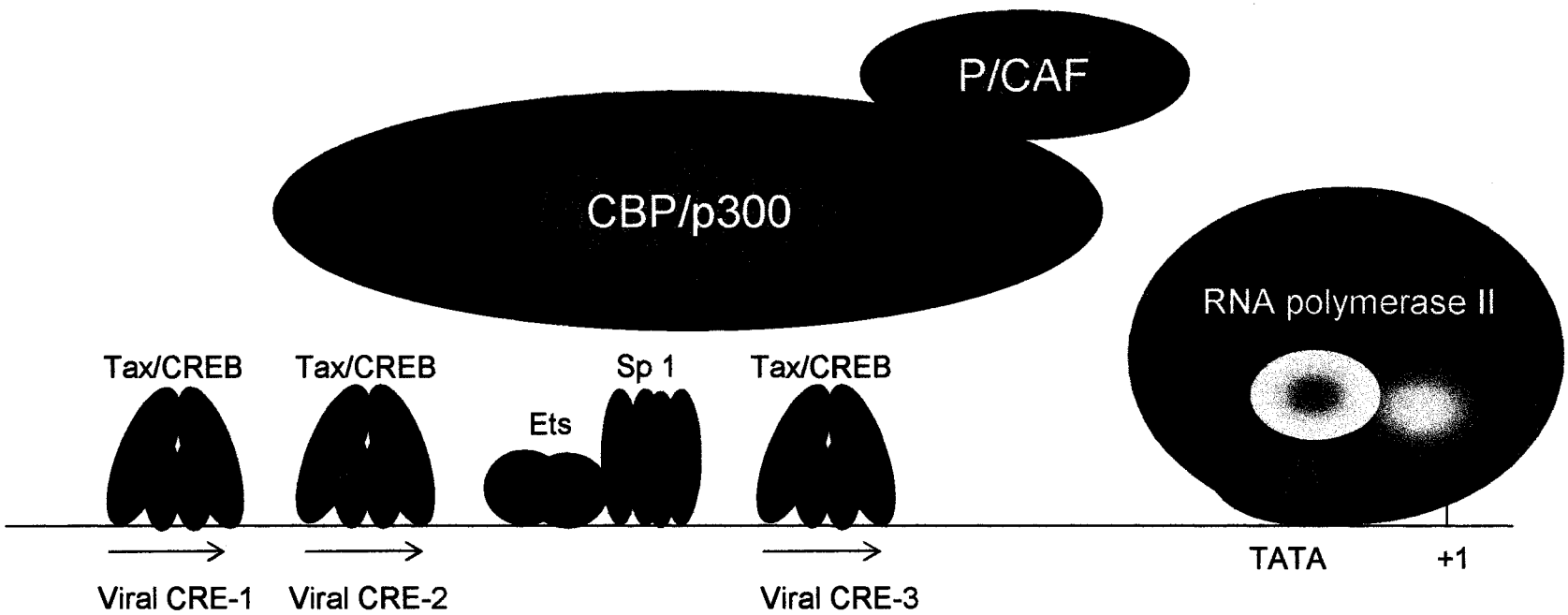


Figure 1.5 **Schematic representation of the transcription compiex on the HTLV-1 promoter.**

important *cis*-acting region of the HTLV-1 promoter. Chromatin immunoprecipitation experiments have revealed other cellular proteins bound to the LTR including CREB-2, ATF-1, ATF-2, c-fos, c-Jun, p300, CBP, HDAC-1, HDAC-2, and HDAC-3 (159, 177). Another important region is between the second and third viral CREs, and is named vSp1 or Ets response region 1 (ERR-1), (see figure 1.6) located between nucleotides -163 to –117 relative to the TATA box (206, 207, 241). This region contains binding sites for Sp1, Ets1, Ets-2, Elf-1, Myb, and Tif-1 (27, 84, 187, 206).

The cellular transcription factor Sp1 is important for HTLV-1 transcription. Sp1 was first identified as a protein that binds GC elements in the SV40 promoter, and was the first mammalian transcription factor to be cloned (130). It contains a zinc finger DNA binding domain at its C-terminus, and a glutamine rich activation domain (48, 130) (reviewed in 129). The gene for Sp1 is expressed in all murine tissues (233), and it is an essential protein in cells as homozygous knockouts of Sp1 in mice result in embryonic lethality (185). Sp1 has been shown to bind to Tax (274). Sp1 has also been shown to bind to the HTLV-1 LTR between the second and third viral CREs, and also at the promoter proximal repeat (see figure 1.6) (17, 207, 289). It has been suggested that Sp1 and CREB compete for binding to the promoter proximal viral CRE (17). Currently, the functional importance of Sp1 with regard to activated HTLV-1 transcription is not known, and this will be discussed further in chapter 4.

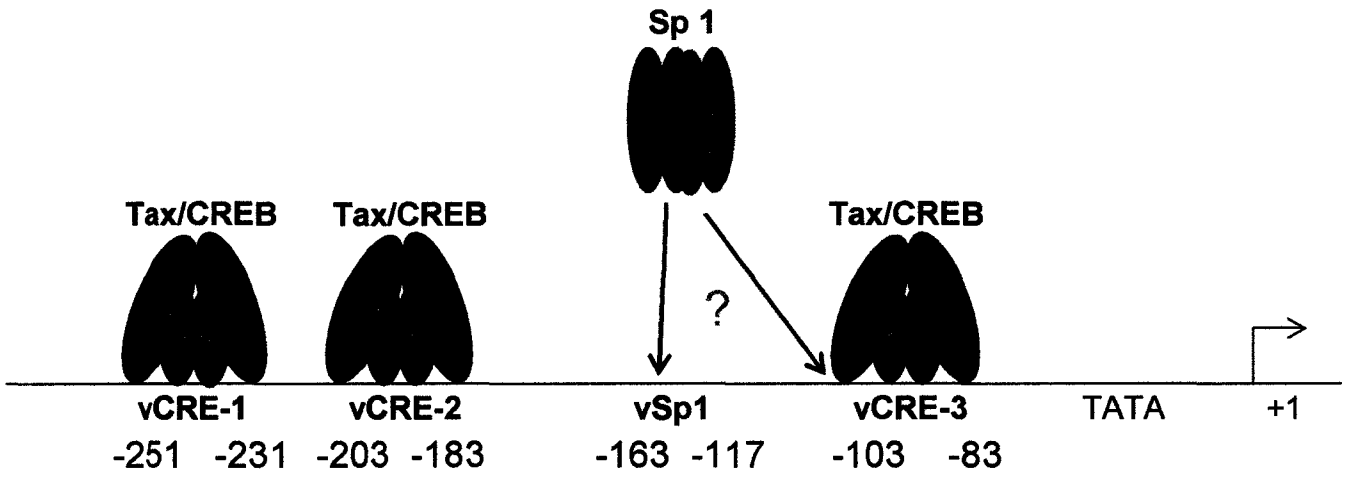


Figure 1.6 **Schematic illustration outlining the putative binding sites for Sp1 on the HTLV-1 promoter.**

1.5 Tax and NF-κB

Tax is predominantly a nuclear protein with a strong nuclear localization signal (258). However, a small amount of the protein is found in the cytoplasm of infected cells, which allows for it to affect cytoplasmic signaling pathways (205). Studies have indicated that cytoplasmic Tax triggers nuclear localization of transcription factors to activate the NF-κB pathway (figure 1.7). HTLV-1 infected cells have been shown to have constitutively elevated NF-κB activity (134, 151, 182, 189, 194).

Members of the NF-κB family have a Rel homology domain responsible for forming homo- and heterodimers that bind to DNA to activate transcription (226). The NF-κB protein family includes p105, p100, p65, p52, p50, c-Rel, and RelB, with the most studied complex being the p50/p65 heterodimer. When inactive, the transcription factors are retained in the cytoplasm by IκB inhibitor molecules containing ankyrin repeats (IκBα, IκBβ, IκBε, p105, p100, and Bcl-3, (for review see 283). A large cytoplasmic multiprotein complex called Iκκ is responsible for inducing the activation of the NF-κB pathway (see figure 1.7). This complex includes two kinases, IKKα and KKβ, plus a regulatory domain named IKKγ (for review see 119). It was also recently discovered that the IKK complex also contains MEKK-1 and NIK (156, 173, 201, 203), which are thought to activate the IKKs by phosphorylation (reviewed in 136). Upon activation by signals including cytokines, virus infection, TNF-α, or stress signals, IκBα is phosphorylated on

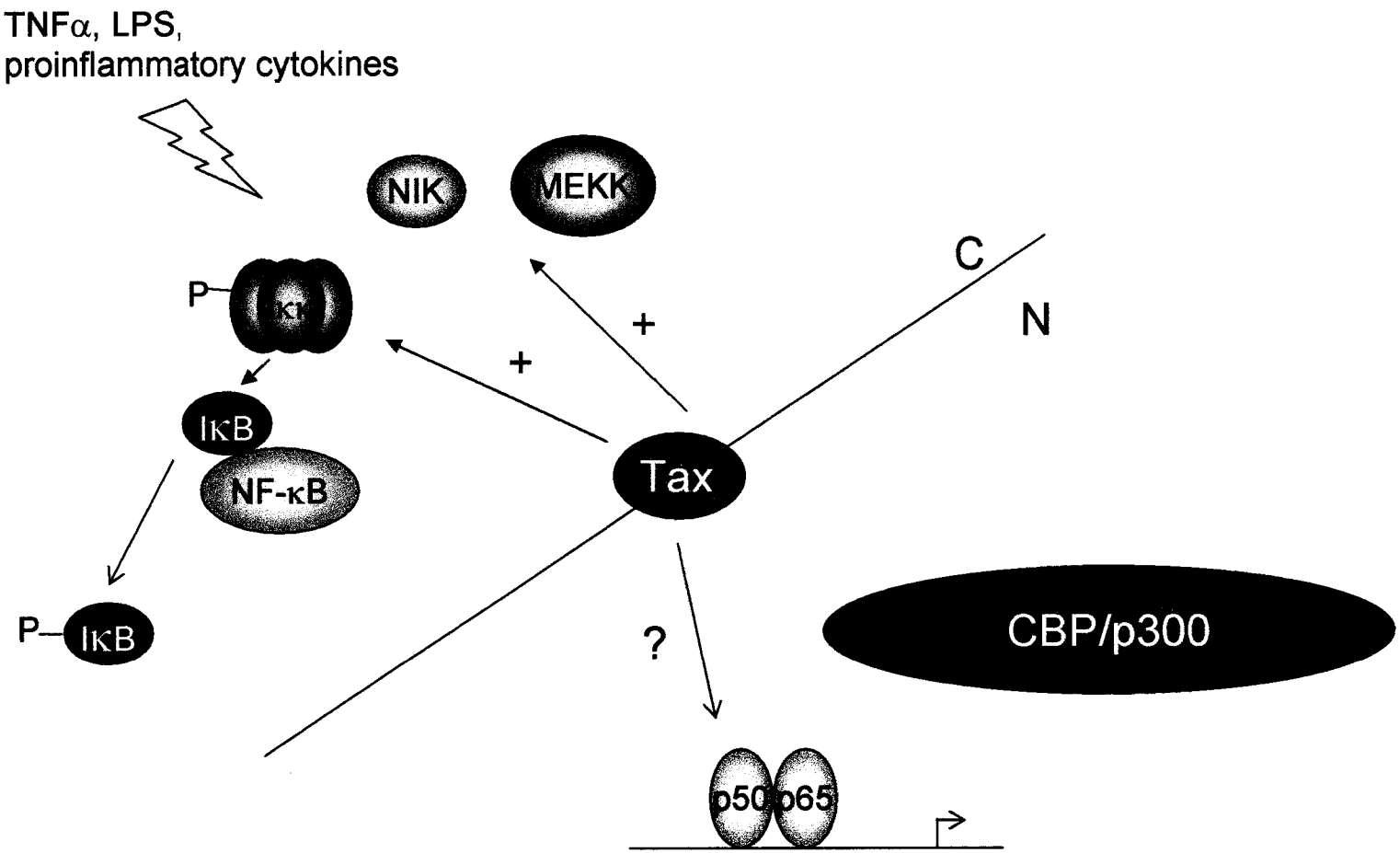


Figure 1.7 **Schematic illustration of the two major pathways of Tax activation of NF-κB-dependent genes.** N: nucleus, C: cytoplasm.

serine residues 32 and 36, while IκBβ is phospyorylated on serine residues 19 and 23 (reviewed in 14). These phosphorylation events target these two proteins for ubiquitination followed by proteasome-mediated degredation. The release of the inhibitors uncovers the nuclear localization signal of NF-κB allowing it to migrate to the nucleus to activate transcription of a wide variety of genes.

Tax has been shown to be involved in the nuclear localization of the NF-κB proteins primarily via upstream events that occur in the cytoplasm. Tax has been shown to bind to many members of the NF-κB family including the regulatory subunit of the IKK complex, IKKγ (42, 99, 124). Furthermore, others described an association of Tax with MEKK1 (123). Tax can also directly bind to the ankyrin motif sequences of IκBα, and p105 (104, 105, 221, 222, 265).

Since Tax is predominantly found in the nucleus, it has also been hypothesized that Tax may affect the transcriptional activities of the NF-κB proteins. Immunomicroscopy revealed that Tax and NF-κB colocalize in nuclear bodies containing RNA polymerase II (23, 245). Tax can also directly bind the subunits p50, p65, and p52 (198, 266). To date, Tax has not been shown to directly alter the action of NF-κB on transcription of genes in the nucleus, and this will be discussed in further detail in chapter 5.

1.6 Statement of Projects

Studies of the effects of Tax on cellular transcription factors are an important way to understand the malignancies caused by HTLV-1infection. Deregulation of the cellular transcription factor p53 is a classic way for a virus to evade cell death. Understanding the ability of Tax to downregulate p53 function is needed to fully understand the path to ATLL. A study on the ability of p53 to bind to a domain of CBP/p300, and the ability of Tax to compete with p53 for binding to this domain is presented in chapter 2.

Transcription of the HTLV-1 promoter is a complex process that involves Tax and many other cellular proteins. One protein, Sp1, has been shown to be involved in HTLV-1 regulation. However, the precise role and promoter element that Sp1 functions through is not clear. A study outlining the function of Sp1 in HTLV-1 transcription is presented in chapter 3.

Treatment options for people infected with HTLV-1 are limited. DNA binding polyamides are an exciting class of drugs that have been shown to inhibit transcription factor binding to DNA. Rationally designed polyamides were made for the HTLV-1 promoter, and their ability to inhibit viral transcription and replication is outlined in chapter 4.

One of the ways Tax deregulates cellular homeostasis is through its ability to upregulate NF-κB responsive genes. The majority of the studies of Tax and NF-κB has been focused on the actions in the cytoplasm. We have instead focused on investigating the ability of Tax to regulate NF-κB responsive promoters in vitro, and this research is presented in chapter 5.

Chapter 2

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. Kirsten Scoggin and I were co first author on this manuscript. I performed the experiments in figures 2.4 and 2.5. 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.

2.1 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 carboxy terminus. The 95 amino acid CR2 region (aa2055-2150) 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.

2.2 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 (81, 82, 87, 252). 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 (281). 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 (142). Loss of p53 activity has been identified in 60% of the human malignancies examined (100, 168), 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 (142, 167). 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 (202), increasing the rate of preinitiation complex assembly (301). 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 (50, 150). 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 (238). Acetylation appears to increase the accessibility of the nucleosomal DNA to transcription factor binding, a critical step in gene activation (155, 284). Interestingly, CBP/p300 have also been shown to acetylate p53 at lysine residues 373 and 382 (96). 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 (60).

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 (280), and an ill-defined carboxy terminal region of CBP/p300 (11, 97, 170, 240). The amino terminal activation domain of p53 has been shown to participate in

each of these coactivator interactions (51, 253, 280). In studies that attempted to elucidate the precise carboxy terminal region of CBP/p300 involved in p53 binding, only the C/H3 domain of CBP (~aa1764-1850; also called TAZ2, and TRAM) has emerged as a site of p53 interaction (209). 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) (51). This result suggests that p53 may make additional contacts within the carboxy terminal region of CBP/p300.

In this study, we set out to further characterize the interaction between p53 and the carboxy 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 (11, 97, 170, 240). We have identified a new p53-interacting domain on CBP (aa2055-2150) and p300 (aa1970-2193) 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 (133, 146, 249). Furthermore, this region has been shown to be an important interaction site for numerous transcription factors, including IRF-3 and HTLV-1 Tax (171, 239). 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 (171). 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 (4, 197, 223, 280).

2.3 Materials and Methods

2.3a Cloning, expression, and purification of recombinant proteins. The expression and purification of GST-C/H1-$KIX_{aa302-683}$, GST-$KIX_{aa588-683}$, GST-$CR1_{aa1514-1894}$, GST-$CR2_{aa1894-2221}$, and GST-$CR3_{aa2212-2441}$ have previously been described (280). 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 (239). 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% [vol/vol] Tween-20, 20% [vol/vol] 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 (280). Tax was expressed from the pTaxH_6 expression plasmid (309), and purified as previously described (80).

In the experiments presented in figure 2.3A, 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 (aa1-107) 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 vs. 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 (aa1-107) was cloned by PCR amplification of the full-length, wild-type p53 cDNA (p53-H-19) (100). The PCR product was inserted into the *NdeI/BamHI* site of pET15b (Novagen).

Drosophila core histones were purified as previously described (32). The yeast NAP-1 cDNA (70) was cloned into pGEX-2T (Amersham Pharmacia Biotech), and the GST-yNAP-1 fusion protein was expressed in *E. coli* and purified by glutathione-agarose affinity chromatography and Q-Sepharose. 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 (113). His_6-tagged wild-type p300 and p300ΔSRC proteins were expressed from recombinant baculoviruses and purified as previously described (146).

2.3b GST pull-down assay. All GST pull-down experiments were performed as previously described (280). Anti-p53 (DO-1 [epitope corresponding to aa 11 to 25]; Santa Cruz Biotechnology), anti-Tax (epitope corresponding to the 13 carboxy-terminal amino acids) and anti-His_6 (H-15; Santa Cruz Biotechnology) antibodies were used in the GST pull-down experiments.

2.3c 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 minutes on ice as previously described (80). Protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel.

2.3d 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 (294). 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.

2.3e Chromatin Assembly and Topological Assay. Nucleosomes were assembled on the Mdm2 P2-G-less plasmid as previously described (77, 112). 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 minutes. Assembly reactions were incubated for 2 hours at 27°C. DNA topological assays were performed as previously described (77). The samples were analyzed on a 1% agarose gel, and the degree of supercoiling was visualized by Sybr Gold (Molecular Probes) staining.

2.3f In Vitro Transcription Assay. The supercoiled Mdm2 P2 G-less plasmid was assembled into chromatin using GST-yNAP-1, dAcf1 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 (USB). Nuclear extract (70 μg) (57), 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 minute 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-α-UTP (3000 Ci/mmol, New England Nuclear). Transcription reactions were processed and analyzed as previously described (163). Molecular weight markers (radiolabeled *Hpa* II digested pBR322) were used to estimate the size of the RNA products.

2.3g 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) 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.

2.3h 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 (Life Technologies, Inc.) and a constant amount of DNA for 5 hours. The cells were allowed to recover for 19 hours 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 HSV-TK promoter, as an internal control.

Expression plasmids for p53 (pC53-SN3) (13), CMV-CR2 (239), and pRC/RSV-CBP (180) have already been described. The luciferase reporter plasmid pG13-Luc (138), has also been described.

2.3 Results

2.4a 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 carboxy terminal half of the coactivator (Fig. 2.1A). 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. 2.1B, lane 6). We found that p53 bound strongly to only one of the three carboxy terminal regions of CBP (Fig. 2.1B, lane 4). This region, which we call carboxy-terminal region 2 (CR2), encompasses CBP amino acids 1894-2221. Consistent with previous studies (51, 209), we found that p53 also bound to C/H3 (TRAM/TAZ2), as our carboxy terminal region 1 protein (CR1; aa 1514-1894) encompasses this domain (Fig. 2.1B, lane 3; see Fig. 2.1A).

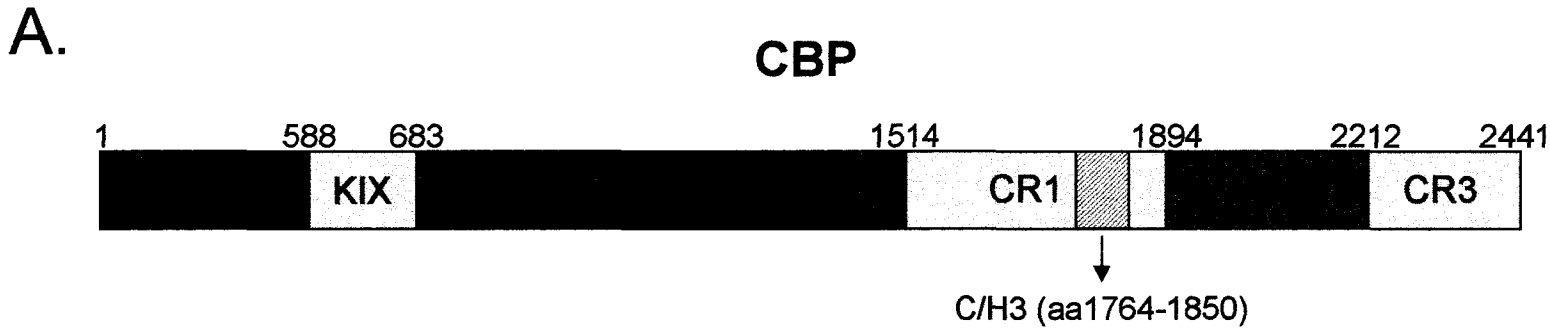


Figure 2.1. **p53 binds strongly to the CR2 domain of CBP.** (A) Schematic representation of the 2441 amino acid cellular coactivator CBP. The regions tested for p53 interaction are indicated.

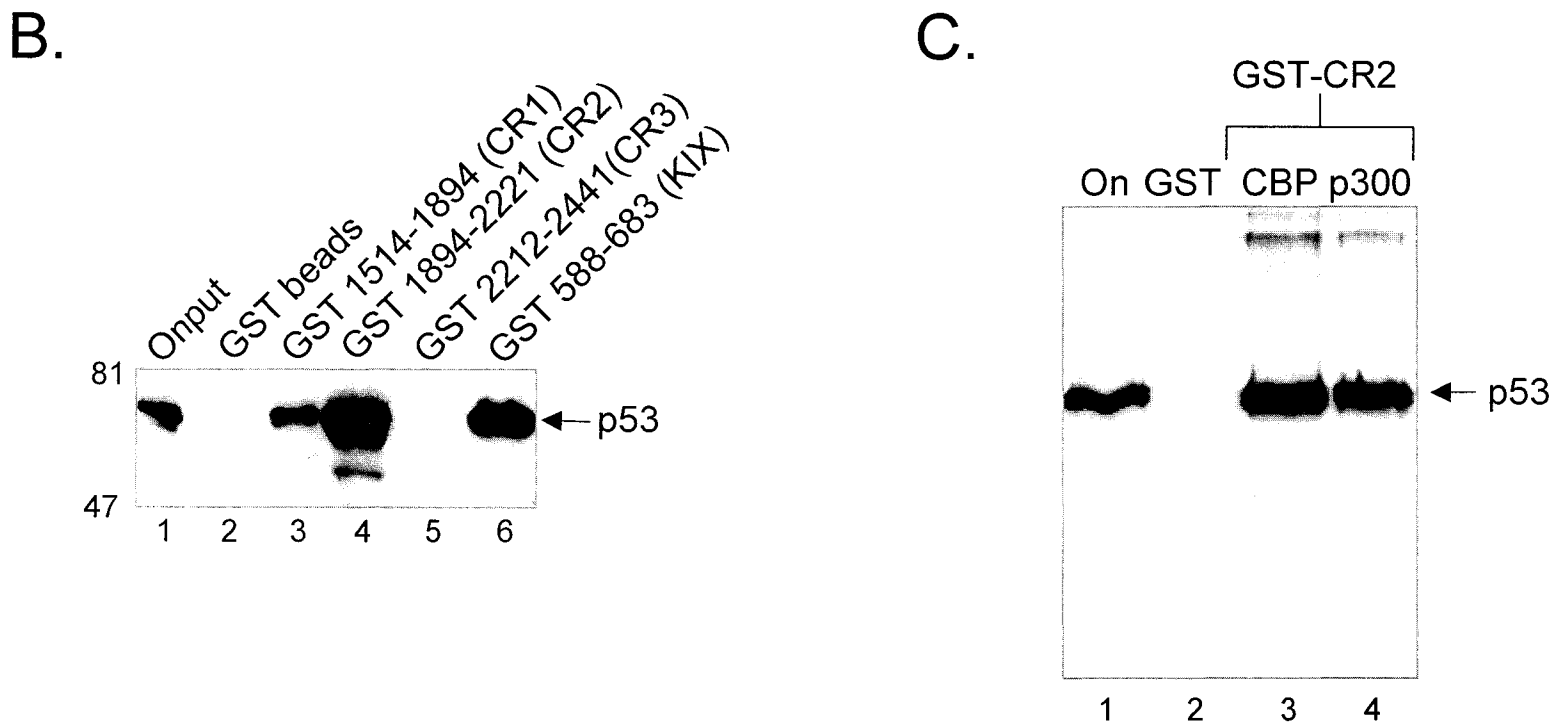


Figure 2.1 (B) p53 binds to the CR2 domain *in vitro*. Full length, purified, recombinant p53 (10 pmol) was incubated with GST alone (lane 2) or the indicated GST-carboxy terminal region fusion proteins (10 pmol each) (lanes 3-5). As a positive control, we also tested p53 binding to GST-KIXaa588-683 (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 (aa2003-2212), or p300 (aa1970-2193) (20 pmol each). p53 was detected using an anti-p53 antibody. Onput p53 (5%) is shown (lane 1). Bound p53 is indicated.

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 (data not shown). Interestingly, the CR2 domain (aa 1894-2221) corresponds closely to the steroid receptor coactivator 1 (SRC1)-interacting domain of CBP (aa 1982-2163) (133, 249, 299). SRC1 is a prominent member of a family of coactivators that utilize CBP/p300 to mediate transcriptional activation of nuclear hormone receptors (86, 166).

CBP and p300 are highly homologous proteins, while their precise role in the regulation of gene expression mediated by either protein is unclear. Since CBP and 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 2.1C shows that p53 binds comparably to the CR2 regions from both CBP and p300 (lanes 3, 4).

2.4b 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 carboxy terminal deletions of GST-CR2 revealed that amino acid 2150 represents the carboxy terminal border competent for wild type interaction with p53 (GST-$CR2_{aa1894-2150}$) (Fig. 2.2A, 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. 2.2A, compare lane 3 with 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. 2.2A, lane 10). This region precisely overlaps with the minimal CBP sequence (aa 2058-2130) required for interaction with SRC-1(249).

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 within a region that forms amphipathic alpha 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: F2101→A/I2102→A; L2068→A/L2071→A; L2072→A/L2075→A; L2140→A/L2143→A. Figure 2.2B shows that only the double point mutant L2068→A/ L2071→A, which resides within the first of the three α-helices, had a significant effect on p53

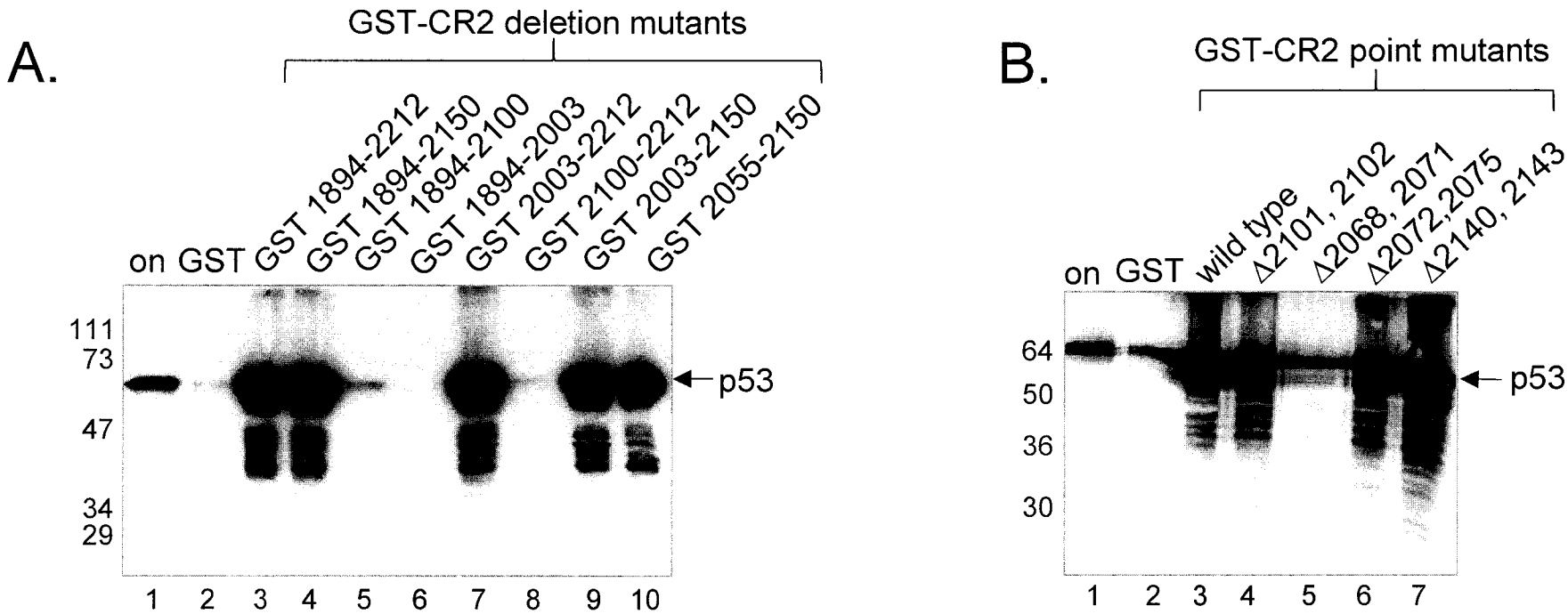


Figure 2.2 **Identification of the minimal region of CR2 competent for p53 binding.** (A) p53 interacts with amino acids 2055-2150 of CBP *in vitro*. Purified p53 (10 pmol) was incubated with GST alone (lane 2) or the indicated GST-CR2 deletion mutants (10 pmol) (lanes 4-10). As a positive control, p53 binding to full-length GST-CR2aa1894-2221 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 L2068→A/L2071→A. Purified p53 (25 pmol) was assayed for its ability to bind to GST alone or the GST-CR2aa2003-2212 double point mutants: F2101→A/I2102→A, L2068→A/L2071→A, L2072→A/L2075→A, or L2140→A/L2143→A. (25 pmol). p53 binding to wild-type GST-CR2aa2003-2212 was tested as a positive control (lane 3). Onput p53 (5%) is shown (lane 1). Bound p53 and protein molecular weight standards are indicated.

C.

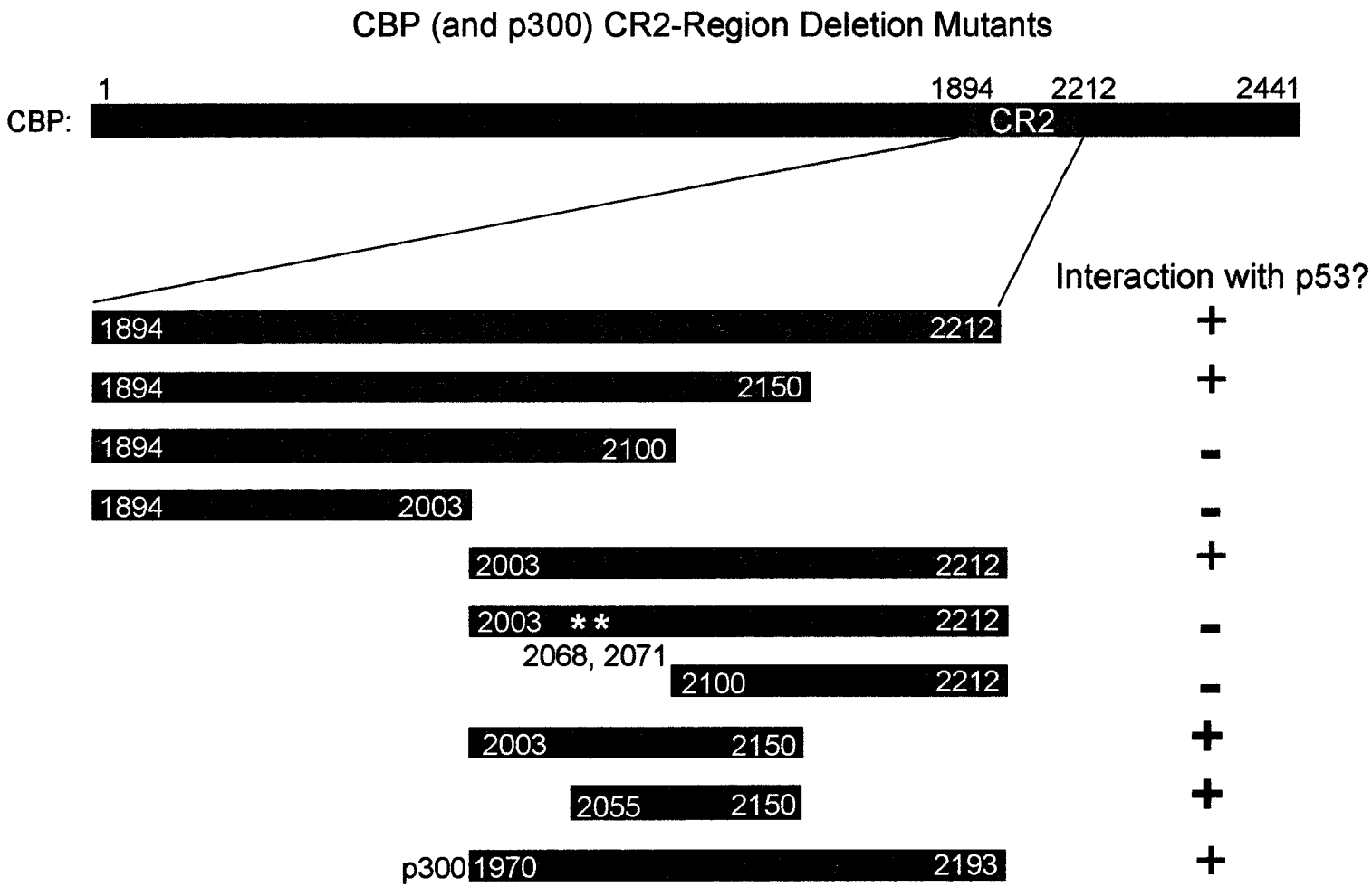


Figure 2.2 (C). Summary of the p53-CR2 interactions.

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 2.2C.

2.4c 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 (data not shown). 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 (aa1-107) were tested for their ability to bind the CR2 domain of CBP. Glutathione beads were bound with the GST-CR2$_{aa1894-2221}$ or with 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 2.3A shows that both the full-length and the amino terminal p53 fragment binds to CR2 (lanes 5, 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 observation that amino and carboxy terminal interactions in p53 are important for p53 function (196).

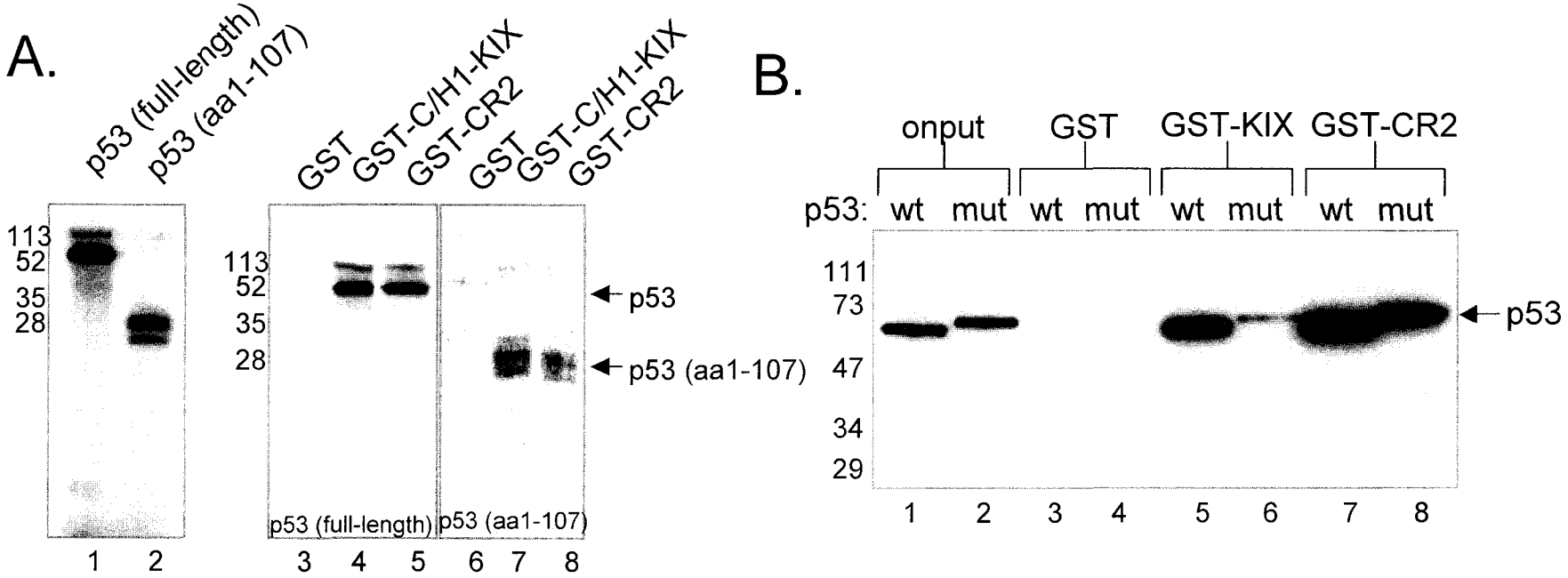


Figure 2.3 **Identification of the minimal domain of p53 responsible for CR2 binding.**
(A) The amino terminus of p53 (aa1-107) is competent for CR2 binding. The full-length p53 35S-labeled *in vitro* translation product (lane 1) was incubated with GST alone (lane 3), GST-C/H1-KIXaa302-683 (lane 4), or GST-CR2aa1894-2221 (lane 5). The p53 (aa1-107) 35S-labeled *in vitro* translation product (lane 2) was incubated with GST alone (lane 6), GST-C/H1-KIXaa302-683 (lane 7), or GST-CR2aa1894-2221 (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, L22→Q; W23→S, is competent for CR2 binding. Purified wild type p53 or activation domain mutant p53 (19) (15 pmol) was incubated with GST alone (lanes 3,4), GST-KIXaa588-683 (lanes 5,6), or GST-CR2aa2055-2150 (lanes 7, 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.

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 (L22→Q; W23→S) into this region (280), and tested the ability of the purified mutant protein to bind the minimal CR2 domain (aa2055-2150). Mutation of these residues has previously been shown to have a dramatic effect on p53 transcription function (172). Figure 2.3B 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. 2.3B, lanes 7,8). As we have previously reported, the double point mutations did significantly reduce p53 binding to the KIX domain (Fig. 2.3B, lanes 5, 6),(280). This data suggests that other amino acids in the p53 tripartite activation domain likely participate in CR2 binding.

2.4d 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 2.4 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

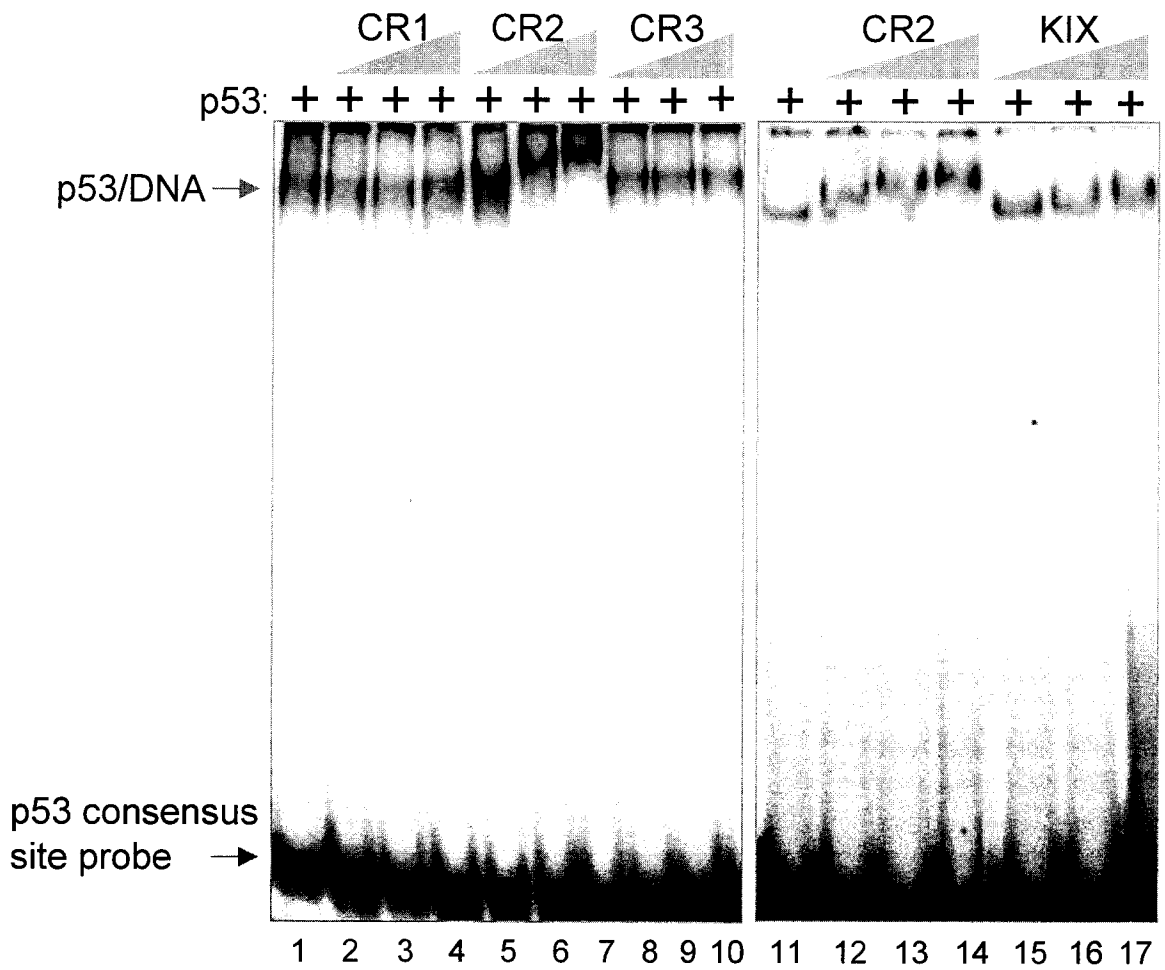


Figure 2.4 **Characterization of CR2 binding to the p53/DNA complex.** Purified, recombinant p53 (0.15 μM) was incubated with a p53 consensus site probe together with increasing amounts of GST-CR1aa1514-1894, GST-CR2 aa2055-2150, GST-CR3 aa2212-2441, or GST-KIX aa588-683 protein (0.20 μM, 0.44 μM, and 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.

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. 2.4, lanes 2-4). The CR3 domain also had no effect on the migration of the p53/DNA complex, consistent with our previous observations (Fig. 2.4, 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 2.4 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 (data not shown). Finally, CR2, as well as the other CBP domains, did not bind DNA in the absence of p53 (data not shown). 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 (data not shown).

2.4e 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 (146). We selected p300 for

these studies, as p53 interacts similarly with the CR2 region of both CBP and p300, and p300 coactivator function has been well characterized in vitro (60, 77, 145, 146, 148). 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 nt guanine-less transcript. This Mdm2 P2 fragment carries two p53 binding sites upstream of the core promoter (127). We chose to analyze transcription in a chromatin context, as several studies have found that analysis of p300 coactivator function in vitro requires nucleosomal templates (60, 77, 145, 148). 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 (113). These assembly proteins are sufficient for the ATP-dependent formation of evenly spaced nucleosomal arrays (111, 294). Figure 2.5A 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); and this ratio was used in subsequent in vitro transcription assays.

A.

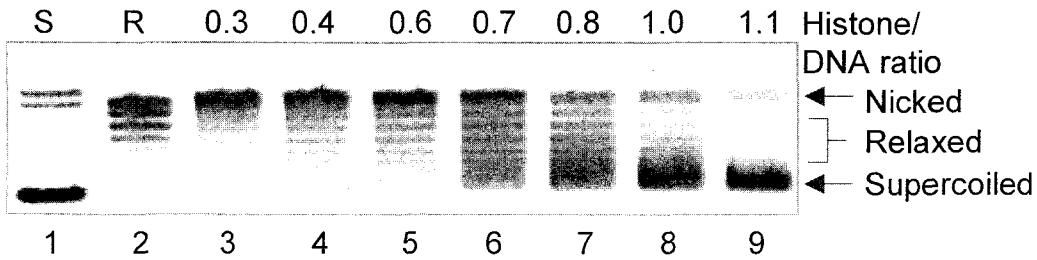


Figure 2.5 **p53 mediated transcriptional activation requires the CR2 domain of p300 *in vitro* and *in vivo*.** (A) One-dimensional DNA topological assays showing the Mdm2 P2 G-less transcription template assembled with *Drosophila* core histones in the presence of dAcf1/ISWI and GST-yNAP-1. The DNA 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.

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 (60). Figure 2.5B 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 p53 produced a significant increase in RNA synthesis from the Mdm2 promoter (18-fold, Fig. 2.5B, lane 4). Under these same conditions, addition of p300ΔSRC, which carries an in frame deletion of CR2 (aa2042 to 2157), activated transcription only 5-fold from these templates; a 3.6-fold reduction in p300 coactivator function (Fig. 2.5B, 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. 2.5B, lanes 6, 7). Figure 2.5C 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 (146).

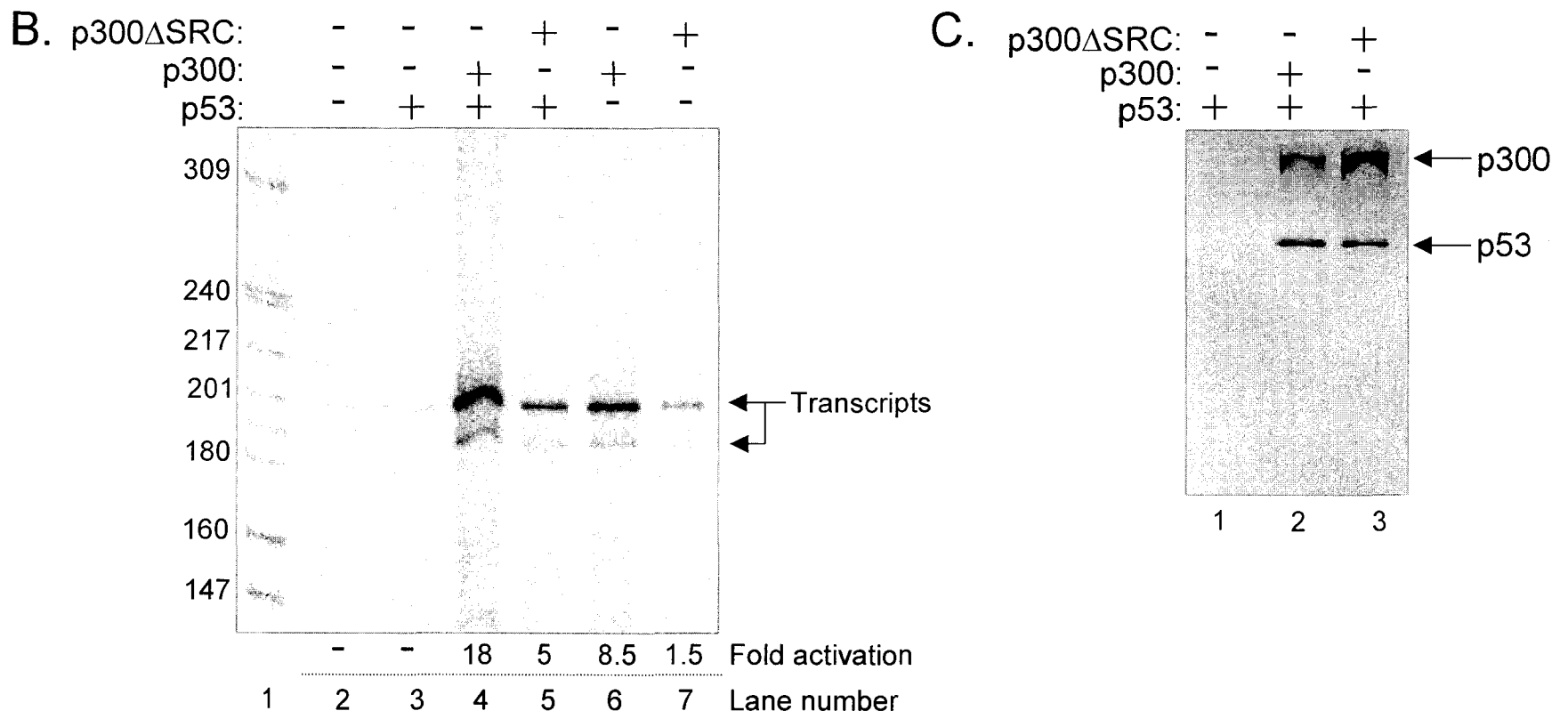


Figure 2.5 (B) Transcriptional activation on Mdm2 P2 G-less chromatin templates was analyzed in the presence of p53 (160 nM, lanes 3-5), wild-type p300 (20 nM, lanes 4, 6), and/or p300ΔSRC (20 nM, lanes 5, 7). Molecular weight size markers, recovery standard, and full length G-less transcripts are indicated. 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) (30nM each) in the presence of 14C-acetyl CoA (100 pmol; 57mCi/mmol). p53 acetylation and p300 autoacetylation are indicated.

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 2.5D 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. 2.5D, 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 and data not shown). These data support a role for the CR2 domain of CBP/p300 in p53 transcription function in vivo.

2.4f 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 (4, 197, 223, 280). Several recent studies suggest that this transcriptional repression may occur as consequence of direct competition for binding to common regions of

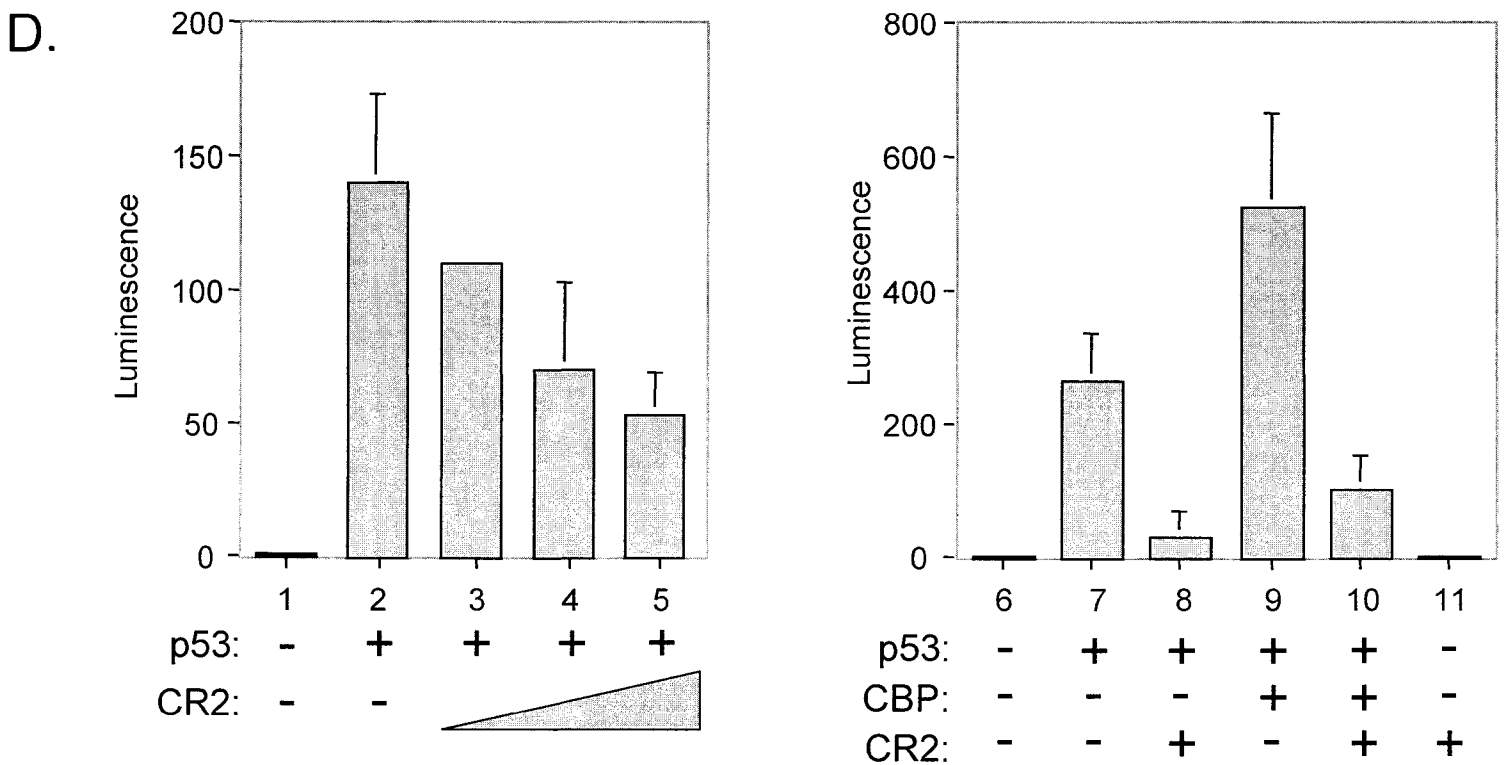


Figure 2.5 (D) The CR2 domain represses p53-activated transcription *in vivo*. In the left panel, the p53 responsive pG13-Luc reporter plasmid (400 ng) was cotransfected with an expression plasmid for p53 (200 ng, lane 2-5), and increasing amounts of an expression plasmid for CMV-CR2 (200 ng, 400 ng, 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 (200ng, lane 7-10). p53 transactivation was assayed in the presence or absence of expression plasmids for full-length CBP (400 ng, lanes 9, 10) and CMV-CR2 (800 ng, lanes 8, 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) +/- the standard deviation.

CBP/p300, thus compromising p53 promoter recruitment of the coactivator (9, 158, 269, 280). Recently, we reported that the HTLV-1 Tax protein binds to the CR2 domain of CBP and p300, and identified CBP aa2003 to 2212 as the minimal region competent for interaction with Tax (239). 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-$CR2_{aa2003-2212}$, 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 2.6 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. 2.6, lanes 7-9). This result is consistent with our observations that both Tax (239) and p53 bind to a similar, overlapping minimal domain of CR2 (aa2003-2212 and aa2055-2150, respectively), and that the CR2 double point mutant L2068→A/ L2071→A reduces interaction with both proteins.

2.5 Discussion

In this report, we show that p53 interacts strongly with the carboxy-terminal region 2 (CR2) of CBP, located between amino acids 2055 and

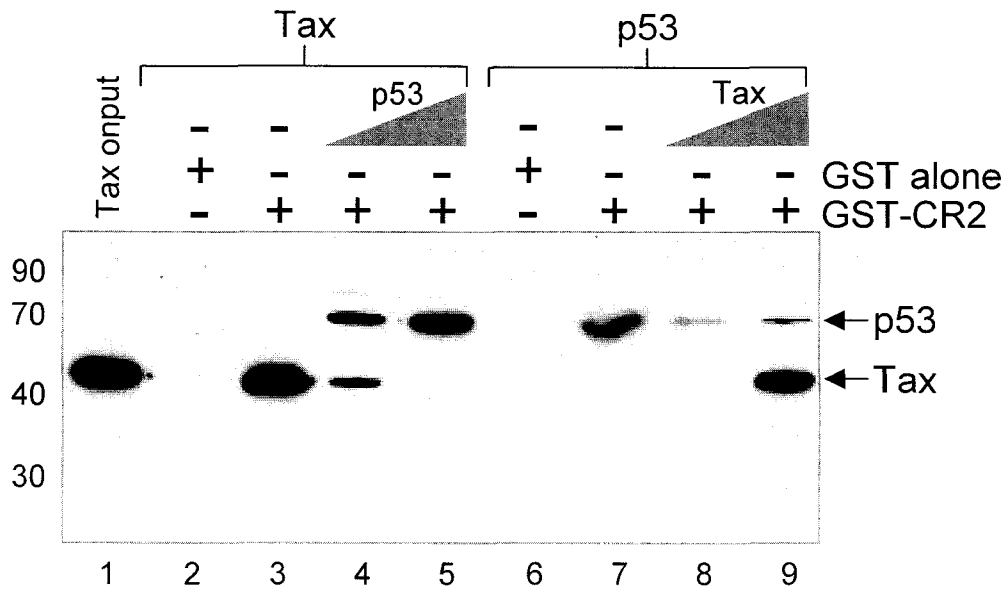


Figure 6. **Tax and p53 binding to CR2 is mutually exclusive.** A constant amount of purified Tax (25 pmol) was incubated with GST alone, or GST-CR2 aa2003-2212 (25 pmol each) in the presence of increasing amounts of purified p53 (25 and 100 pmol, lanes 4, 5). In the reciprocal experiment, a constant amount of p53 (25 pmol) was incubated with GST alone, or GST-CR2 aa2003-2212 (25 pmol each) in the presence of the increasing amounts of purified Tax (25 and 100 pmol, lanes 8, 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.

2150. We also demonstrate that p53 interacts with the corresponding CR2 region of p300, located between amino acids 1970-2193. The CR2 region is distinct from the C/H3 domain, the only previously identified region within the carboxy terminal half of CBP that has been shown to interact with p53 (51, 209). In our assays, p53 interacted more strongly with CR2 than with the region of CBP that encompasses the C/H3 domain (CR1; aa514-1894). 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 (249). This domain also corresponds to the CBP region involved in binding to IRF-3 and HTLV-1 Tax (171, 239). We show that a CR2 double point mutation (L2068→A/ L2071→A), which specifically disrupts the first of the three α-helices that resides within this region (171), 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 (280) and C/H3 domain of CBP (97, 240). We tested whether a minimal region of the p53 activation domain might interact with CR2 using a double point mutant of p53 (L22→Q; W23→S). 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) (93, 94, 97, 209, 240, 280). 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 (4, 36, 73, 195, 197, 223). Several recent studies suggest that this may occur through competition for CBP/p300 (9, 158, 269, 280). We have recently shown that, like p53, Tax also recognizes the CR2 region of CBP/p300 (239), 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 (L2068→A/ L2071→A) (shown in figure 2.2B, and (239). 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.

2.6 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 National Cancer Institute (to J.K.N).

Chapter 2

Supplemental Figures

DNA-Binding Domain	Activation Domain	10 mM AT	20 mM 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 2.7 **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.

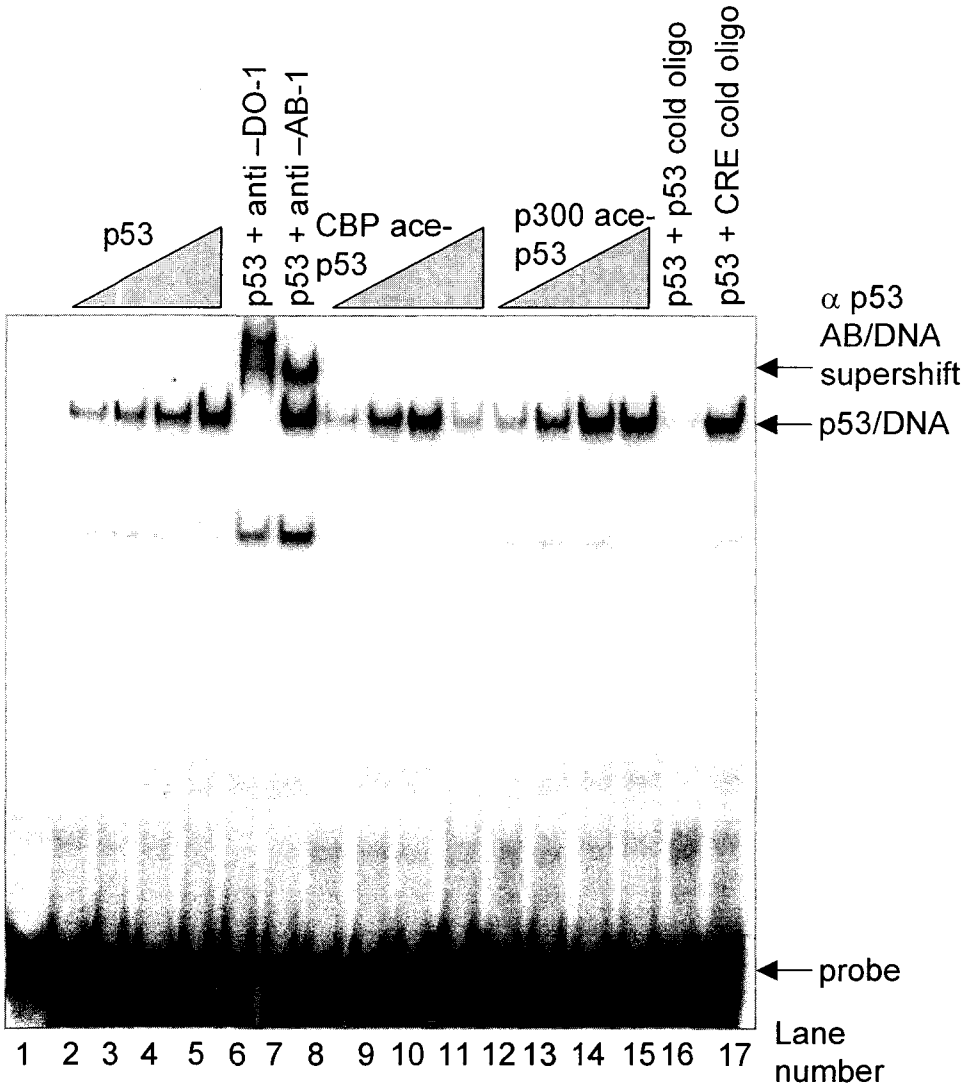


Figure 2.8 **Characterization of CBP-acetylated p53 verses p300-acetylated p53 binding to the p53 consensus probe.** Increasing amounts of CBP-acetylated p53 (60, 120, 240, and 480 ng) (lanes 8 to 11), p300-acetylated p53 (60, 120, 240, and 480 ng)(lanes 12 to 15), and/or purified, recombinant p53 (60, 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 a non-specific CRE cold competitor DNA (lane 17) (each at 100-fold molar excess). The specificity of the p53-DNA complex was also confirmed by addition of p53-specific antibidoes, 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 p53-DNA complex with the p53 consensus site probe is indicated.

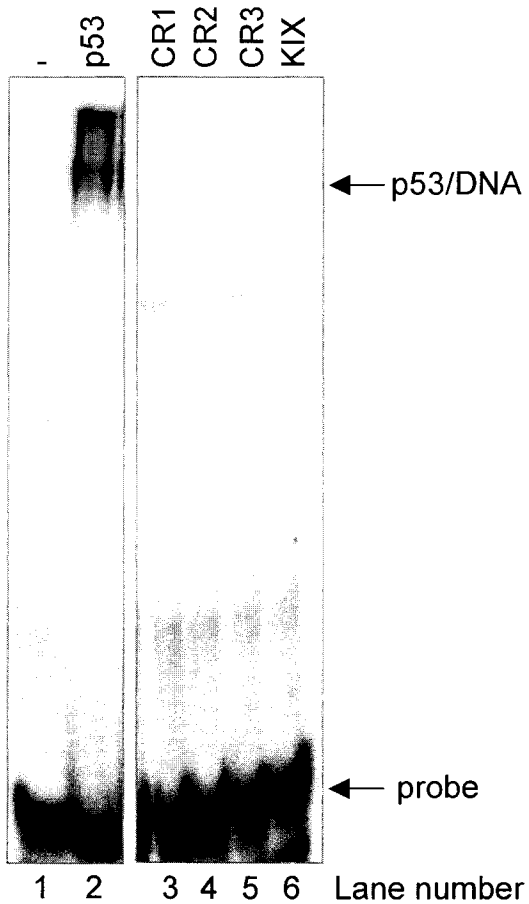


Figure 2.9 **The CBP domains do not bind to the p53 consensus probe.** GST-CR1aa1514-1894, GST-CR2aa2055-2150, GST-CR3aa2212-2441, or GST-KIXaa588-683 protein (200 ng each) were incubated with the p53 consensus probe (lanes 3,4,5, and 6, respectively). Purified, recombinant p53 (100 ng) (lane 2) was 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.

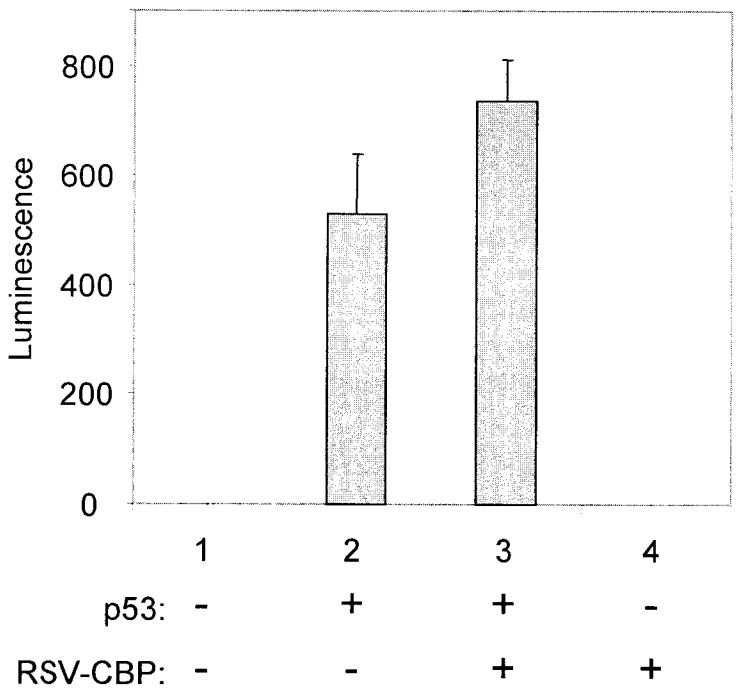


Figure 2.10 **Full-length CBP does not activate transcription from thepG13-Luc promoter in vivo.** The p53 responsive pG13-Luc reporter plasmid (400 ng) was cotransfected with an expression plasmid for full-length CBP (400 ng, lanes 3 and 4), and an expression plasmid for p53 (200 ng, lanes 2 and 3). As a control, cotransfection of an expession 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 3

The High Affinity Sp1 Binding Site in the HTLV-1 Promoter Contributes to Tax-Independent Basal Expression

This chapter was submitted to *Journal of Biological Chemistry* on January 12, 2004. I am the first author, and Jennifer K. Nyborg is the senior author.

3.1 Abstract

Strong transcriptional activation of the human T-cell Leukemia virus, type 1 (HTLV-1) requires many cellular proteins, and the virally-encoded transcription factor Tax. In complex with the cellular ATF/CREB proteins, Tax binds the three viral CREs in the transcriptional control region, and recruits the cellular coactivators CBP/p300 to activate transcription. In addition, HTLV-1 also utilizes several other cellular transcription factors that bind to the promoter to regulate transcription of the provirus. One of these cellular factors, Sp1, has been shown to bind to the viral promoter at two distinct elements; one located within the third viral CRE, and the second located between the second and third viral CREs. The functional significance of Sp1 binding at each of these regions of the viral promoter is not completely understood. We set out to characterize Sp1 binding at these two locations, and to evaluate the functional significance of the Sp1 interaction both in the absence and presence of Tax. We found that Sp1 binds preferentially to the element located between the second and third viral CREs, and modestly activates transcription in vitro and in vivo. Sp1 was detected at the integrated HTLV-1 promoter in vivo, and its binding was unaffected by Tax. Surprisingly, point mutagenesis of the strong Sp1 binding site rendered the HTLV-1 reporter plasmid insensitive to Sp1 activation, and dramatically reduced basal transcription in vivo. These data indicate a role for Sp1 in maintaining basal levels of transcription of the HTLV-1 genome.

3.2 Introduction

Human T-cell leukemia virus type 1 is the etiological agent of an aggressive form of cancer called adult T-cell leukemia/lymphoma (224, 305). The virus is also the causative agent of other diseases, including tropical spastic paraparesis (TSP/HAM), a neurodegenerative disorder similar to multiple sclerosis (reviewed in 184, 286, 307). After infection, HTLV-1 integrates randomly into the host cell genome, and is generally expressed at very low levels. High level expression of the virus requires strong transcriptional activation mediated by the virally-encoded transactivator Tax. Many cellular proteins have also been implicated in transcriptional regulation of the virus. The best characterized promoter elements of the virus are the three imperfectly conserved 21 base pair repeats, called viral CREs. These elements carry an octanucleotide CRE core immediately flanked upstream and downstream by GC-rich sequences. The viral CREs serve as binding sites for Tax in complex with the cellular transcription factor CREB (or other members of the ATF/CREB family of transcription factors) (1, 66, 88, 308). Tax binds to the viral CREs through protein-DNA interactions with the GC-rich sequences (141, 163, 164, 181) and protein-protein interactions with CREB (1, 88). The formation of this promoter-bound Tax-CREB complex is critical for the recruitment of the cellular coactivators CBP and p300 (1, 66, 76, 80, 101, 137, 149, 295, 308).

It has also been reported that the cellular transcription factor Sp1 binds

to the promoter proximal viral CRE, and competes with CREB for binding to this sequence (17, 290). In addition, the region between the second and third viral CREs (–117 to –163) binds several cellular proteins, including Sp1 and Ets (27, 74, 83, 84, 206, 241). Specifically, Sp1 has been shown to bind a GC-rich sequence within this region (74, 206). Studies have also suggested that this region may be responsive to Tax (84, 186). Although Sp1 has been shown to bind these two distinct regions within the HTLV-1 transcriptional control region, the significance of each site remains to be fully elucidated.

Sp1 is a cellular transcription factor involved in a wide variety of processes, and has been shown to bind to more than one thousand different promoters to regulate transcription (46, 129). It was originally identified as the protein that binds GC elements in the SV40 promoter (58). Sp1 is expressed in all tissues types examined (233). It is an essential protein, as homozygous knockouts of Sp1 in mice result in embryonic lethality (185). The carboxy-terminal DNA binding domain of Sp1 contains five zinc fingers that bind with high affinity to sequences with high GC content, called GC-boxes (130). Sp1 has been shown to play a role in transcriptional activation, repression, and maintenance of basal transcription of both cellular and viral genes (48). Sp1 has also been shown to be important for the transcription of genes with promoters that do not contain TATA boxes (24, 126, 143, 178).

In this study, we set out to further characterize the role of Sp1 in HTLV-1 transcription. We were first interested in establishing the recognition elements on the HTLV-1 promoter responsible for high affinity Sp1 binding.

Quantitative DNA binding assays demonstrated that Sp1 had the highest affinity for the region between the second and third viral CRE. Chromatin immunoprecipitation assays revealed that Sp1 was present at the chromosomally integrated HTLV-1 promoter. However, Sp1 only modestly activated HTLV-1 transcription when examined both in vivo and in vitro. Finally, a double point mutation of the site responsible for high affinity Sp1 binding dramatically reduced basal level expression of the virus in vivo, while having no effect on Tax transactivation. These results define a role for Sp1 in Tax-independent, constitutive expression of HTLV-1.

3.3 Materials and Methods

3.3a Electrophoretic mobility shift assay. End-labeled double stranded (d.s.) oligonucleotide probes (0.15 nM) were incubated with increasing amounts of purified Sp1 (Promega) for 30 minutes on ice as previously described (80). Protein-DNA complexes were resolved by electrophoresis on 5% non-denaturing polyacrylamide gels. The top strand sequences of the oligonucleotide probes used in the EMSAs are as follows:

Consensus Sp1: 5'ATTCGATCGGGGCGGGGCGAGC-3';

vSp1: 5'GATCTCCACCAAGAACCACCCATTTCCTA-3' (-149 to -127) (The Sp1 binding GC-box is underlined.);

vCRE-1 (-251 to -231): 5'-GATCTCTAAGGCTCTGACGTCTCCCCCCGGA-3';

vCRE-2 (-203 to -183): 5'-GATCTGCTAGGCCCTGACGTGTCCCCCTGAA-3';

vCRE-3 (-103 to -83): 5'-GATCTCTCAGGCGTTGACGACAACCCCTCAC-3'. (The octanucleotide CRE sequence is underlined in each vCRE.) A d.s. oligonucleotide that encompassed both the vCRE-3 and vSp1 sites (-76 to -155) in their natural context in the HTLV-1 promoter was also used. For the determination of relative binding affinities, we ensured that the concentration of free Sp1 approximated total concentration of Sp1 by keeping the amount of labeled DNA probe constant at a low level. Both bound and free probe were quantitated to determine the percent of DNA in complex with Sp1. Analysis of the data was performed with ImageQuant software and Kaleidagraph.

3.3b In vitro transcription templates. All DNA templates have been previously described (8). The pHTLV-1/G-less cassette carries the full promoter (upstream to -306) driving expression of a 380 bp G-less cassette. The p4TxRE/G-less cassette carries four reiterated copies of the third viral CRE cloned immediately upstream of the HTLV-1 core promoter (-52) driving expression a 380 bp G-less cassette.

3.3c Chromatin assembly. Nucleosomes were assembled on DNA templates as previously described (112). 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 *Drosophila* NAP-1 (8:1 dNAP-1/core histones) on ice for 30 minutes. The supercoiled plasmids were assembled into chromatin using dAcf1 and *Xenopus* histones, at a 0.6:1.0 histone to DNA ratio, overnight at 27°C (77).

3.3d In vitro transcription assays. Following chromatin assembly, preinitiation complexes were formed on 150 ng of the plasmid DNA, as previously described (77). All reactions contained 100 µM acetyl CoA (USB). CEM cell nuclear extract (70 µg) was added immediately following the addition of the activators and/or coactivator. Following a 60 minute 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-α-UTP (3000 Ci/mmol, New England Nuclear). Transcription reactions were processed and analyzed as previously described (163). Molecular weight markers (radiolabeled *Hpa* II digested pBR322) were used to estimate the size of the RNA products.

3.3e Chromatin immunoprecipitation (ChIP) assays. The ChIP assays were performed as described (159). Formaldehyde cross-linked chromatin from 10^6 (SLB-1) or 10^7 (CHOK1-Luc) cells/antibody was used for immunoprecipitation. Cross-linking reactions were quenched with 125 mM glycine, cells were lysed, and chromatin was sonicated to obtain an average

DNA length of 500 bp. Following centrifugation, the chromatin was diluted 10-fold, and precleared with a protein A agarose slurry containing salmon sperm DNA and BSA (Upstate Biotechnology). Precleared chromatin (1 ml) was incubated with 1 µg to 5 µg of antibody overnight at 4°C, followed by immunoprecipitation with protein A agarose. Protein A agarose was precoated with the appropriate secondary antibody when the Tax monoclonal antibody was used. Immunoprecipitated complexes were washed and eluted twice with 200 µl of elution buffer. The protein-DNA cross-links were reversed by heating at 65°C overnight, and 10% of the recovered DNA was used for PCR amplification (27-30 cycles).

3.3f Antibodies. For the ChIP assays, antibodies against Sp1 and CBP were purchased from Santa-Cruz. Tax monoclonal antibody (Hybridoma 168B17-46-92) was obtained from the NIH AIDS Research and Reagent Program.

3.3g ChIP primers. The HTLV-1 promoter primer set for PCR amplification of chromatin from SLB-1 cells is as follows: -290/-31, 5'-TTCCGAGAAACAGAAGTCTG-3'/5'-CTCCTGCTAGTTTATTGAGC-3'. The HTLV-1 promoter primer set for PCR amplification of chromatin from CHOK1-Luc cells is as follows: -349/-81, 5'-GTGAGGGGTTGTCGTCA-3'/5'-AATGACCATGAGCCCCA-3'.

3.3h Cell culture. CEM cells, Jurkat T-cells, and HTLV-1 transformed SLB-1 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin. Hamster CHOK1-Luc cells (211) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin-streptomycin, and 500 µg/ml of G418 (Geneticin; Invitrogen).

3.3i Mammalian expression plasmids and transient transfection assays. Sp1 was expressed from the pCMV-Sp1 plasmid (courtesy of Robert Tjian). The HTLV-1 Tax expression plasmid has been previously described (29). The reporter plasmid, (pHTLV-1/Luc), carries the HTLV-1 promoter driving the luciferase gene. For transient cotransfection assays, cells were grown to a density of 10^6 cells/ml and transfected with Lipofectamine (Life Technologies, Inc.) and a constant amount of DNA for 5 hours. The cells were incubated for 19 hours 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 HSV-TK promoter, as an internal control.

3.3j Site directed mutagenesis. The pHTLV/ΔvSp1-Luc reporter plasmid was prepared by PCR-based site directed mutagenesis. The top strand of the primer set is as follows:

5'GGGAAGCCACCAAGAACCAAACATTTCCTCCCC-3'. The two underlined nucleotides in the GC-box were changed from C (wild-type) to A. The mutation was verified by DNA sequence analysis of the full promoter and 5' end of the luciferase gene.

3.4 Results

3.4a Quantitative analysis of Sp1 binding to HTLV-1 promoter elements

Previous studies have shown that Sp1 binds to two distinct sites in the HTLV-1 promoter, one located within a short GC-rich region between the second and third viral CRE (74, 206), and the second located within the third (promoter proximal) viral CRE (17, 290). We were interested in determining the relative binding affinities of Sp1 for each of these sites. For these studies, we used the electrophoretic mobility shift assay (EMSA) to characterize Sp1 binding. Although Sp1 binding was previously shown at the third viral CRE, we tested all three viral CREs (vCRE-1, -2, -3) since they each contain highly conserved GC-rich sequences. We also tested Sp1 binding to the GC-rich region located between the second and third viral CREs (vSp1; -149 to –127) (see Fig. 3.1A). As a control we tested Sp1 binding to the consensus site. In these experiments, the amount of radioactively labeled DNA was kept constant, and purified recombinant Sp1 was titrated over a wide concentration range. The binding reactions were analyzed on a non-denaturing polyacrylamide gels (Fig. 3.1B). To determine the apparent binding affinities,

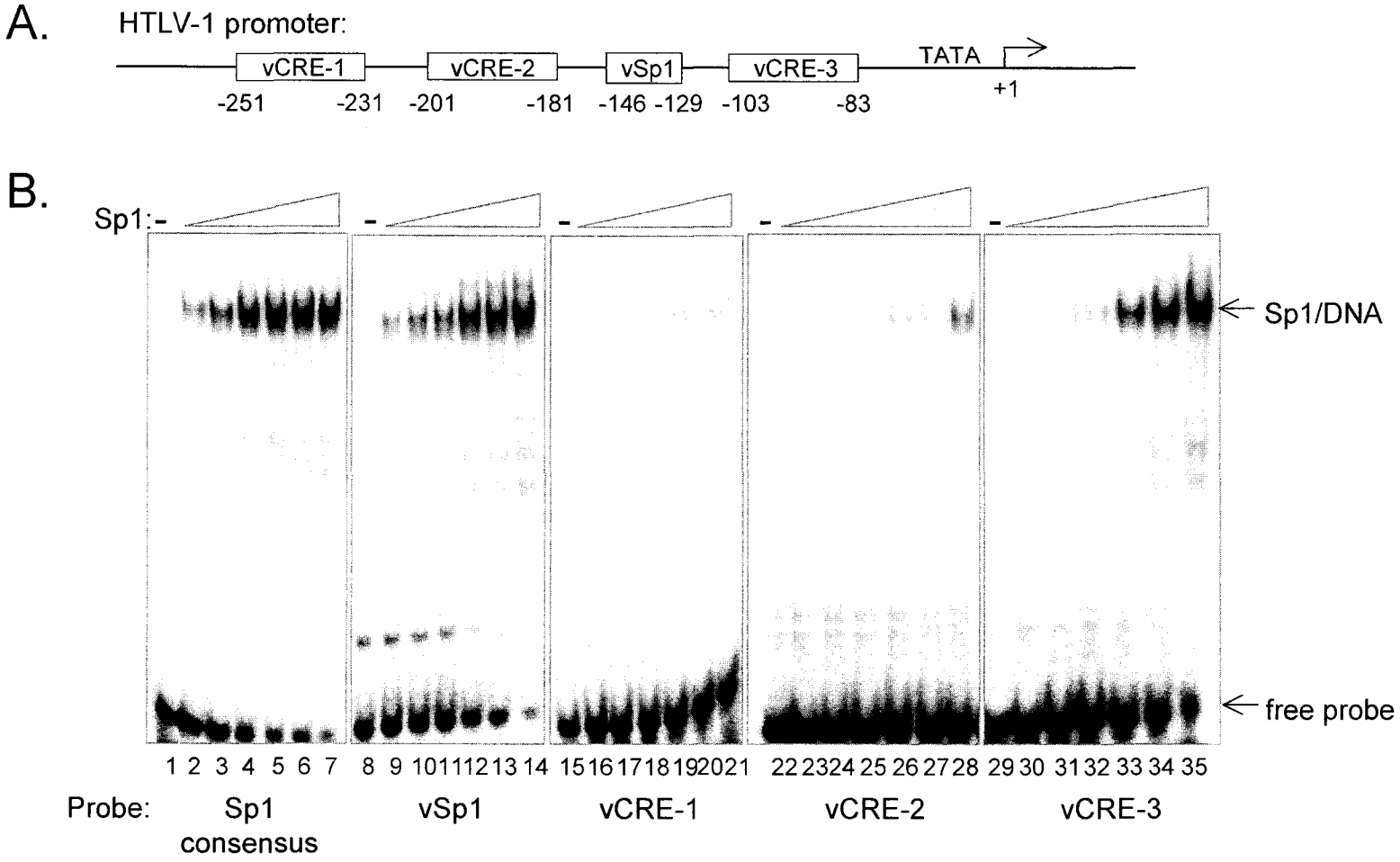


Figure 3.1. **Sp1 binding at HTLV-1 promoter elements.** (A) Schematic representation of the HTLV-1 promoter. (B) Increasing concentrations of purified, recombinant Sp1 (3 nM to 96 nM) was added to binding reactions containing the consensus Sp1 site, the viral Sp1 (vSp1) site, and each of the three viral CREs (vCRE-1, -2, -3) (0.15 nM). Protein-DNA complexes were resolved on a non-denaturing 5% polyacrylamide gel. Free probe and Sp1/DNA complexes are indicated.

the fraction of probe bound versus the total Sp1 concentration was graphed (Fig. 3.1C). The concentration of protein required for 50% binding was used to determine the apparent K_D of Sp1 for the indicated DNA sequence (Table 3.1).

An EMSA of Sp1 binding at each of these sequences is shown in figure 3.1B. As expected, Sp1 bound the consensus site with the highest apparent affinity (10 nM), with Sp1 binding to the vSp1 site only ~2-fold less than that observed for the consensus site (23 nM) (Fig. 3.1B, lanes 1-14, Fig. 3.1C, Table 1). Surprisingly, Sp1 bound the vSp1 site with 3-fold higher affinity than that observed for the vCRE-3 site (75 nM), suggesting that the vSp1 site may represent the most physiologically relevant Sp1 binding site on the HTLV-1 promoter. Sp1 binding to vCRE-3 was lower than expected, and is much lower than the reported 5 nM binding affinity of CREB for this same element (30).

In the experiment presented in figure 3.1B, Sp1 binding was analyzed on short oligonucleotide probes. We were therefore interested in determining whether Sp1 retained its preference for vSp1 (over vCRE-3) using a larger using a larger DNA fragment that carries these two Sp1 sites in their natural promoter context (vSp1/vCRE-3). We performed an EMSA using this 79 base pair fragment that encompasses the region from -76 to -155 of the HTLV-1 promoter (see Fig. 3.2A). Figure 3.2B shows purified Sp1 binding to this DNA fragment (lane 1), and this binding was effectively competed by the addition of 100-fold molar excess of the vSp1 oligo (lane 2). This Sp1/DNA

C.

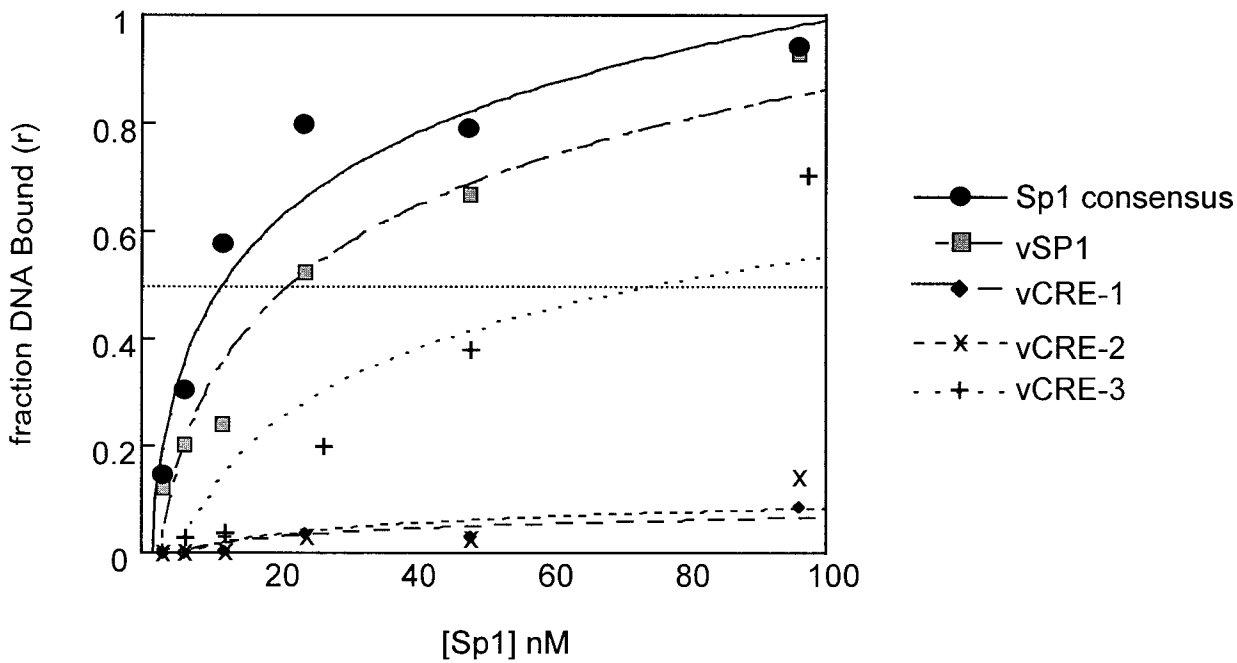


Figure 3.1. **Sp1 binding at HTLV-1 promoter elements.** (C) A graph showing the fraction of probe bound, from EMSAs shown in B, plotted as a function of Sp1 concentration.

Table 3.1

Recognition element	Apparent K_D*
Consensus Sp1 site	10 nM
vSp1 site	23 nM
vCRE-1	N.A.
vCRE-2	N.A.
vCRE-3	75 nM

* Apparent K_D is defined as the concentration of Sp1 at the midpoint of binding

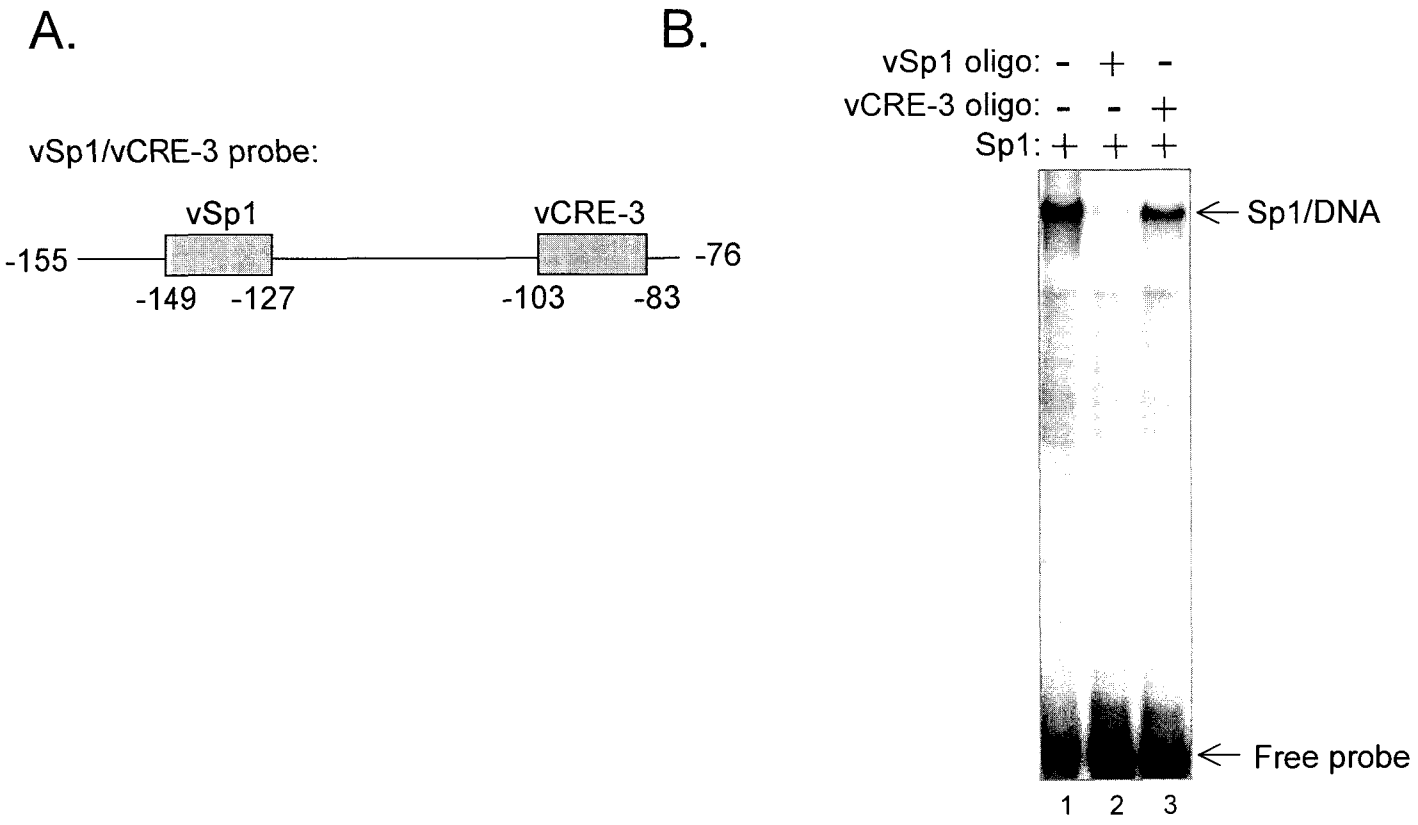


Figure 3.2. **Sp1 preferentially binds the vSp1 site.** (A) Schematic of the vSp1/vCRE-3 probe used in the EMSA. (B) Purified, recombinant Sp1 (28 nM) was added to binding reactions containing the vSp1/vCRE-3 probe. A 100-fold molar excess of the vSp1 (lane 2) or vCRE-3 (lane 3) unlabeled oligonucleotides were added to the binding reactions, as indicated. Protein-DNA complexes were resolved on a non-denaturing 7% polyacrylamide gel.

complex was not efficiently competed with 100-fold molar excess of the vCRE-3 oligo (lane 3). These data corroborate the preferential binding of Sp1 to the vSp1 site in the HTLV-1 promoter.

3.4b Sp1 binds to the HTLV-1 promoter in vivo

Although it is clearly established that Sp1 binds to the HTLV-1 promoter in vitro, we were interested in investigating whether Sp1 binds to the proviral promoter under physiological conditions in the HTLV-1 productively infected human T-cell line SLB-1. We used the chromatin immunoprecipitation assay (ChIP) to investigate the binding of Sp1 in these cells. Cross-linked SLB-1 chromatin was immunoprecipitated with an antibody to Sp1 and the purified genomic DNA was amplified with primers specific to the HTLV-1 promoter (-31/-290). As shown in figure 3.3A, Sp1 is detected at the HTLV-1 promoter in SLB-1 cells (lane 4). As controls, we also immunoprecipitated Tax and the coactivator CBP, and found that both proteins were present on the HTLV-1 promoter as previously described (Fig. 3.3A, lane 5, 6) (159). These data indicate that Sp1 binds to the integrated provirus in vivo.

Since SLB-1 cells express large amounts of Tax protein, the previous experiment revealed Sp1 binding to the HTLV-1 promoter in the presence of Tax. However, since Sp1 has been shown to be involved in basal transcription, we were interested in comparing Sp1 binding in the absence and presence of Tax. For these experiments, we used a cell line that

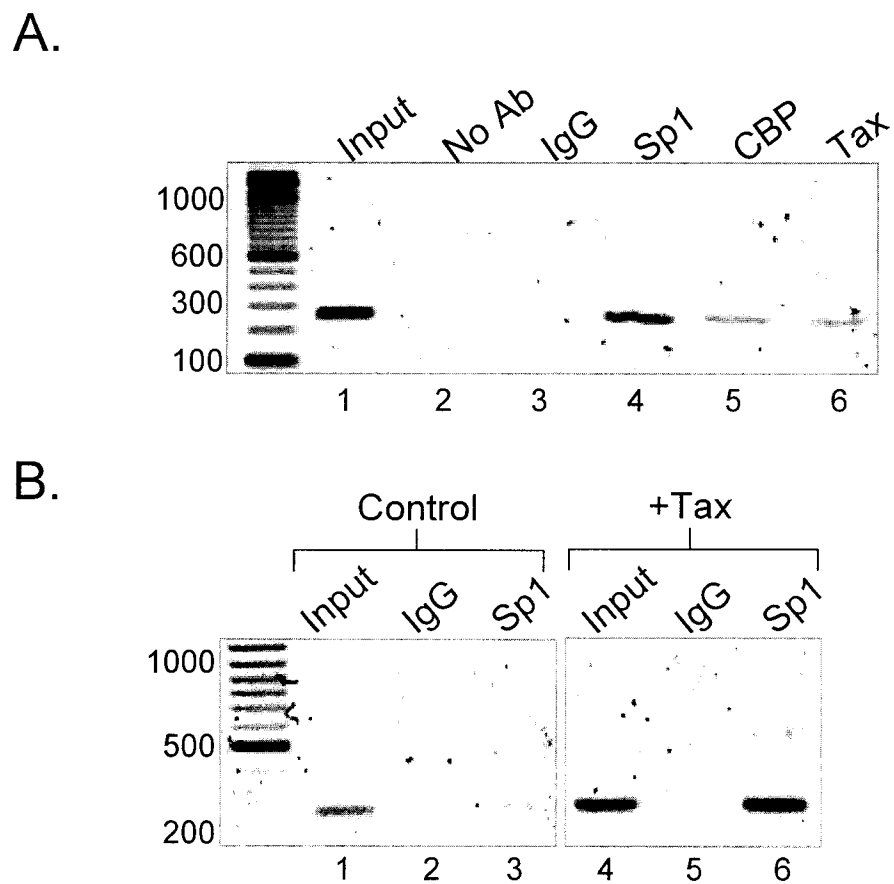


Figure 3.3. **Sp1 binds the chromosomally-integrated HTLV-1 promoter.** (A) ChIP analysis showing Sp1 binding to the HTLV-1 promoter in infected SLB-1 cells. PCR results from coimmunoprecipitation reactions using no antibody, preimmune rabbit serum (IgG), and antibodies against Sp1, CBP, and Tax are shown. Each panel shows amplification of 0.4% of the total input chromatin (input). Purified DNA was analyzed by PCR using primer sets that amplify the viral promoter (-31 to -290). DNA size standards are indicated. (B) ChIP analysis showing Sp1 proteins bound to the integrated HTLV-1 promoter in CHOK1-Luc cells (44) in the absence and presence of transfected Tax. PCR results from input, preimmune serum (IgG), and antibody against Sp1 are shown. The cells were either transfected with pUC (as a control), or the HTLV-1 Tax expression plasmid, as indicated. Transfection efficiencies of approximately 50% were obtained with these cells. Purified DNA was analyzed by PCR using primer sets that amplified the viral promoter (-349/-81).

contained the HTLV-1 5' promoter, driving luciferase expression, stably integrated in the genome of the cell line CHOK1 (211). In the absence of Tax, the HTLV-1 promoter in this cell line is transcriptionally repressed, exhibiting low levels of luciferase activity (data not shown, and 211). Upon transfection of a plasmid that expresses Tax, strong activation of the HTLV-1 promoter was observed (211). Figure 3.3B revealed the binding of Sp1 to the inactive HTLV-1 promoter (lane 3). Upon transfection of a Tax expression plasmid, the relative amount of Sp1 found at the HTLV-1 promoter was unchanged (lane 6). Since the transfection efficiency of these cells was approximately 50%, we would expect that substantial changes in Sp1 binding would be detected in this assay. Real-time PCR of these ChIP assays corroborated these findings (data not shown). These data suggest that Sp1 is persistently bound at the HTLV-1 transcriptional control region, and its binding is unaffected by Tax transactivation.

3.4c Functional significance of Sp1 binding to the HTLV-1 LTR

To determine the functional role of Sp1 in living cells, we examined the transcriptional effects of Sp1 on the HTLV-1 promoter in transient transfection assays. In the ChIP assays shown in figure 3.3, we detected Sp1 binding at the chromosomally intergrated HTLV-1 promoter in CHOK1-Luc cells, indicating that as expected these cells contain endogenous Sp1. We were interested in determining whether enforced overexpression of Sp1 would activate the viral promoter in these cells. Figure 3.4A shows that the highest

amount of an Sp1 expression plasmid increased HTLV-1 transcription approximately 5-fold. We also tested Sp1 activation of HTLV-1 in the human T-cell line Jurkat. For these experiments, we cotransfected the HTLV-1 luciferase reporter plasmid together with the Sp1 expression plasmid. Comparison of figures 3.4A and B reveals that the modest effect of Sp1 on HTLV-1 transcription is similar in both cell types tested. We were also interested in testing whether Sp1 might cooperate with Tax in transcriptional activation from the HTLV-1 promoter. Figure 3.4C shows that cotransfection of expression plasmids for Sp1 had essentially no effect on the extent of Tax transactivation.

To further examine the role of Sp1 in HTLV-1 transcription, we measured the function of purified, recombinant Sp1 in an in vitro transcription assay. We used a DNA template carrying the natural HTLV-1 promoter driving synthesis of a 380 nucleotide guanine-less transcript (see Fig. 3.5A). We chose to analyze the activity of Sp1 in a chromatin context, as we found Sp1 had no activity on non-nucleosomal DNA (data not shown). Chromatin assembly of the HTLV-1 G-less template was performed using the recombinant *Drosophila* assembly proteins Acf1/ISWI, GST-yNAP-1, and purified *Xenopus* core histones, as previously described (77, 112, 113). These assembly proteins are sufficient for the ATP-dependent formation of evenly spaced nucleosomal arrays. Topological assays were performed to determine the optimal ratio of core histones to DNA (data not shown). We performed in vitro transcription assays on the chromatin templates using a

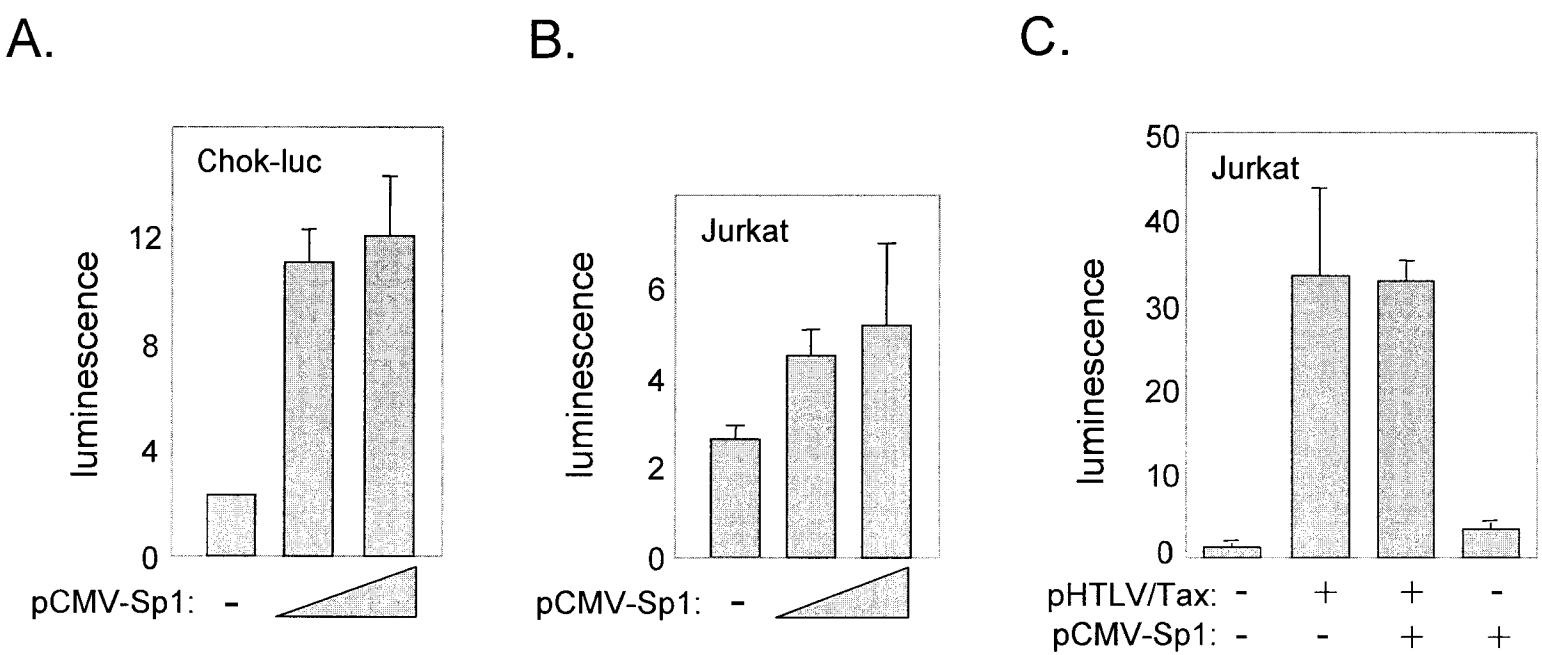
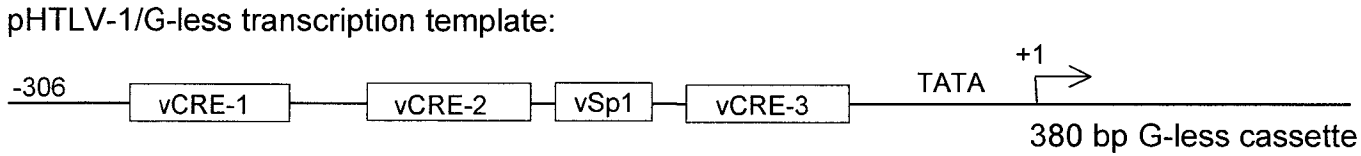


Figure 3.4. **Sp1 modestly activates HTLV-1 in transient transfection assays** (A) An expression plasmid for Sp1 (pCMV-Sp1; 400 ng and 800 ng, respectively) was transfected into CHOK1-Luc cells. Luciferase expression was driven by the integrated HTLV-1 5' promoter (44). The values shown are the mean fold activation (in duplicate) +/- the standard deviation. (B) The pHTLV-1/Luc reporter plasmid (200 ng) was cotransfected into Jurkat cells with increasing amounts of the expression plasmid for Sp1 (pCMV-Sp1; 400 ng and 800 ng, respectively). (C) The pHTLV-1/Luc reporter plasmid (200 ng) was cotransfected into Jurkat cells with the expression plasmid for Tax (pHTLV/Tax; 200 ng) and/or the expression plasmid for Sp1 (pCMV-Sp1; 300 ng), as indicated.

A.



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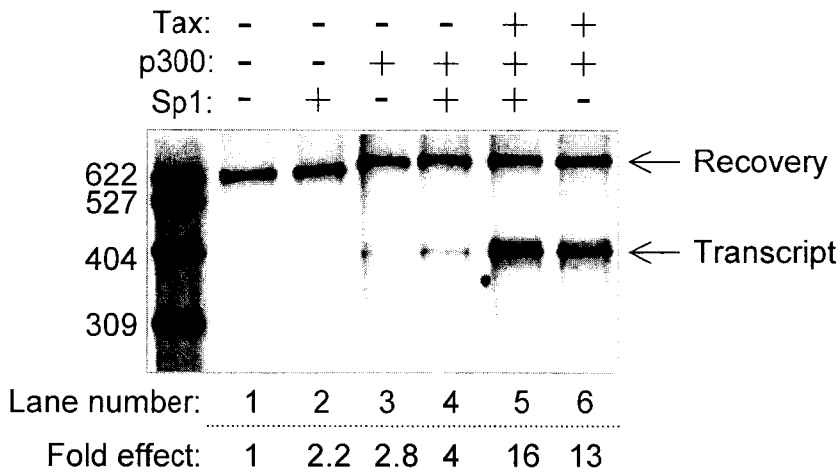


Figure 3.5. **Sp1 only modestly activates HTLV-1 transcription from recombinant chromatin templates in vitro.** (A) Schematic representation of the HTLV-1 promoter (pHTLV-1/G-less) used in the in vitro transcription assays. (B) Transcription from the pHTLV-1/G-less chromatin template was analyzed in the presence of Sp1 (16 nM), p300 (20 nM), and Tax (280 nM), as indicated. All reactions contained acetyl CoA (100 μM). Molecular weight size markers, recovery standard, and the full-length transcripts are indicated. The fold activation was calculated relative to basal transcription.

nuclear extract from CEM cells (a human T-lymphocyte cell line) as a source of RNA polymerase and general transcription factors. Figure 3.5B shows that the addition of Sp1 modestly activated (2-fold) HTLV-1 transcription, relative to transcription observed in the absence of activators (compare lanes 1, 2). The addition of the cellular coactivator p300 only slightly enhanced the activation observed in the presence of Sp1, suggesting that p300 and Sp1 do no cooperate in transcriptional activation (Fig. 3.5B, lane 4). Strong transcription activation was observed in the presence of purified Tax (Fig. 3.5B, lane 6); the addition of Sp1 did not significantly enhance Tax transactivation (lane 5). These data suggest that the transcriptional activation seen in the presence of Tax and Sp1 is not synergistic.

Since we have identified the vSp1 site as the high affinity binding site for Sp1 on the HTLV-1 promoter, we were interested in testing whether this site contributes to the 2-fold Sp1 activation we observed in the in vitro transcription assay shown above. To perform this experiment, we used a DNA template that carries four reiterated copies of the third viral CRE, cloned upstream of the first 52 base pairs of the core HTLV-1 promoter (see Fig. 3.5C). This transcription template carries the four lower affinity Sp1 binding sites (vCRE-3) previously identified (17, 290), but does not contain the vSp1 site. This construct is highly responsive to Tax/CREB activation (76, 77). The 4TxRE/G-less plasmid was assembled into chromatin, and transcription was assayed in the presence of purified Sp1. Figure 3.5D shows that Sp1 has no effect on this template (compare lanes 1 and 2). As expected,

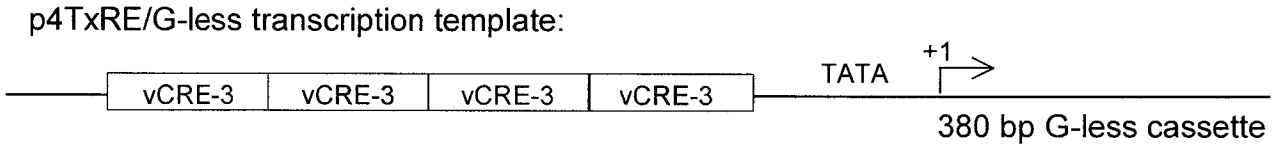
p4TxRE/G-less was strongly activated by Tax (Fig. 3.5D, lane 5). These results suggest that the vSp1 site is the major *cis*-acting element for Sp1 function on the HTLV-1 promoter.

3.4d Sp1 plays a role in constitutive expression of HTLV-1 in vivo

As shown above, functional assays with Sp1 revealed only modest activation of HTLV-1 transcription both in vitro and in vivo. We reasoned that this modest activation may be due to near saturating levels of endogenous Sp1 present in the Jurkat cells and the CEM nuclear extracts. This endogenous Sp1 may have been sufficient for HTLV-1 transcription, and thus masked the effects of exogenous Sp1. To evaluate endogenous Sp1 levels, we performed western blot analysis. Figure 3.6A reveals that both Jurkat and CEM whole cell extracts contain comparable levels of Sp1. This endogenous Sp1 may therefore play a significant role in activating HTLV-1 transcription in each of the functional assays performed above, rendering the HTLV-1 promoter essentially insensitive to exogenous Sp1.

To circumvent this problem, and directly test the role of Sp1 in HTLV-1 transcription, we used site-directed mutagenesis to create a double point mutation in the high affinity vSp1 site. This site was selected, as we show above that Sp1 binds to this region with a 3-fold higher affinity than vCRE-3. This mutation left the remainder of the HTLV-1 reporter plasmid fully intact. We then compared this construct, named pHTLV/ΔvSp1-Luc, with the wild type reporter plasmid in transient transfection assays. Surprisingly, the basal

C.



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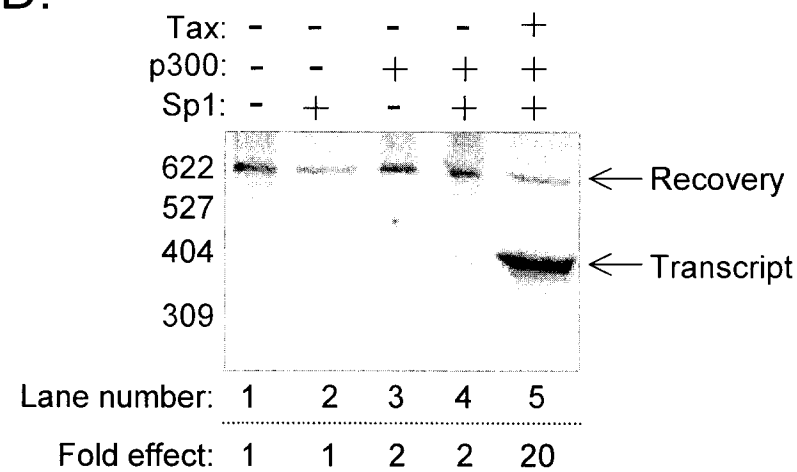


Figure 3.5. **Sp1 only modestly activates HTLV-1 transcription from recombinant chromatin templates in vitro.** (C) Schematic representation of the HTLV-1 4TxRE/G-less promoter used in the in vitro transcription assays. (D) Transcription from the p4TxRE/G-less chromatin template was analyzed in the presence of activators and acetyl CoA as describe in figure 5B. Molecular weight size markers, recovery standard, and full-length G-less transcripts are indicated.

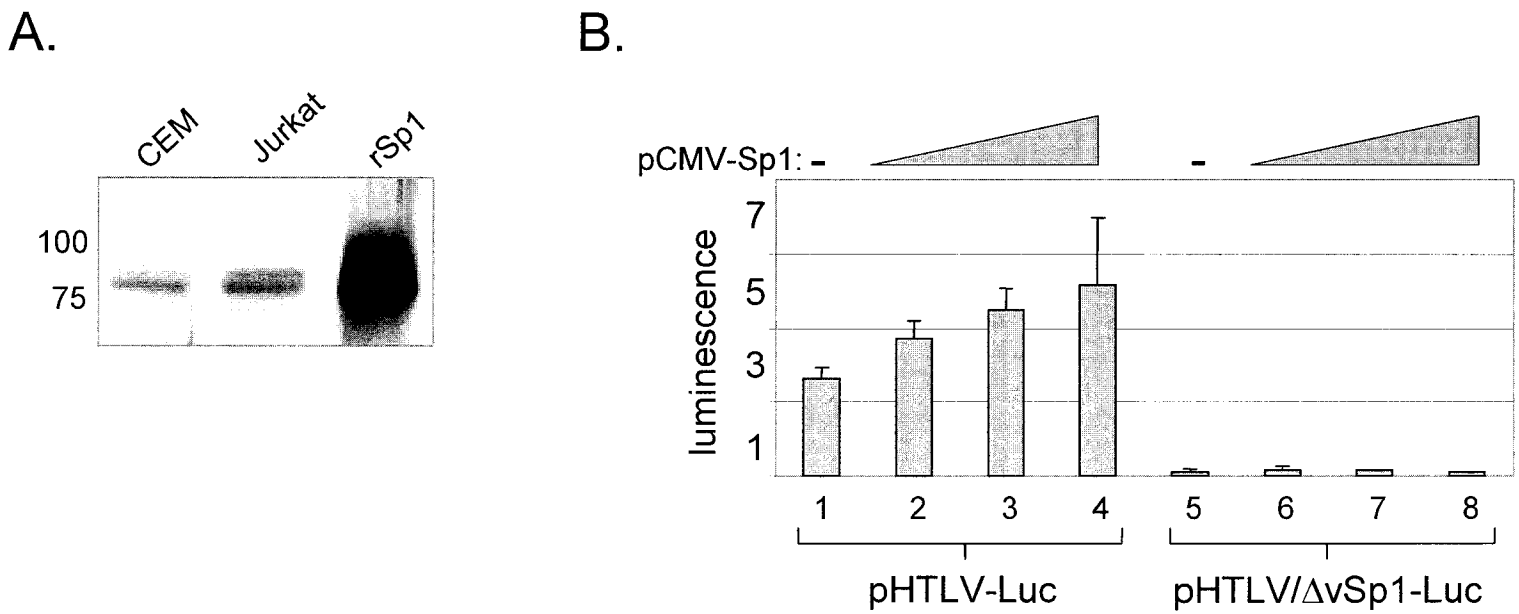


Figure 3.6. **A double point mutation in the vSp1 site significantly reduces basal HTLV-1 transcription.** (A) Western Blot of endogenous cellular Sp1 protein. Whole cell extracts of CEM and Jurkat cells (50 µg) were analyzed by western blot using an Sp1 antibody. Recombinant Sp1 protein (100 ng) was added as a positive control. (B) The pHTLV-1/Luc and pHTLV-1/ΔSp1-Luc reporter plasmids (200 ng each) were cotransfected with increasing amounts of the pCMV-Sp1 expression plasmid (200, 400, and 800 ng respectively), as indicated. The values shown are the mean fold activation (in duplicate) +/- the standard deviation.

C.

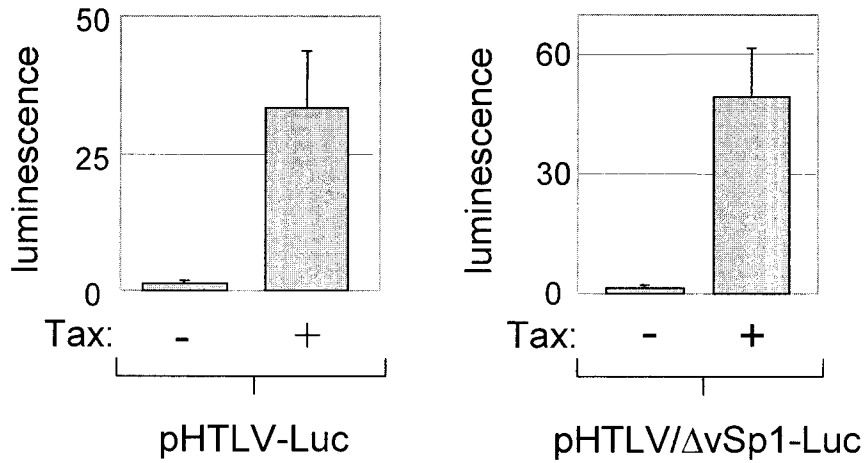


Figure 3.6. **A double point mutation in the vSp1 site significantly reduces basal HTLV-1 transcription.** (C) The pHTLV-1/Luc and pHTLV/ΔSp1-Luc reporter plasmids (200 ng each) were cotransfected with an expression plasmid for Tax (pHTLV/Tax, 200 ng), as indicated. The values shown are the mean fold activation (in duplicate) +/- the standard deviation.

level of transcription from pHTLV/ΔvSp1-Luc was significantly reduced (15-fold) as compared to the wild type HTLV-1 reporter plasmid (Fig. 3.6B). Furthermore, pHTLV/ΔvSp1-Luc was unresponsive to increasing amounts of the Sp1 expression plasmid (Fig. 3.6B, lanes 5-8). To ascertain whether this mutation in vSp1 influenced Tax transcription function, we transfected an expression plasmid for Tax. Figure 3.6C shows that both the wild-type and pHTLV/ΔvSp1-Luc reporter constructs are similarly responsive to Tax. These data suggest that Sp1, through specific binding to the vSp1 site, plays a prominent role in basal level expression of HTLV-1. Furthermore, the data also indicate that the vSp1 does not participate in Tax transactivation.

3.5 Discussion

Previous studies have shown that Sp1 binds to two distinct promoter elements in the HTLV-1 promoter. The first is a GC-box located between the second and third viral CREs (74, 206). This site was originally identified by DNase I footprinting, and purified Sp1 was shown to bind between nucleotides –149 and –127 of the HTLV-1 promoter (vSp1) (206). This sequence is functionally responsive to a transfected Sp1 expression vector in Sp1-deficient Schneider cells, and cooperates with the cellular transcription factor Ets in HTLV-1 transcriptional activation (74). The second Sp1 binding site is located within the upstream GC-rich sequence of the third viral CRE (vCRE-3) (17, 290). This sequence has also been shown to bind purified Sp1, and is functionally responsive to Sp1. Unfortunately, the respective

contribution of these two sites to HTLV-1 transcription by Sp1 has not been addressed.

In this study, we set out to compare the relative binding affinities of Sp1 for these two regions. Quantitative electrophoretic mobility shift assays revealed that Sp1 binds with 3-fold higher affinity to the vSp1 site, relative to the vCRE-3 site; a result corroborated by oligonucleotide competition assays. The affinity of Sp1 for the vSp1 site was within two-fold of that observed for a consensus Sp1 site, indicating relatively high affinity binding. To establish the physiological relevance of Sp1 binding, we also tested whether Sp1 bound to the chromosomally integrated HTLV-1 promoter in vivo. Using the chromatin immunoprecipitation assays, we detected Sp1 binding on the integrated HTLV-1 promoter in two cell lines. Sp1 binding was comparable in the absence and presence of Tax, suggesting that under conditions of strong HTLV-1 transcriptional activation, Sp1 binding remains unchanged. Together, these binding data suggest that Sp1 plays a physiologically relevant role in the regulation of basal HTLV-1 transcription, and that the effects of Sp1 are likely mediated primarily through the vSp1 site.

The two previous studies that defined the promoter proximal viral CRE as the major Sp1 binding site did not simultaneously examine Sp1 binding at vSp1 (17, 290), and therefore may have been unaware of the significance of this element. Although the affinity of Sp1 for vCRE-3 is 75 nM (as compared with 23 nM for vSp1), under conditions of high Sp1 protein concentrations, it is probable that Sp1 also binds to the third viral CRE. However, since CREB

displaces Sp1 from this sequence (17), and since the binding affinity of CREB for vCRE-3 is only 5 nM, it is unlikely that Sp1 occupies this site under most conditions in vivo.

We also examined the transcriptional effects of Sp1 both in transient transfection assays, and in in vitro transcription assays using chromatin-assembled DNA templates. We found that in all cases, Sp1 only modestly activated HTLV-1 transcription. However, we found that endogenous Sp1 was present in the cell lines and extracts used in the assays, and reasoned that this may account for the low level activation observed by Sp1 addition. To address this issue, we prepared a double point mutation in the GC-box at the vSp1 binding site (CC -135/-136→AA), and found that basal transcription levels in the transient transfection assay were dramatically reduced. Interestingly, this mutation had no effect on Tax transactivation.

This data supports a prominent role for Sp1 in basal HTLV-1 expression, and raises the question as to whether Sp1 participates in activated viral transcription. We have not observed synergistic effect of Sp1 on Tax transactivation in vivo or in vitro (data not shown), however it is has been shown that Sp1 cooperates with the cellular transcription factors Ets and p53 in Tax-independent activated transcription (74, 272). Perhaps the significantly higher levels (15-fold) of basal transcription observed with the wild type HTLV-1 promoter, relative to the vSp1 mutant HTLV-1 promoter, reflects synergy between Sp1 and other cellular factors binding within this region. Together, the data indicate that Sp1, specifically via the vSp1 site, is

required for supporting physiological expression of the HTLV-1 genome in the absence of Tax.

3.6 Acknowledgments

We thank Isabelle Lemasson and Nick Polakowski and for their significant intellectual contributions to this research. We also thank Teh Jeang for the CHOK1-Luc cells and Robert Tjian for the Sp1 expression plasmid. This work was supported by NIH grant R01 CA55035.

Chapter 3 Supplemental Figures

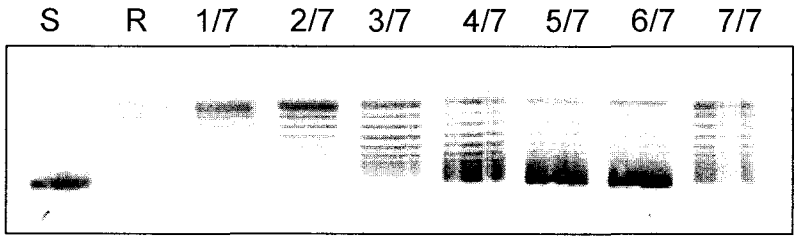


Figure 3.7. **Chromatin assembly of HTLV-1 G-less transcription template.** (A) One-dimensional DNA topological assays showing the HTLV-1 G-less transcription template assembled with *Xenopus* core histones in the presence of dAcf1/ISWI and dNAP-1. The DNA topoisomers were resolved on an agarose gel, 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. Experiment by Isabelle Lemasson.

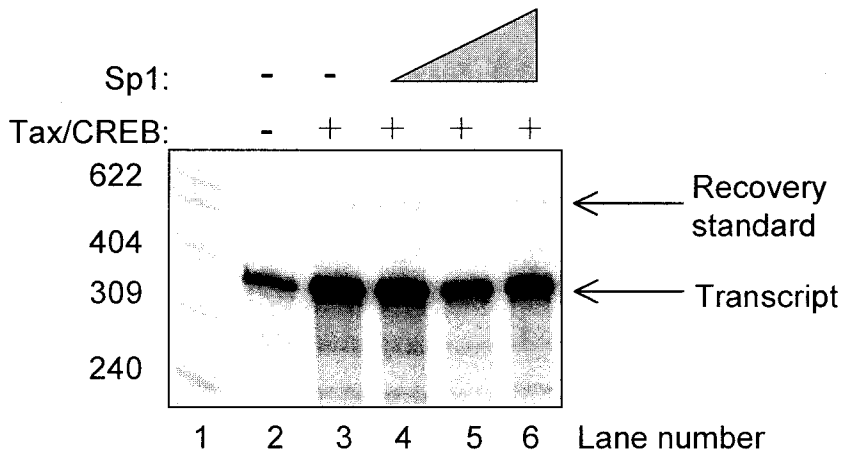


Figure 3.8. **Sp1 does not strongly activate transcription on naked DNA.** Transcriptional activation on HTLV-1 G-less chromatin templates was analyzed in the presence of Tax (100ng) CREB (100 ng), and Sp1(??). Molecular weight size markers, recovery standard, and full length G-less transcripts are indicated.

Chapter 4

Paradoxical Effects of DNA Binding Polyamides on HTLV-I Transcription

This chapter was submitted to *Frontiers in Bioscience* in January, 2004. This work was done in collaboration with Peter Dervan's laboratory at Cal Tech. I am first author, followed by Eric J. Fechter who designed and synthesized the polyamides. I performed all of the experiments shown in the paper with the exception of the polyamide footprinting and the determinatioin of the binding affinities. Peter B. Dervan is third author, and Jennifer K. Nyborg is senior author.

4.1 ABSTRACT

Human T-cell leukemia virus type-1 (HTLV-1) depends on the virally encoded transcription factor Tax for efficient viral replication and gene expression. In a complex with CREB, Tax contacts the minor groove of the promoter DNA at guanine and cytosine rich sequences that flank three off consensus cyclic-AMP response elements (CREs). In this study, we used six Tax-directed pyrole-imidazole polyamides specifically designed to block Tax binding to DNA at each GC sequence of the three viral CREs. We found that four of these polyamides disrupt binding of the Tax/CREB complex in vitro, and that these same molecules also inhibit Tax-mediated transcription in vitro on chromatin-assembled templates. However, of these four Tax/CREB-specific polyamides, only one polyamide appears to be uniquely Tax specific. We show that polyamides can enter the nuclei of HTLV-1 infected T-cells, and two of the four polyamides down-regulated virion production in these cells. Together, these data illustrate the importance of studying polyamide inhibition of gene expression in vitro and in vivo, as the function of the polyamides in living cells is not fully understood. Finally, our data indicates that targeted disruption of the Tax/CREB complex, or other complexes which assemble on the HTLV-1 promoter, may provide a novel approach for inhibiting viral replication in vivo.

4.2 INTRODUCTION

Human T-cell leukemia virus type-1 (HTLV-1) is a retrovirus responsible for an aggressive and fatal malignancy called adult T-cell leukemia (ATLL) (224, 305), and a neurodegenerative disorder called tropical spastic paraparesis (TSP/HAM) (165, 184, 286, 307). In an infected T-cell, the virus is believed to remain latent until an ill-defined signal triggers expression of the virally-encoded transcription factor called Tax. A prominent role for the transcription factor Tax is has been established in the etiology of both ATLL and TSP/HAM. Perhaps integral to these effects on pathogenesis, Tax has also been shown to promote high-level virion production, and to accelerate cell division of the HTLV-I-infected cell (288).

To stimulate transcription, Tax binds to three imperfectly conserved 21 base pair repeats in the HTLV-1 promoter called viral CREs (cyclic AMP response elements) (165). The viral CREs are composed of an off consensus cAMP response element immediately flanked by GC-rich sequences. Both the CRE core and the GC flanking sequences are essential for efficient transcription. To stimulate transcription, Tax makes protein-protein contacts with the transcription factor CREB, while also making protein-DNA contacts with the minor groove of the GC rich sequences of the viral CRE (1, 88, 141, 164, 264, 303, 308). The Tax-CREB complex then recruits the cellular co-activator CBP/p300 to the promoter (77, 80, 149, 239). The

ternary complex containing Tax, CREB and CBP/p300 is essential for high levels of HTLV-I transcription (30, 88, 216, 264, 303, 308).

Polyamides are small molecules containing imidazole and pyrrole heterocyclic amino acids that bind in the minor groove of DNA (273, 291). They can be designed to bind to the DNA with very high sequence specificity, and have been shown to inhibit transcription factor binding to a variety of cis-acting elements (52). Previously, we have used polyamides to target the minor groove GC-rich sequence of the third viral CRE of HTLV-I (163). This study showed that designed polyamides specifically displaced Tax from the viral CRE DNA, providing additional strong evidence that Tax binds DNA specifically at these sequences (163). Furthermore, at concentrations where the polyamides specifically blocked the Tax-DNA interaction, they also inhibited Tax transactivation in vitro from a promoter carrying reiterated copies of the third viral CRE. This study was limited in scope, as the polyamides bound their target sequences with low affinities, and only the third viral CRE was targeted by polyamides. Sequence variation among the three viral CREs prohibited inhibition of Tax binding at all three sites.

In this study, six hairpin polyamides (**U1**, **D1**, **U2**, **D2**, **U3**, and **D3**) were designed to target an upstream (U) or a downstream (D) Tax binding site at one of all three viral CREs within the HTLV-1 promoter. In addition, a seventh polyamide (**C**) was synthesized as a control that bound the HTLV-1 promoter, but at sequences between the viral CREs. We hypothesized that these polyamides would block the binding of Tax at each of the individual viral

CREs simultaneously, and thus serve as inhibitors of Tax function on the natural HTLV-I promoter both in vitro and in vivo. If these molecules are effective at disruption of Tax binding, they may inhibit replication of the virus in vitro, and by extension, in cell culture experiments.

Of the hairpin polyamides **U1**, **D1**, **U2**, **D2**, **U3**, and **D3,** three of these molecules reduced binding of the Tax/CREB complex, and only one demonstrated specific inhibition of Tax transactivation in vitro. We show that polyamides readily enter the nucleus of living HTLV-1-infected T-cells, and that two of the polyamides downregulated HTLV-1 virion production in living cells. However, the two polyamides that inhibited viral replication in cell culture had no Tax-specific effects in vitro, suggesting that the mechanisms of polyamide inhibition are not clearly understood, and emphasizing the importance of investigating polyamide function in living cells. Finally, our data suggest that further development of sequence specific DNA binding polyamides may provide a novel approach to disrupting replication of the virus.

4.3 MATERIALS AND METHODS

4.3a Purification of Recombinant Proteins. Tax was expressed from the pTaxH_6 expression plasmid, and purified as previously described (80, 309). His_6-tagged p300 was expressed from recombinant baculovirus in Sf9 cells and purified as previously described (146). Full-length recombinant CREB

was expressed and purified as previously described (65, 80). Expression and purification of GST-KIX (a.a. 471-719) has been previously described (80). Purified proteins were dialyzed against TM buffer (50mM Tris-HCL [pH 7.9], 100mM KCl, 12.5 mM $MgCl_2$, 1mM EDTA [pH 8.0], 1mM dithiothreithol, 20% [v/v] glycerol) and stored at –70°C. Drosophila NAP-1 (dNAP-1) (His_6 tagged) was expressed from recombinant baculovirus in Sf9 cells cultured in spinner flasks. The protein was purified by Ni^{2+}-agarose batch binding and elution followed by Source 15Q column chromatography (110, 112). FLAG-tagged ISWI and Acf1 were co-expressed from baculovirus in Sf9 cells and complex was purified by anti-FLAG affinity batch binding and eluted as previously described (113). The four core histones were individually expressed in *E. coli* and purified to homogeneity as previously described (179). Histone octamers were prepared by denaturation, then renaturation with high levels of salt, followed by purification of the octamers by gel filtration and ion exchange chromatography. CEM and SLB-1 nuclear extract were purified as previously described (57).

4.3b In Vitro Transcription. The HTLV-1 promoter G-less cassette template, and –52 G-less cassette template, have been previously described (8). The basal HTLV-1 promoter construct carried the –52 to +1 HTLV-1 promoter sequences followed by a 190 bp G-free cassette (8). The supercoiled plasmids were assembled into chromatin using dNAP-1, ACF and *Xenopus* histones, at a 0.57:1.0 histone:DNA ratio (112). Following

assembly, preinitiation complexes were formed on the equivalent of 150 ng of the plasmid DNA in the absence or presence of Tax (100 ng), p300 (150 ng), and polyamide. All reactions contained 100 μM acetyl CoA. CEM (a human T lymphocyte cell line) nuclear extract (50 μg) was added immediately following the addition of the activator and coactivator. Following a 60 minute preincubation reaction, RNA synthesis was initiated by the addition of 250 μM ATP, GTP and CTP, and 12 μM UTP plus 0.8 μM ^{32}P-alpha-UTP (3000 Ci/mmol). Transcription reactions were processed and analyzed as previously described (163). Molecular weight markers (radiolabeled *Hpa* II digested pBR322) were used to estimate the size of the RNA products.

4.3c Electrophoretic Mobility Shift Assay. Protein-DNA complexes were resolved by non-denaturing polyacrylamide gel electrophoresis. EMSAs were performed by incubation of the indicated amount of purified proteins and polyamides. The appropriate ^{32}P-end-labeled DNA (4 fmol) probe was used in a 20-μl reaction mixture. The polyamides were incubated with the DNA for 20 min at room temperature before protein addition. The proteins were added, the reaction tube was incubated on ice for 30 min, and its contents were analyzed on 5% nondenaturing polyacrylamide gels (acrylamide/N,N'-methylenebisacrylamide, 36.5:1 [wt/wt]) in buffer containing 0.04 M Tris-HCl, 0.306 M glycine (pH 8.5), and 0.1% (v/v) Nonidet P-40. The gels were visualized with a PhosphorImager.

4.3d ELISA. Kits were purchased from Zeptometrix Corporation and experiments were carried out according to the manufacturers protocol. HTLV-1-transformed T-cell lines (SLB-1 and MT2) were incubated with 2.5 μM of the indicated polyamides. Cells were counted daily, kept in log-phase growth, and assayed for viability with trypan blue. Polyamides at these concentrations did not produce any adverse affects on cell growth. At days 3 and 7 culture supernatants were harvested and diluted 1:1000 for testing of viral p19 antigen.

4.3e Cell Culture. HTLV-1 transformed T-cells (SLB-1 and MT-2) were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin.

4.3f Polyamide Synthesis. Polyamides were synthesized using solid-phase methods (21). Polyamides were resuspended in H_2O and the concentration was assayed by spectrophotometer. Chemical structures of each of the polyamides are as follows: ImImPy-$(R)^{H_2N}\gamma$-ImPyPyCONHMe (**U1**), ImIm-β-Im-$(R)^{H_2N}\gamma$-PyPy-β-PyPyPyCONHMe (**D1**), ImImPyPy-$(R)^{H_2N}\gamma$-ImImPyPyCONHMe (**U2**), ImImPyPy-$(R)^{H_2N}\gamma$-ImPy-β-PyPyPyCONHMe (**D2**), ImIm-β-Im-$(R)^{H_2N}\gamma$-PyImPyPyCONHMe (**U3**), ImImImPy-$(R)^{H_2N}\gamma$-PyPyPyPyCONHMe (**D3**), and ImPyImPy-$(R)^{H_2N}\gamma$-ImPyPyPyCONHMe (**C**),

where Im = imidazole, Py = pyrrole, β = β-alanine, and $(R)^{H_2N}\gamma(R)$-diaminobutyric acid.

4.4 RESULTS

4.4a Synthesis and Design of Polyamides

The HTLV-1 promoter consists of three imperfectly conserved 21 bp repeats referred to as viral CRE 1, 2, and 3 (see figure 4.1A). These sequences are highly similar in nucleotide sequence, but non-identical. Expression of HTLV-1 via the viral CREs is dependent upon the virally-encoded Tax protein, and all three viral CREs have been show to contribute to Tax-activated transcription (21, 128, 232, 242). To further investigate a role for polyamides in the inhibition of Tax-mediated viral transcription, we targeted each of the three viral CREs with specific, rationally designed polyamides (figure 4.1B). Polyamides were designed to bind both of the 5' and 3' GC-rich regions on the three viral CREs where Tax makes DNA contacts in the minor groove (141, 163, 164). The polyamides were named **U1** (upstream GC flank-viral CRE 1), **D1** (downstream GC flank-viral CRE 1), **U2** (upstream GC flank-viral CRE 1), **D2** (downstream GC flank-viral CRE 2), **U3** (upstream GC flank-viral CRE 3), and **D3** (downstream GC flank-viral CRE 3). Since polyamides bind exclusively in the minor groove of the DNA, these molecules were expected to compete with Tax for the binding to these sites on the viral promoter, as previously shown for the third, promoter

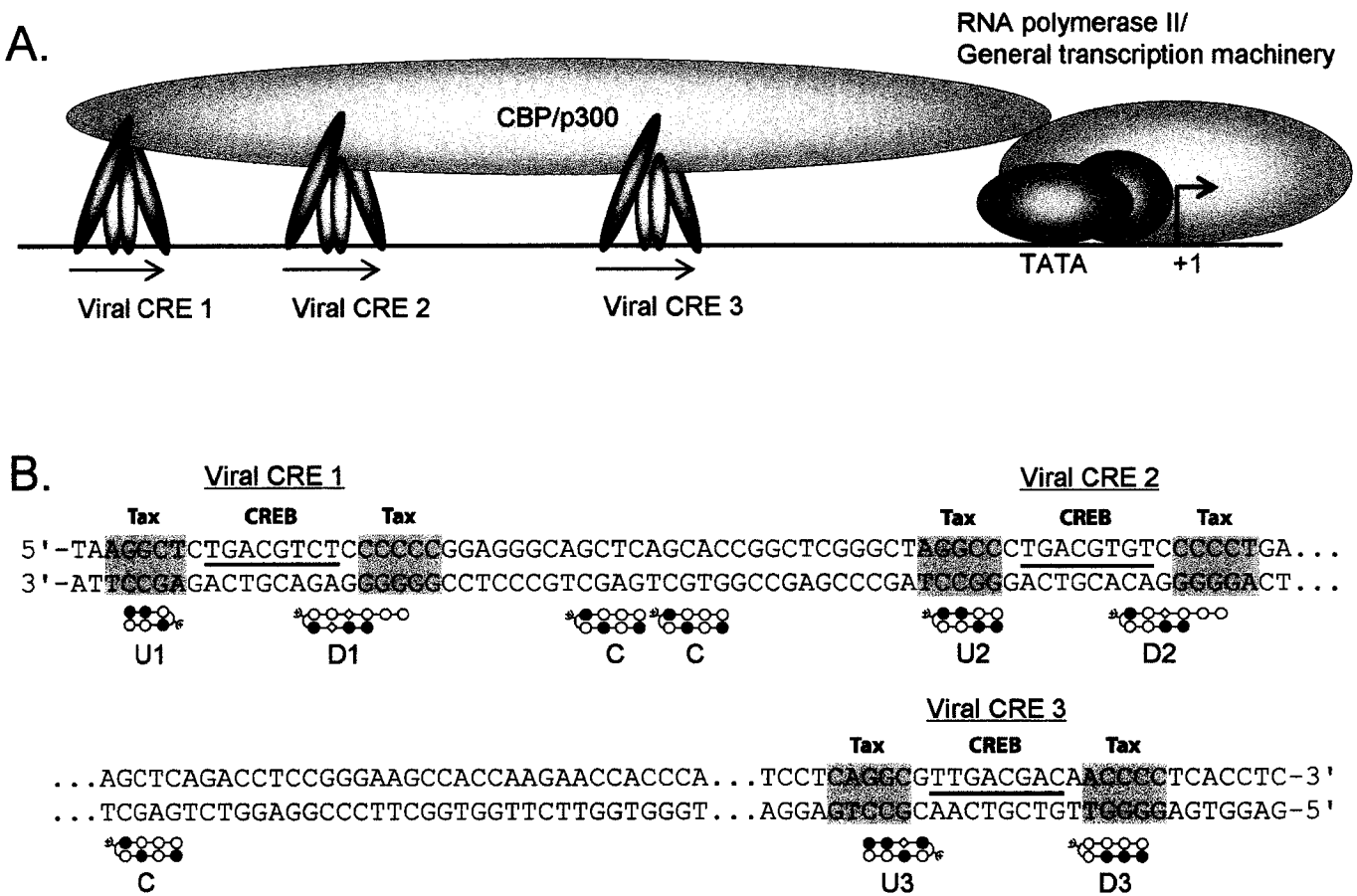


Figure 4.1 **Design of polyamides for the HTLV-1 promoter.** (A) Schematic of the HTLV-1 promoter showing Tax and CREB binding at the three viral CREs. (B) Nucleotide sequence of the viral CREs. Sequences targeted by polyamides (shown in grey), schematics of the six polyamides, and the positions of Tax/CREB binding are shown.

Table 4.1
Equilibrium binding constants of polyamides to targeted sites on the HTLV -1 promoter

Molecule	K_d
U1	2 nM
D1	-
U2	0.2 nM
D2	0.04 nM
U3	0.3 nM
D3	1.2 nM
C	0.6 nM

proximal viral CRE (163). A control polyamide (**C**), which recognizes the sequence 5'-WGWGCW-3', was synthesized and does not target any viral CRE region, but rather the intervening sequences between the viral CREs. The polyamides were characterized by DNase I footprinting to assess specificity and affinity for their respective target sites (data not shown). All polyamides bound their target sites with $K_d \leq 2$nM (Table 4.1) with the exception of the hairpin **D1**, which was relatively non-specific.

4.4b Characterization of Polyamide Binding to Viral Promoter DNA

The electrophoretic mobility shift assay (EMSA) was used to assess polyamide inhibition of Tax/CREB binding to their respective viral CRE DNA. In these experiments, individual viral CREs were incubated with a single targeted polyamide, recombinant Tax, CREB, and the KIX domain of CBP/p300. The KIX domain was used in these assays, as it enhances and stabilizes the Tax/CREB complex (76, 80, 149), thus enabling better visualization of potential binding inhibition by the polyamides. The polyamides were titrated into binding reactions, and their effect on Tax/CREB binding was quantitated and graphed (figure 4.2). The polyamides that inhibited Tax/CREB binding at 100 nM were **U1** (figure 4.2B), **D2** (figure 4.2C), and **U3** (figure 4.2D). It is possible that targeting only one of the two GC-flanks was not sufficient to fully disrupt the Tax/CREB/KIX complex from the DNA. The remaining polyamides, **D1**, **U2**, **D3**, and **C**, inhibited Tax/CREB binding to the viral CREs only at their highest concentrations (1 μM).

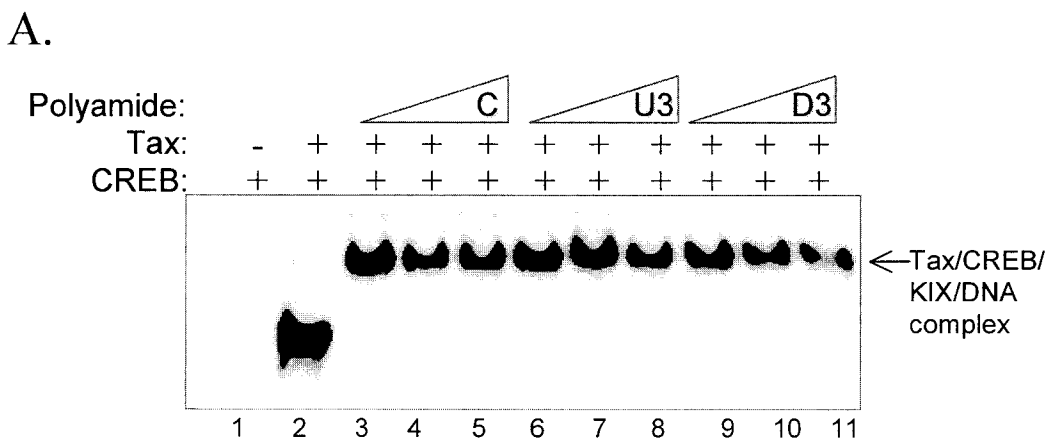
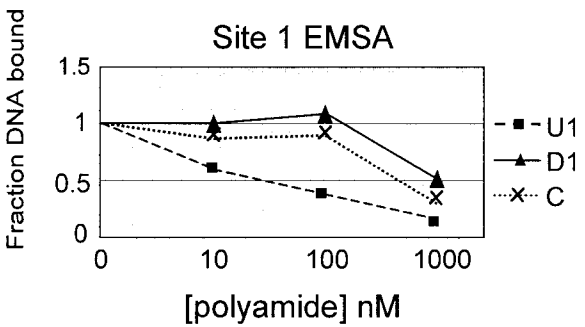
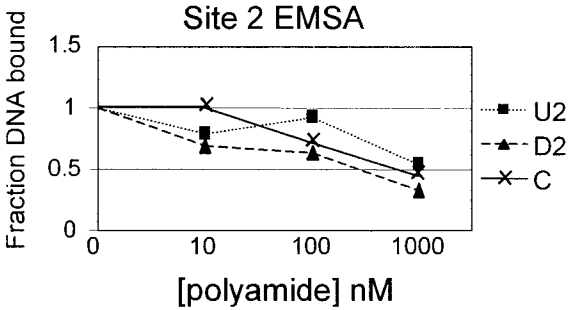


Figure 4.2 **Viral CRE targeted polyamides inhibit Tax/CREB binding to DNA.** (A) Representative EMSA reveals Tax-polyamide competition for the viral CRE 3. Binding reactions for each EMSA was carried out using the relevant viral CRE probe, Tax/CREB/KIX, and polyamide. Reactions contained purified, recombinant Tax (375 fM), CREB (1 fM), and GST-KIX aa 471-719 (200 fM) and increasing amounts (10, 100, 1000 nM) of the indicated polyamide. Protein-DNA complexes were resolved on a non-denaturing 5% polyacrylamide gel. The percent of protein bound as compared to free probe was quantified using ImageQuant, and the results were graphed and shown in panels B, C, and D.

B.



C.



D.

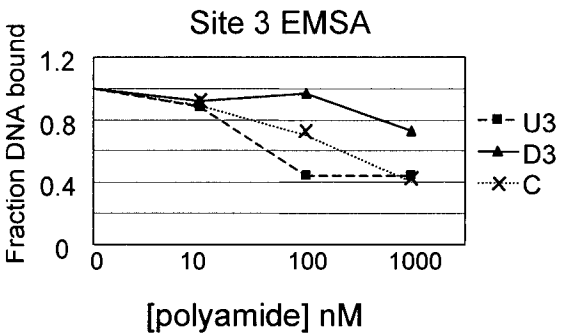
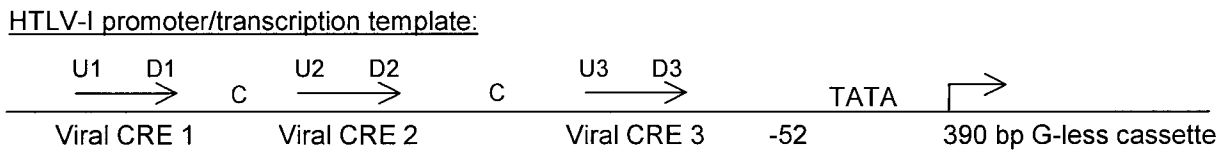


Figure 4.2 **Viral CRE targeted polyamides inhibit Tax/CREB binding to DNA.** Binding reactions for each EMSA was carried out using the relevant viral CRE probe, Tax/CREB/KIX, and polyamide. Reactions contained purified, recombinant Tax (375 fM), CREB (1 fM), and GST-KIX aa 471-719 (200 fM) and increasing amounts (10, 100, 1000 nM) of the indicated polyamide. The percent of protein bound as compared to free probe was quantified using ImageQuant, and the results were graphed and shown in panels B, C, and D.

4.4c Effects of Polyamides on Tax-Mediated Transactivation

To characterize Tax function in the presence of polyamides, we tested polyamide effects on Tax-mediated transactivation via the HTLV-1 promoter. We performed in vitro transcription assays using a plasmid carrying the natural HTLV-1 promoter cloned upstream of a 390 bp G-less cassette. A schematic representation of the HTLV-I promoter/transcription template is shown in figure 4.3A. Previous studies have shown that chromatin-based in vitro transcription systems are much more responsive to Tax (76, 77, 177). Therefore, we chose to analyze the effects of the polyamides in the context of chromatin, as this system better recapitulates the in vivo environment. Furthermore, polyamides have previously been shown to bind directly to nucleosomal DNA (263). Chromatin assembly of the HTLV-1 G-less plasmid was performed using recombinant *Drosophila* assembly proteins Acf1/ISWI, Nap-1, and purified *Xenopus* core histones, as previously described (77, 112). These assembly proteins are sufficient for the ATP-dependent formation of evenly spaced nucleosomal arrays. Topological analysis assays determining the optimal ratio of histones to DNA were performed prior to the in vitro transcription studies (data not shown). We performed in vitro transcription assays on the HTLV-1 responsive chromatin template using nuclear extracts from CEM cells (a human T-lymphocyte cell-line) as a source of basal transcription factors, ATF/CREB proteins, and RNA polymerase II.

A.



B.

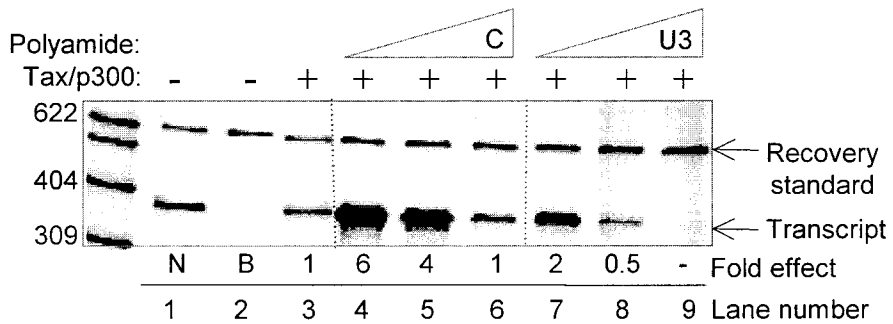


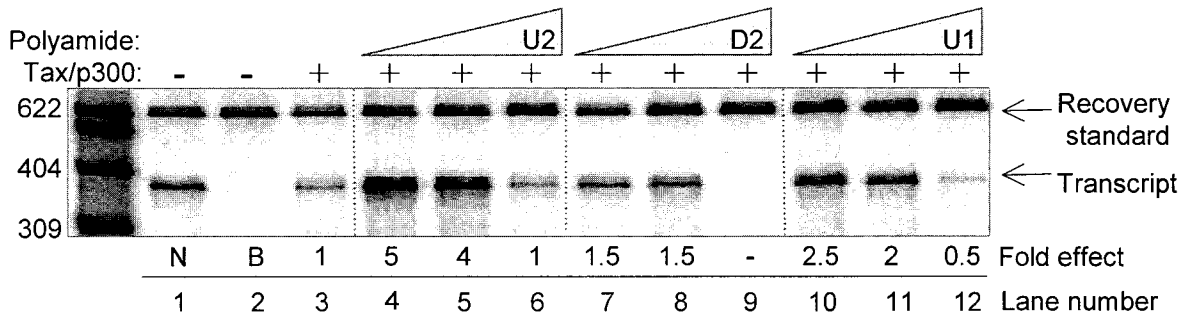
Figure 4.3 **Tax mediated transcription in vitro is inhibited by viral CRE targeted polyamides.** (A) Schematic representation of the HTLV-1 promoter/transcription template used in the in vitro transcription assays. (B) Transcriptional activation on the HTLV-1 G-less, chromatin assembled template, was analyzed in the presence of Tax (70 fM), p300 (30 fM), and 50 μg of CEM nuclear extract. Polyamides were titrated at 10 nM, 100 nM, and 1 μM, respectively. Molecular weight size markers, recovery standard, and the full-length G-less transcripts are indicated. The fold change, relative to the reaction containing Tax/p300 in the absence of polyamide, is indicated. The experiments shown in panels C and D were carried out as described in panel B, except that 300 nM of each polyamide was used in the experiment shown in panel D.

All experiments were performed in the presence of acetyl CoA. Activator, coactivator, nuclear extract, and polyamides were all added following the assembly of nucleosomes on the transcription template. Addition of polyamides prior to preinitiation complex formation produced the same results as compared with adding the polyamides with the activator proteins during preinitiation complex formation (data not shown).

Figure 4.3B shows that the addition of Tax and p300 strongly stimulates transcription from the chromatin-assembled HTLV-I (compare lanes 2 and 3). Transcription on naked, or unassembled DNA, is also shown (figure 4.3B lane 1). Polyamides were then titrated into the transcription reactions at concentrations of 10 nM, 100 nM, and 1000 nM, respectively. Of the five polyamides tested (**C**, **U3**, **U2**, **D2**, and **U1**), only **U3** inhibited Tax transactivation at 100 nM polyamide concentration (figure 4.3B, lane 8). **D2** and **U1** inhibited Tax transactivation at the highest concentrations of polyamide, whereas **U2** and **C** had no inhibitory effect at any of the concentrations tested (figure 4.3B and 4.3C). Surprisingly, **U2** and **C** polyamides showed enhancement of Tax transactivation at the lower concentrations (figure 4.3B, lanes 4,5; figure 4.3C, lanes 4, 5). Because this polyamide activation was not observed on naked DNA (data not shown), it may be due to nucleosome disruption, thus resulting in transcriptional activation.

We were next interested in determining whether multiple polyamides produced a more dramatic inhibitory effect on HTLV-I transcription. Figure

C.



D.

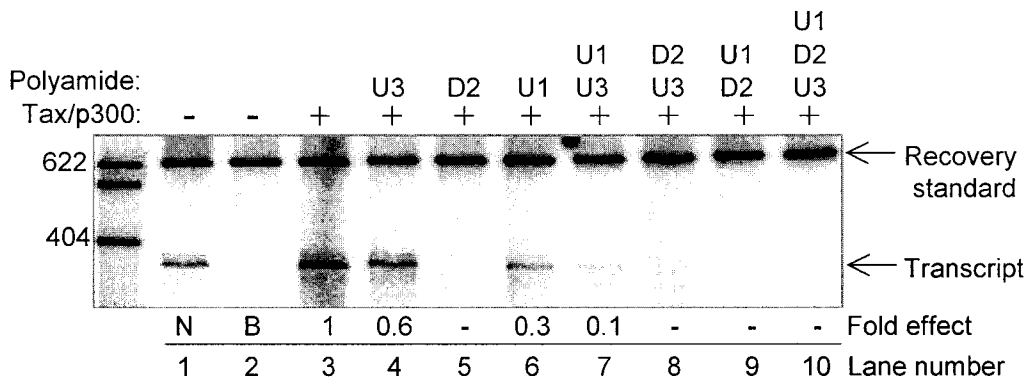


Figure 4.3 **Tax mediated transcription in vitro is inhibited by viral CRE targeted polyamides.** (C) Transcriptional activation on the HTLV-1 G-less, chromatin assembled template, was analyzed in the presence of Tax (70 fM), p300 (30 fM), and 50 µg of CEM nuclear extract. Polyamides were titrated at 10 nM, 100 nM, and 1 µM, respectively. Molecular weight size markers, recovery standard, and the full-length G-less transcripts are indicated. The fold change, relative to the reaction containing Tax/p300 in the absence of polyamide, is indicated. The experiments shown in panels C and D were carried out as described in panel B, except that 300 nM of each polyamide was used in the experiment shown in panel D.

4.3D shows the effect of combining polyamides in in vitro transcription assays at a concentration of 300 nM each. Transcriptional down-regulation was again observed in the presence of **U3**, **D2**, and **U1**, and the combination of **U3** and **U1** showed further inhibition than either polyamide alone (figure 4.3D, lanes 4, 6, 7). These data suggests that a combinatorial approach may be essential to efficient inhibition of Tax function.

4.4d Effects of Polyamides on Basal and CREB Activation

To evaluate the specificity of the polyamides on Tax-dependent transcriptional activation, we were interested in testing the effect of the polyamides in CREB-mediated transcription from the HTLV-I promoter. Since the polyamides were designed to target the GC-rich sequences flanking the CRE, and since they bind in the minor groove, these molecules were predicted to have no effect on CREB binding. We added recombinant CREB to chromatin-assembled DNA and titrated the polyamides into the reaction. Interestingly, we found polyamides **D2** and **U1** strongly inhibited CREB-mediated transcription (figure 4.4A lanes 8, 9, and 12). Only one of the polyamides (**U3**) had no effect on CREB-dependent transcription from the HTLV-1 promoter. These data suggest that the transcriptional inhibitor effects of **D2** and **U1** are not Tax-specific. Furthermore, they provide support for the specificity of polyamide **U3** in Tax-mediated transcriptional activation.

To further address the specificity of the polyamides, we used an HTLV-1 promoter construct carrying only the first 52 bases upstream of the

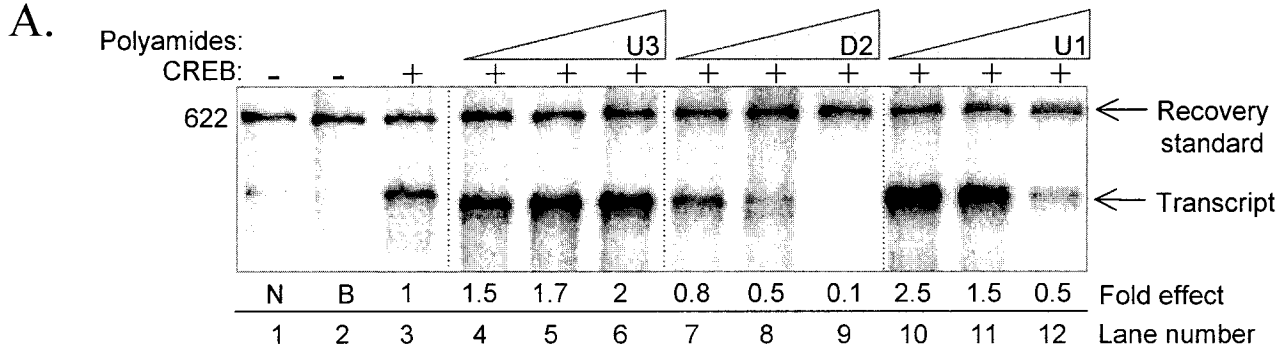


Figure 4.4 **Viral CRE-targeted polyamides inhibit CREB-mediated and basal transcription.** (B) Transcription from the HTLV-1 G-less DNA templates, in the absence of chromatin, was analyzed in the presence 50 µg of CEM extract. Molecular weight size markers, recovery standard, and full-length G-less transcripts are indicated. The fold activation indicated was calculated relative to transcription in the absence of polyamide. Polyamides were titrated at 10 nM, 100 nM, and 1 µM, respectively.

B.

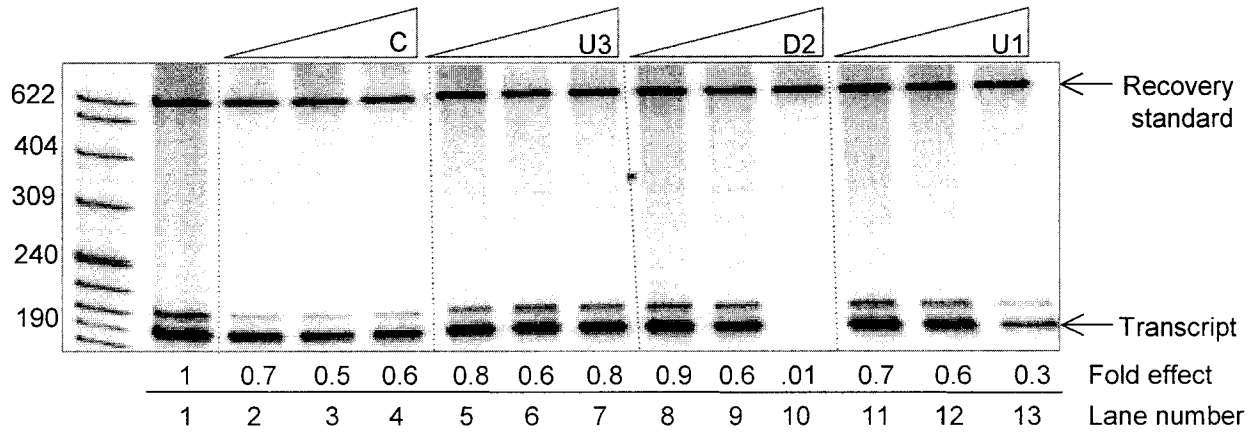


Figure 4.4 **Viral CRE-targeted polyamides inhibit CREB-mediated and basal transcription.** (A) Transcriptional activation on HTLV-1 G-less chromatin assembled templates was analyzed in the presence of purified recombinant CREB (80 nM) and 50 μg of CEM nuclear extract. Molecular weight size markers, recovery standard, and full-length G-less transcripts are indicated. The fold change was calculated relative to the reaction containing CREB in the absence of polyamide. Polyamides were titrated at 10 nM, 100 nM, and 1 μM, respectively. (B) Transcription from the HTLV-1 G-less DNA templates, in the absence of chromatin, was analyzed in the presence 50 μg of CEM extract. 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 absence of polyamide. Polyamides were titrated at 10 nM, 100 nM, and 1 μM, respectively.

core HTLV-1 promoter, and lacking all three viral CREs (see figure 4.3A). This promoter is not Tax responsive, and thus allowed us to monitor basal transcription from the HTLV-1 core promoter. These transcription assays were performed in the absence of chromatin, as the core HTLV-1 promoter is not responsive to transcriptional activators. Consistent with the above results, we found that polyamides **D2** and **U1** down-regulated basal HTLV-1 transcription, whereas polyamide **U3** had no effect. This observation furthers identifies polyamide **U3** as the only polyamide that specifically inhibits Tax-mediated transcription. All of the others appear to inhibit non-specifically, or have no effect.

4.4e Polyamides Enter the Nucleus of Living HTLV-1 Infected T-cells

We were also interested in correlating our in vitro polyamide studies with viral expression in HTLV-1 infected cells. It has been shown that polyamides enter the nucleus of certain types of T-cells (22). Therefore, we tested the ability of our HTLV-1 infected cell lines to take up fluorescently labeled polyamides into their nuclei. A sequence specific hairpin polyamide-Bodipy conjugate (figure 4.5A) was incubated with two HTLV-1 infected cultured T-cell lines, SLB-1 (figure 4.5B) and MT2 (figure 4.5C). Figure 4.5 shows that both cell lines exhibited nuclear uptake at all concentrations tested. Cells were also incubated with a mitochondrial staining dye to confirm cell viability. Figure 4.5C (right panel) shows that the polyamides do not cause cell death or apoptosis. These experiments indicate that the

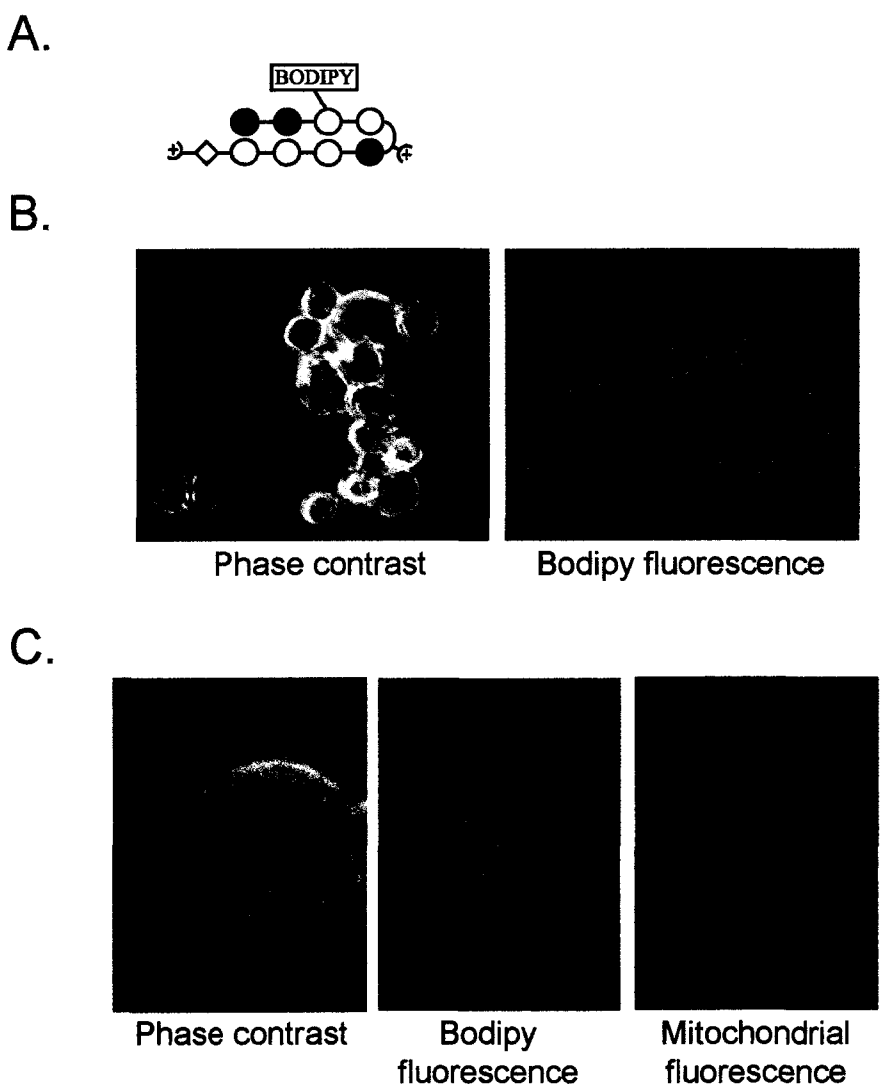


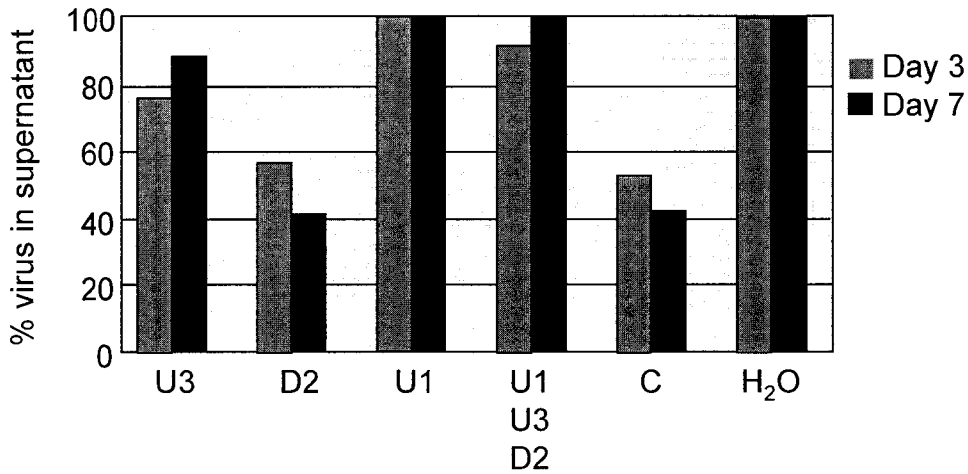
Figure 4.5 **Polyamides enter the nucleus of living HTLV-1 infected T-cells.** Cells were incubated with a bodipy conjugated polyamide (5 μM) for 20 hours under normal growth conditions for these cell lines. The cells were visualized by confocal microscopy. (A) Structure of Polyamide-Bodipy Conjugate used for cellular uptake study. (B) MT-2 cells. Left panel, phase contrast image; right panel, bodipy fluorescence. (C) Oil of immersion image of an SLB-1 cell. Left panel, phase contrast image; middle panel, bodipy fluorescence; right panel, mitochondrial fluorescence following incubation with TMRM, a dye specific for active mitochondria.

polyamides enter the nucleus of living HTLV-1 infected T-cells, and therefore are available for potential effects on gene expression.

4.4f Polyamides Inhibit Viral Replication in Cell Culture

With the knowledge that polyamides efficiently localize to the nucleus of HTLV-1 infected cells, we began studying the effect of polyamides on HTLV-1 expression in living cells. If certain polyamides blocked Tax/CREB binding to the HTLV-1 promoter, could they then downregulate viral replication, inhibiting virion production? To test this, we incubated both SLB-1 and MT2 cells with the small library of polyamides, and measured virion levels by ELISA monitoring p19. Both of these HTLV-1 infected cell lines carry multiple proviruses, and express large amounts of Tax (159). We used a polyamide concentration of 7.5 μM, as this is below the level of polyamide toxicity (56). We tested the polyamides that were previously shown to be successful at inhibition of Tax/CREB binding and HTLV-1 transcription in vitro. Polyamides, **D2** and the **C** produced the most dramatic effects on virion production in both cell types examined (figure 4.6A, B). Remarkably, neither of these polyamides were shown to have specific effect on Tax-activated transcription in vitro (see figures 4.3 and 4.4). In contrast, polyamide **U3**, which had the most dramatic Tax-specific effects in vitro, had essentially no effect in living cells (figure 4.6).

A.



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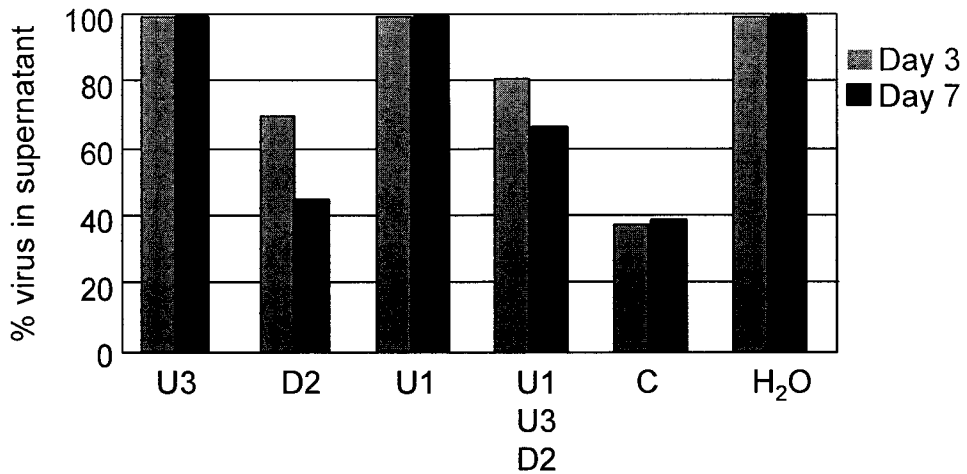

Figure 4.6 **Polyamides inhibit virion production in living HTLV-1 infected cells.** Polyamides were incubated with living cells to a final concentration of 7.5 μM for 3 or 7 days, as indicated. Culture supernatant was harvested and analyzed by ELISA testing for viral p19 antigen. (A) SLB-1 cells. (B) MT-2 cells.

4.5 DISCUSSION

Sequence specific DNA binding polyamides have the potential to be effective molecules for inhibiting protein binding to DNA (52). Their specificities and affinities approach those of naturally occurring DNA binding proteins, thus making them good candidates for a chemical approach to modulation of transcription. They have been shown to effectively inhibit the binding to factors of promoters such as HIV (47), TBP binding (59), Polymerase II (53), TFIIIA, (89), Human Papilloma virus E2 binding (237), NF-κB (293), and MMTV (296). Although in vitro studies displaying their effectiveness at inhibiting transcription factor binding are positive and promising, their function in vivo is not fully understood.

A previous study from our laboratory showed that two minor groove binding polyamides were effective at inhibiting HTLV-1 Tax protein from binding to its promoter DNA sequence (163). In the present study, we designed and synthesized six different polyamides targeted to GC-rich viral CRE sequences in the HTLV-1 promoter. These studies were carried out with the ultimate goal of disrupting Tax binding and transcriptional activation in vitro, and inhibiting viral replication in vivo. We demonstrated that three of the six polyamides disrupt Tax/CREB binding, whereas only one (**U3**) inhibited Tax-specific transcription. The others either had no effect, or were non-specific in their effects. Interestingly, two polyamides (**D2** and **C**) were effective at inhibiting viral replication in cell culture, neither of these

polyamides were shown to be Tax specific in vitro. Interestingly, the control polyamide **C**, which was designed to bind the HTLV-1 promoter but *not* to bind the viral CREs, turned out to be one of the most potent inhibitors of HTLV-1 in living cells. Analysis of polyamide **C** binding to the HTLV-1 promoter by DNase footprinting shows that this polyamide binds between the viral CREs. Although Tax does not bind in this region, it is possible that another factor may interact in this region that could play a prominent role in transcription. Multiple cellular factors have previously been shown to bind between the second and third viral CREs (206, 241). Alternatively, it may be that polyamide **C** exerts its effect as an architectural cofactor, perhaps stiffening the DNA in regions that should be bent in a higher order protein-DNA complex.

Since we do not fully understand the biology of HTLV-1 transcription, we may not be able to accurately predict the effect of rationally designed DNA binding ligands on viral regulation. Perhaps a better way to search for polyamides that successfully inhibit HTLV-1 replication will be to screen a library of molecules for their effectiveness at specifically reducing viral replication. Following the initial screening and identification of "active" polyamides, investigation into the molecular mechanisms of their mode of inhibition may lead to interesting new discoveries about the biology of HTLV-1 transcription. This approach may also lead to the identification of effective polyamides that may be useful as pre-clinical lead structures for anti-HTLV-1 therapeutics.

4.6 ACKNOWLEDGMENTS. We thank Dr. Barbara Bernstein for help with confocal imaging. We also thank Joel Gottesfeld for helpful discussions. This work was supported by NIH grants R01 CA55035 (to J.K.N.), and R01GM51747 (to P.B.D.).

Chapter 4 Supplemental Figures

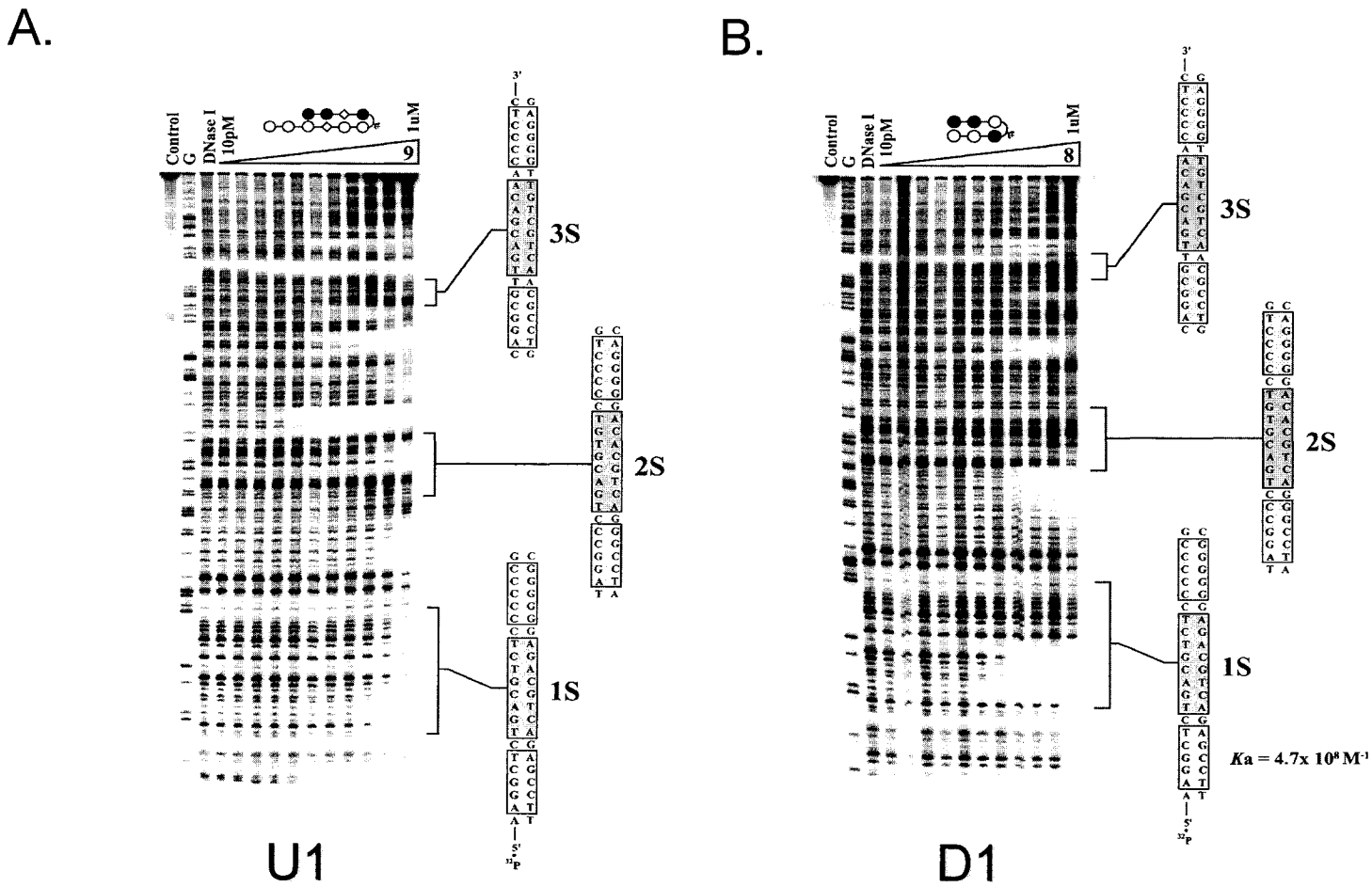

Figure 4.7 **Binding reactions of Polyamides to HTLV-1 promoter.** Quantitative DNase I footprint titration experiments on the PCR-amplified 5'-^{32}P-labeled fragment of HTLV-1. (A) Polyamide U1 was titrated from 0pM, 30pM, 100pM, 300pM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, to 1uM. (B) Polyamide D1 was titrated from 0pM, 30pM, 100pM, 300pM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, to 1uM. Experiments by Eric Fechter.

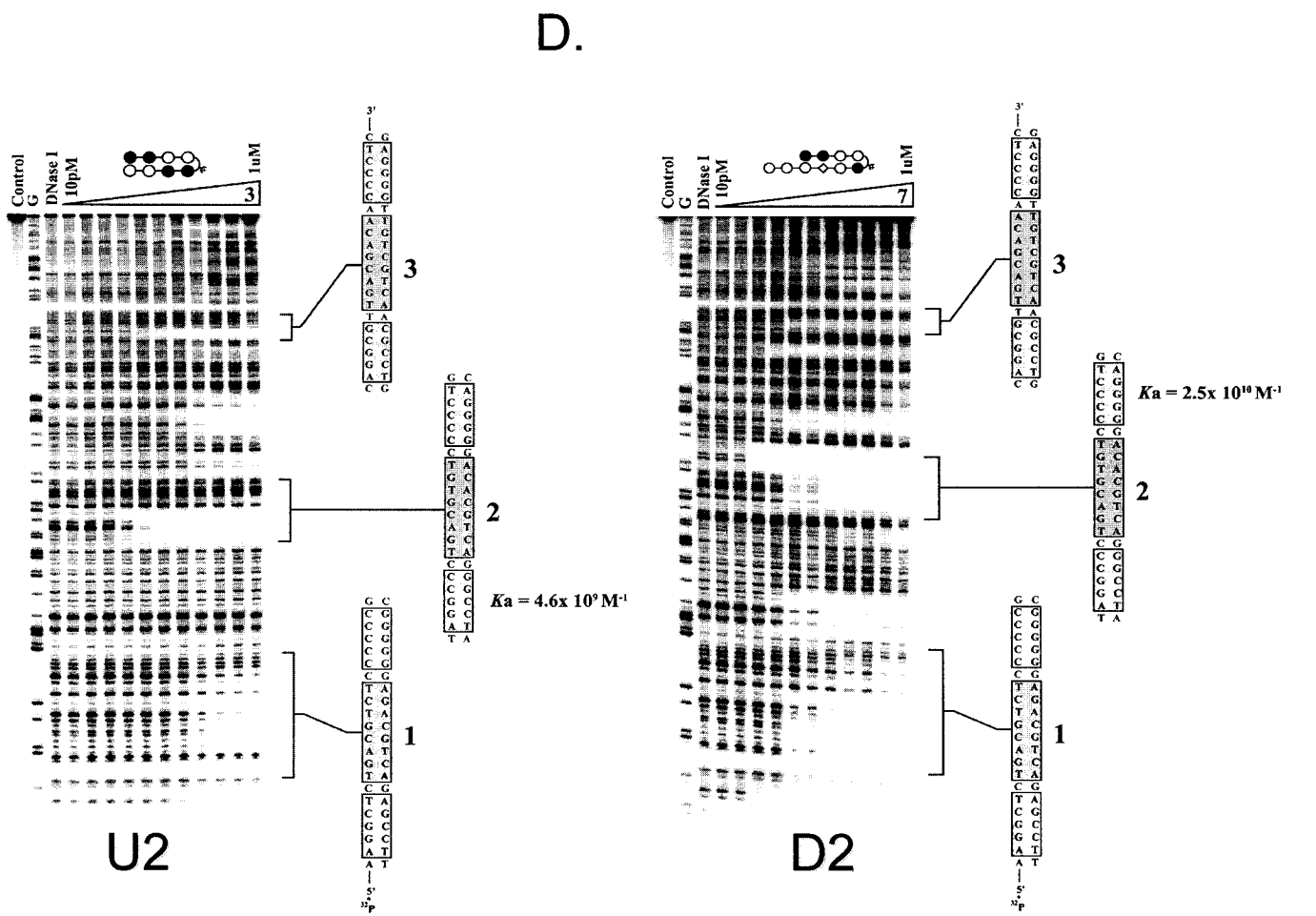

Figure 4.7 **Binding reactions of Polyamides to HTLV-1 promoter.** Quantitative DNase I footprint titration experiments on the PCR-amplified 5'-^{32}P-labeled fragment of HTLV-1. (C) Polyamide U2 was titrated from 0pM, 30pM, 100pM, 300pM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, to 1uM. (D) Polyamide D2 was titrated from 0pM, 30pM, 100pM, 300pM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, to 1uM. Experiments by Eric Fechter.

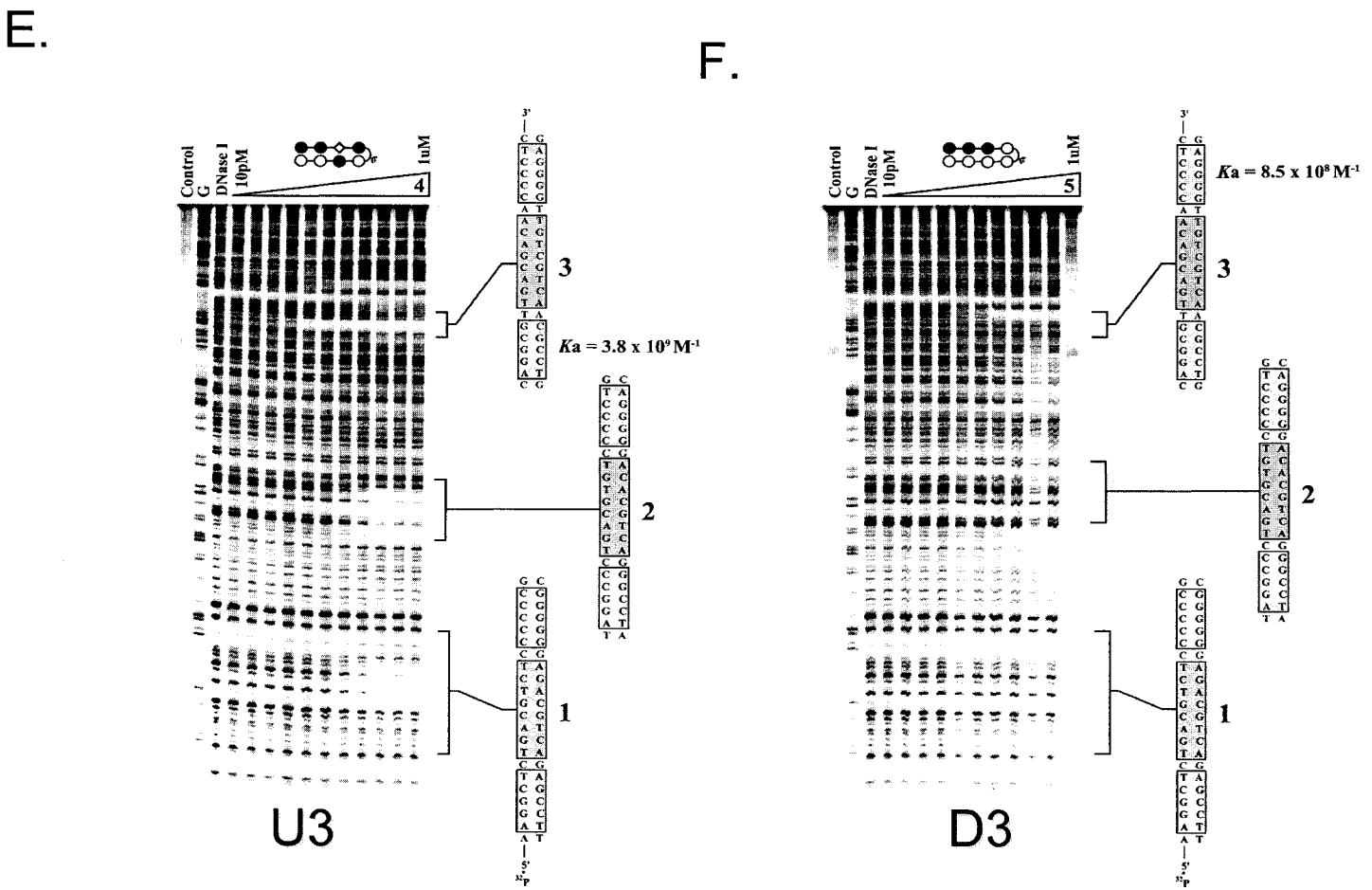

Figure 4.7 **Binding reactions of Polyamides to HTLV-1 promoter.** Quantitative DNase I footprint titration experiments on the PCR-amplified 5'-^{32}P-labeled fragment of HTLV-1. (E) Polyamide U3 was titrated from 0pM, 30pM, 100pM, 300pM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, to 1uM. (F) Polyamide D3 was titrated from 0pM, 30pM, 100pM, 300pM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, to 1uM. Experiments by Eric Fechter.

G.

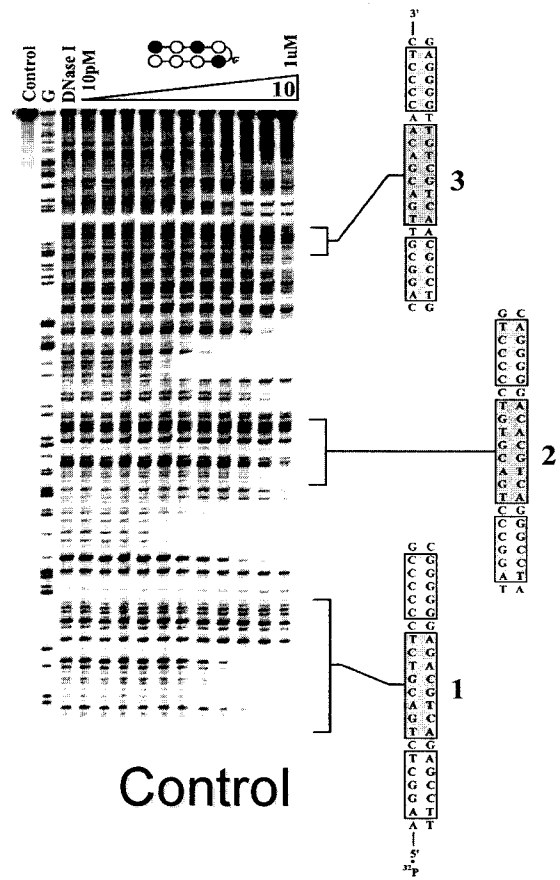

Figure 4.7 **Binding reactions of Polyamides to HTLV-1 promoter.** Quantitative DNase I footprint titration experiments on the PCR-amplified 5'-^{32}P-labeled fragment of HTLV-1. (G) Polyamide Control was titrated from 0pM, 30pM, 100pM, 300pM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, to 1uM. Experiments by Eric Fechter.

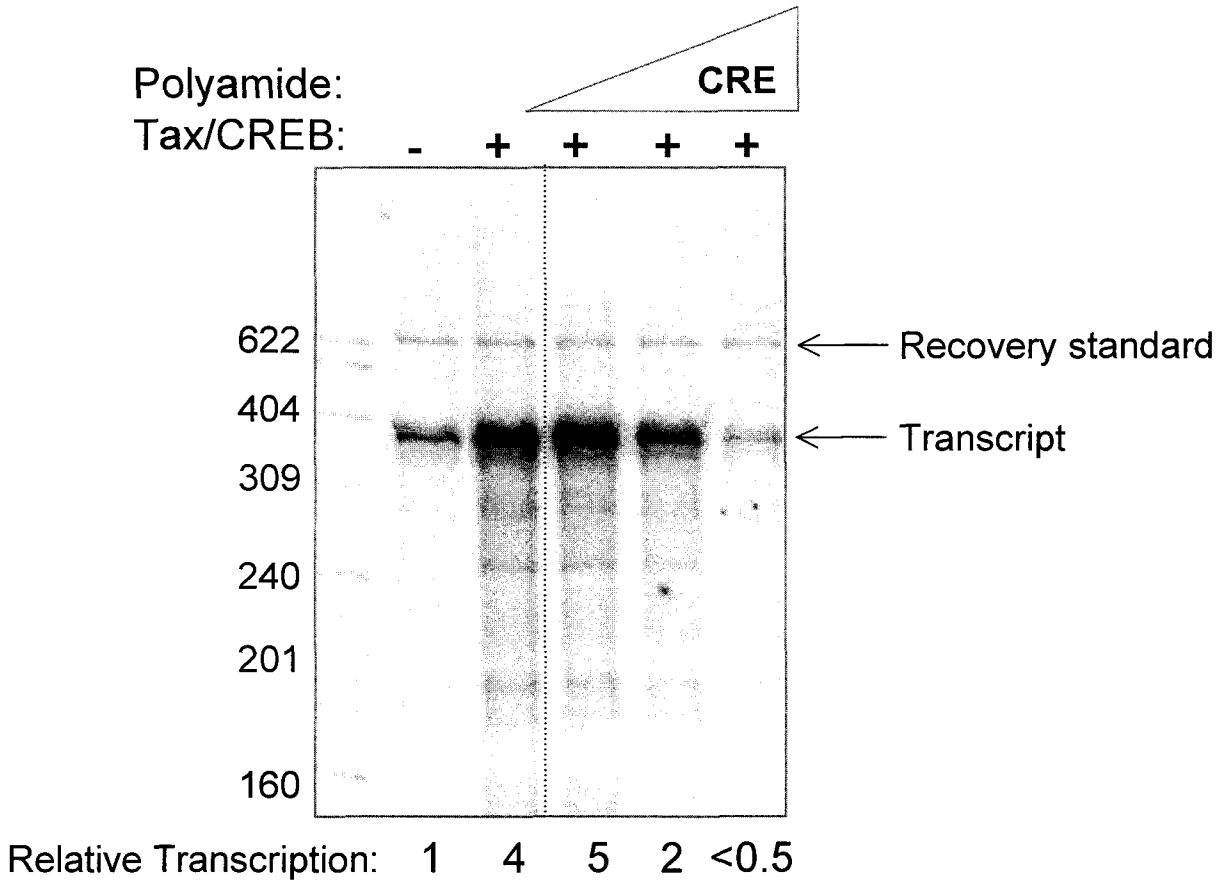

Figure 4.8 **Polyamides inhibit transcription on naked DNA.** Transcriptional activation on HTLV-1 G-less chromatin templates was analyzed in the presence of Tax (100ng) and CREB (100 ng). Molecular weight size markers, recovery standard, and full length G-less transcripts are indicated.

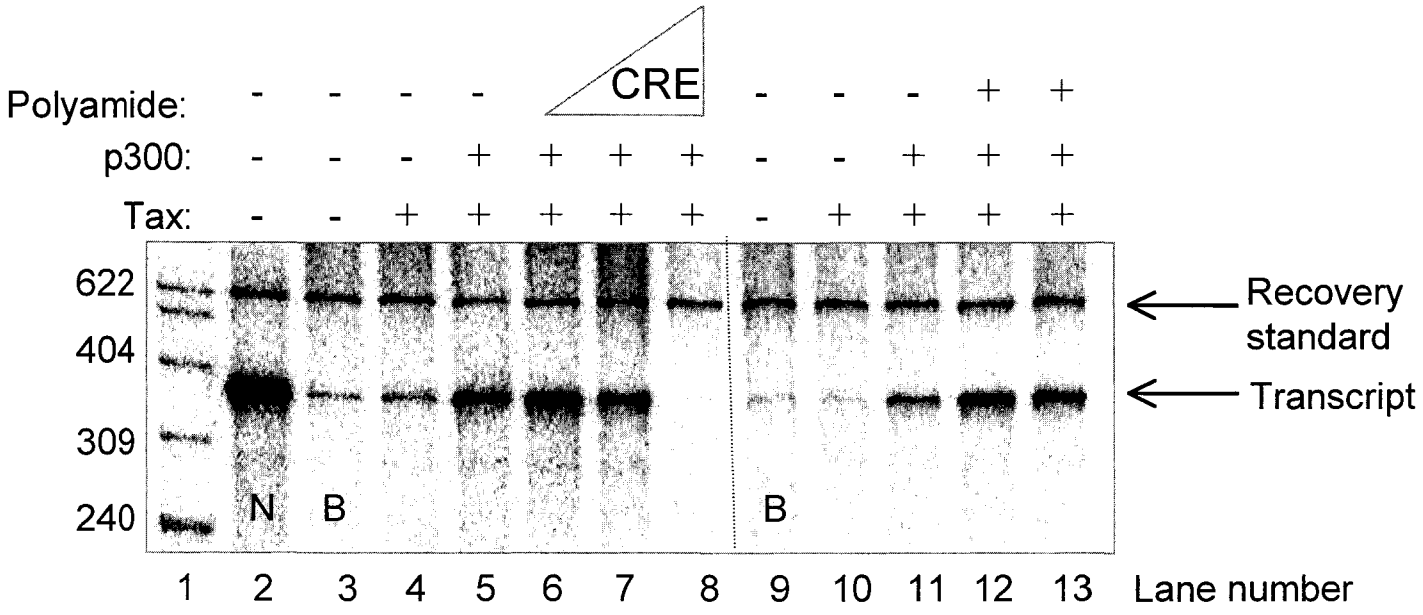

Figure 4.9 **Polyamides do not activate transcription on naked DNA.** Transcriptional activation on HTLV-1 G-less chromatin templates was analyzed in the presence of Tax (100ng) and p300 (150 ng). Molecular weight size markers, recovery standard, and full length G-less transcripts are indicated. Polyamide was added to a concentration of 10 nM, 100 nM, and 1000 nM in lanes 6-8. Polyamide was incubated with DNA before addition of activators to a concentration of 100μM in lanes 12 and 13.

Chapter 5

Tax does not affect the transcriptional activity of NF-κB in vitro

5.1 Abstract

Human T-cell leukemia virus (HTLV-1) is the causative agent of an aggressive malignancy called adult T-cell leukemia/lymphoma. HTLV-1 encodes a transcriptional activator called Tax, which is responsible for high levels of transcription of the HTLV-1 genome. Malignant transformation is linked to the pleiotropic functions of Tax due to its ability to interact with a variety of cellular transcription factors. This results in deregulated expression of cellular genes involved in programmed cell death and cell cycle progression. Of the cellular transcription factors targeted by Tax, the NF-κB proteins are widely studied and are believed to play a prominent role in mediating Tax-deregulation of cellular gene expression. The majority of the studies in the past have focused on the ability of Tax to target NF-κB regulatory proteins in the cytoplasm, which leads to an increase in the levels of p50/p65 in the nucleus. However, other studies have suggested that Tax works directly at the promoter of NF-κB responsive genes in concert with these proteins. We investigated this second mechanism by focusing on the events that occur directly at the promoters of NF-κB-responsive genes in an in vitro transcription system. We found Tax had no effect on NF-κB-mediated transcription in vitro, suggesting that the primary mechanism of Tax deregulation of NF-κB occurs by targeting NF-κB regulatory proteins in the cytoplasm.

5.2 Introduction

HTLV-1 Tax is responsible for high levels of expression of HTLV-1. It also plays a role in deregulating the expression of many cellular genes. Of the cellular transcription factors targeted by Tax, the family of NF-κB proteins is one of the major pathways by which Tax deregulates cellular gene expression. This deregulation may ultimately contribute to malignant transformation. Studies of mice transgenic for expression of Tax suggest that the transforming properties of Tax are due to its ability to deregulate the NF-κB pathway (114).

NF-κB belongs to a family of cellular proteins involved in the activation of genes regulating cell growth and proliferation, inflammation, and apoptosis (reviewed in 12). Regulation of the transcriptional activity of NF-κB occurs through the complex interplay between signal transduction pathways involving many cellular proteins. The NF-κB family is composed of a variety of homo- and hetero-dimeric transcription factors including NF-κB1 (p50 and p105), NF-κB2 (p52 and p100), RelA (p65), RelB, and c-Rel (reviewed in 283). These proteins share a conserved Rel homology domain that confers DNA binding ability, dimerization, and nuclear transport capabilities. The proteins were first discovered based on their ability to bind the enhancer elements in the kappa light-chain genes in murine B-lymphocytes (247). In its inactive form, NF-κB is sequestered in the cytoplasm by its interaction with an inhibitor complex called IκB. A large kinase complex (IKK) is responsible for the

phosphorylation of members of the inhibitor IκB complex. The IKK protein complex is composed of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ). Upon stimulation of cells by many signals including TNF-α, IKK phosphorylates IκBα at serine residues 32 and 36. IκBα is subsequently polyubiquitinated and targeted to the 26S proteasome for degradation (40, 54). This releases IκB from NF-κB complex, allowing NF-κB proteins to enter the nucleus where they activate cellular gene expression. The most studied dimer of the NF-κB protein family is the p50/p65 heterodimer. Once it is transported into the nucleus, it has been shown that the p50/p65 heterodimer can recruit the cellular coactivator CBP/p300 through its KIX domain (78, 219, 312). Phosphorylation of p65 by protein kinase A may play a role in the recruitment of CBP/p300, and this has been shown to increase transcriptional activation of the NF-κB complex (312).

The study of Tax regulation of NF-κB has primarily been focused on the ability of Tax to affect the nuclear localization of NF-κB. Tax has been shown to bind to many components of the NF-κB pathway including the following members of the IKK complex; IκB, MEKK1, and NIK, (for reviews see 106, 262). These Tax targets primarly function through maintaining the phosphorylation of the inhibitor IκB, which leads to its degradation (42, 75, 277). Tax has also been shown to interact with members of the NF-κB proteins through their Rel homology domain (152, 266). However, studies in vivo have suggested a role of Tax in the regulation of NF-κB in the nucleus.

Both stably infected HTLV-1 T-cells, and cells transiently expressing Tax have increased levels of NF-κB in the nucleus (151). Immunocytochemistry and confocal microscopy revealed that Tax and NF-κB proteins (p50 and p65) colocalize in the nucleus (23), and Tax has also been shown to increase the DNA binding activity of NF-κB (10, 222).

Thus far, the majority of the studies looking at the effects of Tax on NF-κB have focused on the ability of Tax to interact with members of the NF-κB family in the cytoplasm, and have failed to fully elucidate a direct role, if any, for Tax in the transcriptional regulatory function of NF-κB at responsive promoters. In this study, we have studied the effect of Tax on the ability of NF-κB to bind to DNA and activate transcription in vitro. Using DNA binding assays and in vitro transcriptions in the context of chromatin we show that Tax does not affect the ability of NF-κB to activate transcription.

5.3 Materials and Methods

5.3a Expression and purification of recombinant proteins. CREB, pTax-His_6, GST-CR2, and GST-CH1-KIX were expressed and purified as previously described (66, 309, 80, and 239, respectively). NF-κB constructs were provided by Dimitris Thanos at Columbia University, and were expressed and the proteins were purified as previously described (270). The GST-p65 construct was a provided by Warner Greene at the Gladstone Institute,and was purified using glutathione-agarose chromatography (239).

Purified proteins were stored at -70°C in TM buffer (50 mM Tris-HC[pH 7.0], 100 mM KCl, 12.5 mM MgCl2, 1 mM EDTA[pH 8.0], 1 mM dithiotrhreiotol, 0.1% [vol/vol] Tween-20, 20% [vol/vol] glycerol).

Drosophila core histones were purified as previously described (32). Drosophila NAP-1 (dNAP-1-His6) was expressed from recombinant baculovirus (110) and purified first by nickel chelate chromatography followed by +Source 15Q-column chromatography as previously described (77, 112). We co-expressed FLAG-tagged ISWI and Acf1 from baculoviruses, and purified the complex by anti-FLAG affinity batch binding and elution as previously described (113). FLAG-tagged CBP and PCAF were expressed from recombinant baculoviruses and purified using anti-FLAG resin as previously described (297). His_6-tagged p300 protein was expressed from recombinant baculovirus and purified using nickel-chelate chromatography as previously described (146).

5.3b Electrophoretic mobility shift assay. The ^{32}P-γ-ATP labeled double stranded oligonucleotide probe (0.4 nM) was incubated with increasing amounts of purified proteins for 30 minutes on ice as previously described (80). The oligonucleotide sequence used is as follows: (5'-GAT CGC TGG GAC TTT CCA GGA-3') Protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel.

5.3c Transcription templates. The HTLV-1 promoter G-less cassette template, 4TxRE G-less template and –52 G-less cassette templates have been previously described (8). The 4TxRE plasmid contained four copies of the third 21 base pair repeat cloned upstream of the first 52 bases of the HTLV-1 promoter. The NF-κB transcription template was made by ligating the NF-κB oligo (5'-GAT CGC TGG GAC TTT CCA GGA-3') into a plasmid containing the first 52 bases of the HTLV-1 core promoter driving the expression of a 190 nucleotide G-less transcript. After ligation, the promoter was sequenced to confirm the insertion of three NF-κB oligonucleotides cloned in tandem upstream of the core HTLV-1 promoter.

5.3d Chromatin assembly and topological assay. Nucleosomes were assembled on DNA templates as previously described (112). The supercoiled plasmids were assembled into chromatin using dNAP-1, dAcf1 and *Xenopus* histones, at a 0.57:1.0 histone:DNA ratio. Briefly, histone octamers were preassembled with drosophila-NAP-1 (8:1 dNAP-1/core histones) on ice for 30 minutes. 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. Assembly DNA reactions were incubated overnight at 27°C. Samples were analyzed on a 1% agarose gel, and the degree of supercoiling was visualized by Sybr Gold (Molecular Probes) staining.

5.3e In vitro transcription assay. Following chromatin assembly, preinitiation complexes were formed on 150 ng of the plasmid DNA. All reactions contained 100 μM acetyl CoA (USB). Nuclear extract (70 μg), prepared from CEM cells (a HTLV-1 negative human T-lymphocyte cell line) was added immediately following the addition of the activator and/or coactivator. Following a 60 minute 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-α-UTP (3000 Ci/mmol, New England Nuclear). Transcription reactions were processed and analyzed as previously described (163). Molecular weight markers (radiolabeled *Hpa* II digested pBR322) were used to estimate the size of the RNA products.

5.3f Biotin-streptavidin DNA pull-down assays. Double-stranded DNA fragments containing a single NF-κB site (and biotinylated at the 5'-end of one strand, [Integrated DNA Technologies]) were annealed and bound to streptavidin-agarose (Novagen) in TM buffer (50 mM Tris, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 0.1% Tween 20, 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]). The immobilized DNA (2 pmol) was incubated with p50 (10 pmol), p65 (10 pmol), Tax (10 pmol), or p300 as indicated in the relevant figures. The reaction mixtures were incubated in binding buffer (25 mM Tris, 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 0.05% Tween 20, 50 mM NaCl, 2 mM DTT, 1 mM

PMSF) for 1 h at 4°C, washed three times in binding buffer, resuspended in SDS sample dyes, boiled, and analyzed by SDS-PAGE (10% polyacrylamide). Bound proteins were detected by Western blot analysis.

5.3g In vitro acetylation assay. Acetylation of recombinant proteins p53 (200 ng), p50 (200 ng), and p65 (200 ng) was assayed by incubation with purified, recombinant p300 (150 ng). The proteins were incubated together in a solution containing ^{14}C-acetyl CoA (100 pmol; 57mCi/mmol, Amersham) in a solution containing 50 mM Tris, (pH8), 10% glycerol, 10 mM sodium butyrate, 1 mM DTT, and 1 mM PMSF. Samples were incubated at 30ºC for 60 minutes. Proteins were separated by 10% SDS-PAGE, fixed, and acetylation was visualized by PhosphorImager analysis.

5.3h In vitro phosphorylation assay. Phosphorylation reactions were performed as previously described (80).

5.3i GST pull-down assay. All GST pull-down experiments were performed as previously described (280). Briefly, 12.5 µl of glutathione-agarose beads were equilibrated in 0.5x Superdex buffer (Superdex buffer is 25 mM HEPES [pH 7.9], 12.5 mM $MgCl_2$, 10 µM $ZnSO_4$, 150 mM KCl, 20% [vol/vol] glycerol, 0.1% Nonidet P-40, and 1 mM EDTA). The purified GST fusion protein was incubated with the beads for 1 to 2 h at 4°C and then washed with Superdex buffer. The second protein(s) was then added to the washed beads and

incubated for 1 to 2 h (or overnight) at 4°C. The beads were washed as before, and bound proteins were eluted with SDS sample dyes. Bound proteins were separated by electrophoresis on a 10% or 12% SDS gel, transferred to nitrocellulose, and probed with the appropriate antibody.

5.4 Results

5.4a NF-κB and Tax do not co-occupy DNA

The aim of this project was to investigate whether Tax directly affects the ability of NF-κB to activate transcription in vitro. We chose to begin by studying the binding of NF-κB to its consensus binding site, and to assay the effects of purified Tax on NF-κB binding to DNA. We expressed and purified full-length p50 and p65 as previously described (270). The ability=ies of the p50 homodimer, p65 homodimer, and p50/p65 heterodimer to bind to a ^{32}P labeled NF-κB consensus oligonucleotide were then tested. Figure 5.1 A shows the results of an electrophoretic mobility shift assay (EMSA) with two amounts of each protein on the NF-κB site. All protein complexes tested were shown to bind to DNA (Fig. 5.1 A). We then tested the effects of purified recombinant Tax on the binding activity of each of the NF-κB proteins after they have bound to their consensus NF-κB oligonucleotide. Titration of Tax into each of the binding reactions (p50, p65, p50/p65) had little to no effect on their binding to DNA (Fig. 5.1 B). Addition of Tax to the p50 homodimer

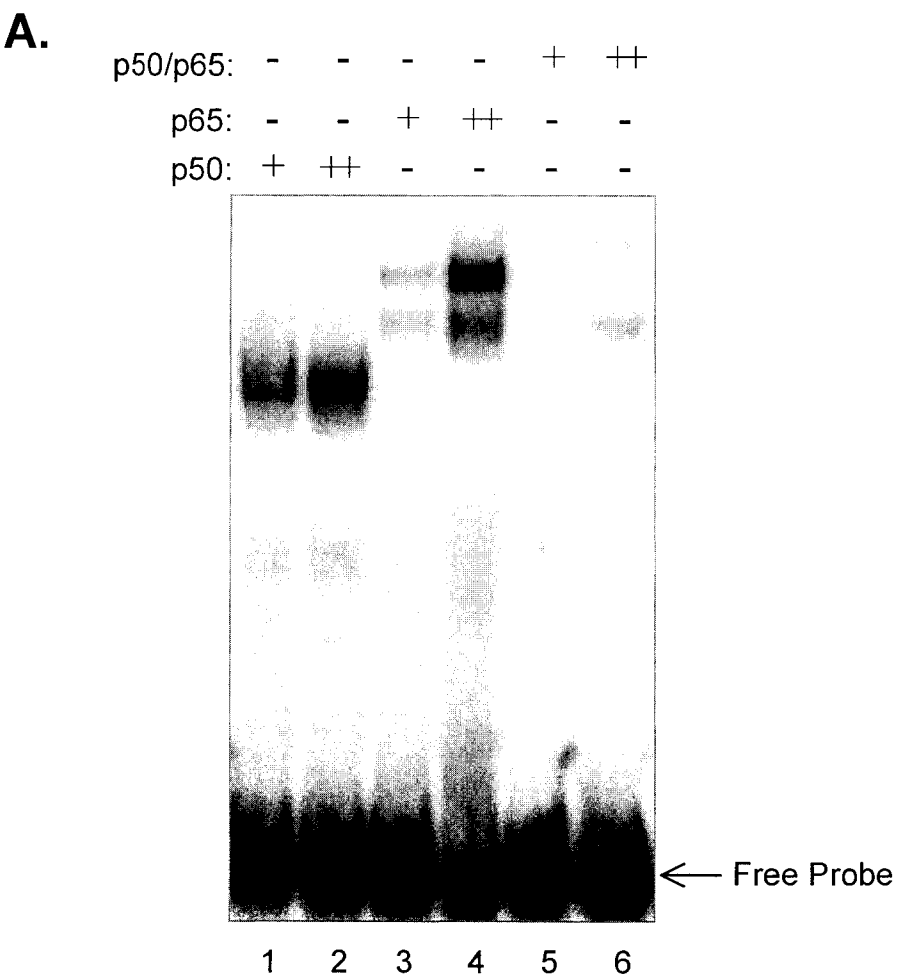

Figure 5.1 (A) **Confirmation of p50 and p65 protein DNA binding activity.** Purified, recombinant p50 (50, 100 ng), p65 (60, 120 ng), and p50/p65 (75, 150 ng) was incubated with a NF-κB consensus site probe. Protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The position of the protein-DNA complex with the NF-κB consensus site probe is indicated.

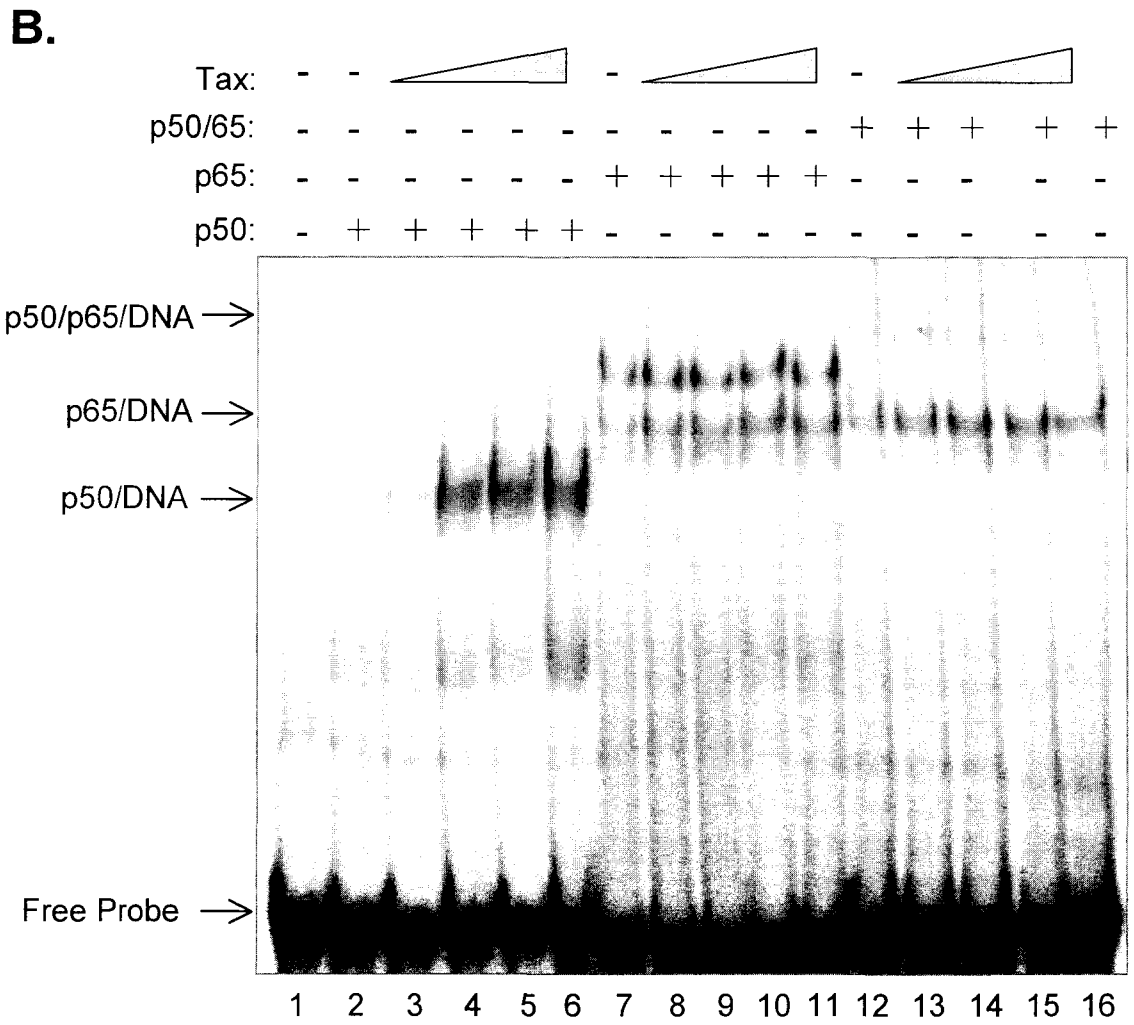

Figure 5.1 (B) **Tax does not interact with NF-κB /DNA complexes.** Purified, recombinant p50 (100 ng), p65 (60 ng), and p50/p65 (150 ng) was incubated in the presence of increasing amounts of Tax (50 to 400 ng) on a NF-κB consensus site probe. Protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The position of the protein-DNA complex with the NF-κB consensus site probe is indicated.

suggested that Tax increased the binding of p50, however these results were not reproducible (lanes 3-6).

5.5b Tax interacts with p65 in solution

It has been previously published that Tax binds to the Rel homology domain of NF-κB proteins (266). We were interested in confirming these results with our purified proteins, and we were most interested in the interaction of Tax with the p65 subunit of NF-κB, since it has been shown to be most important in transcriptional activation (310). The ability of Tax and p65 to bind in solution was tested in a GST pull-down assay (Fig. 5.2). Purified, recombinant Tax protein was titrated with constant amounts of GST-p65, and the results were visualized by western blot. As a positive control, Tax was bound to a region of CBP called CR2 (amino acids 2003-2212) (lane 7) (239). These experiments indicate that Tax directly binds GST-p65, confirming the published results (lane 4-6) (266).

5.5c Tax is not recruited to NF-kB/DNA complex

The hypothesis that Tax interacts with the NF-κB proteins directly was further tested in biotinylated DNA pull-down assays. In these experiments, an NF-κB oligonucleotide was biotinylated at one end, and the molecule was bound to streptavidin-agarose beads. This enabled us to bind proteins to an immobilized bead and wash off unbound proteins. The bound proteins were analyzed by Western blot. Figure 5.3 A shows that addition of purified Tax

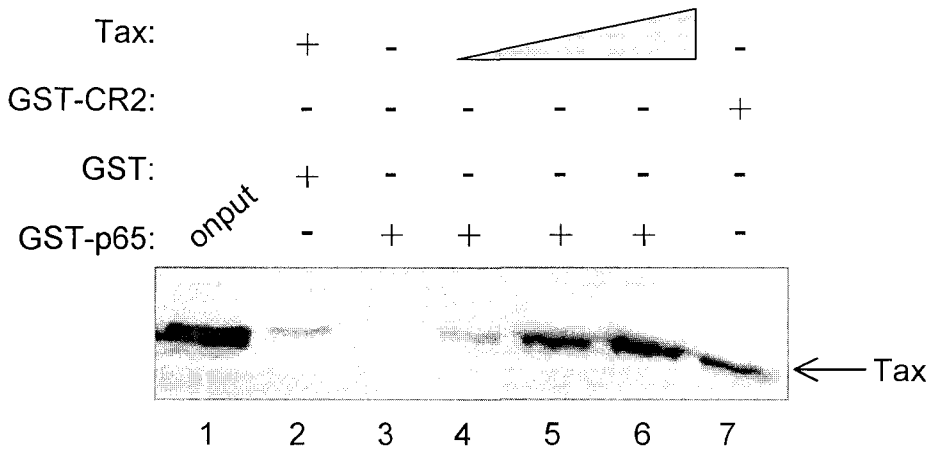

Figure 5.2 **Tax interacts with p65 in solution.** Purified recombinant Tax (12-48 pmol) was incubated with GST alone or GST-p65 (24 pmol each). As a control, Tax binding to GST-CR2 and GST alone was also assayed (24 pmol each). The bound proteins were separated by SDS-10% PAGE, transferred to nitrocellulose, and detected using anti-His_6 antibody.

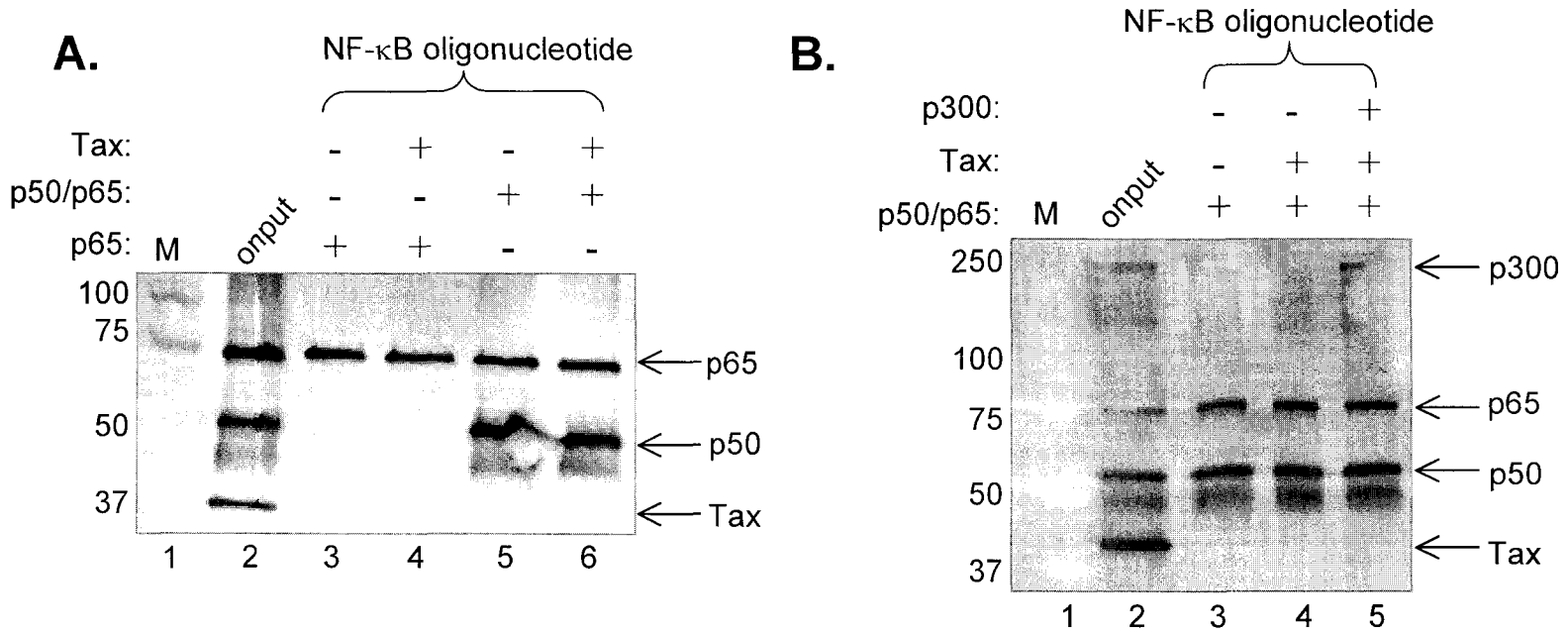

Figure 5.3 **Tax is not recruited to NF-κB/DNA complex**. (A) Binding reaction mixtures contained a DNA fragment carrying a single NF-κB site linked to a streptavidin-agarose bead. p65 (450 ng), p50/65 (400 ng), and Tax (600 ng) were added to the immobilized DNA. Binding reaction mixtures contained a biotinylated DNA fragment carrying a single NF-κB site bound to a streptavidin-agarose bead. (B) p65 (450 ng), p50/65 (400 ng), Tax (600 ng), and p300 (1.5 μg) were added to the immobilized DNA as indicated. Reactions were analyzed by western blotting.

into reaction mixtures containing the p65 homodimer, or the p50/p65 heterodimer, produced no change in NF-κB binding, nor did Tax enter into the complex (compare lanes 3 to 4, 5 to 6). Since Tax and NF-κB both bind to CBP/p300 (80, 149, 239, 310), we next tested whether p300 would serve as a "bridging molecule" to recruit Tax to the NF-κB site. We added purified recombinant p300 to biotinylated DNA pull-down reactions containing the NF-κB heterodimer p50/p65 and Tax. Figure 5.3 B shows that Tax is not recruited to the NF-κB/DNA complexes in the presence of p300 (compare lanes 3 to 4 and 5).

5.4d The role of post-translational modifications of NF-κB

Previous studies have shown that p50/p65 can be post-translationally modified by acetylation (39), and phosphorylation (311, 312). These modifications have been shown to be important in altering the DNA binding activity of many transcription factors like p53 (96). Phosphorylation of p65 at serine residue 276 has been previously linked to increased affinity for the KIX domain of CBP, and thus enhances transcriptional activation of NF-κB (78, 219, 312). Since Tax also binds the KIX domain of CBP/p300 (80, 149, 158, 239, 295), we wanted to test whether the p65 modification influences complex formation with Tax. We hypothesized that Tax interaction with NF-κB may require post-translational modifications like phosphorylation or acetylation.

In vitro phosphorylation by PKA revealed that p50 (Fig. 5.4 lane 1 and 3) and p65 (lane 2 and 4) are both weakly phosphorylated by the catalytic

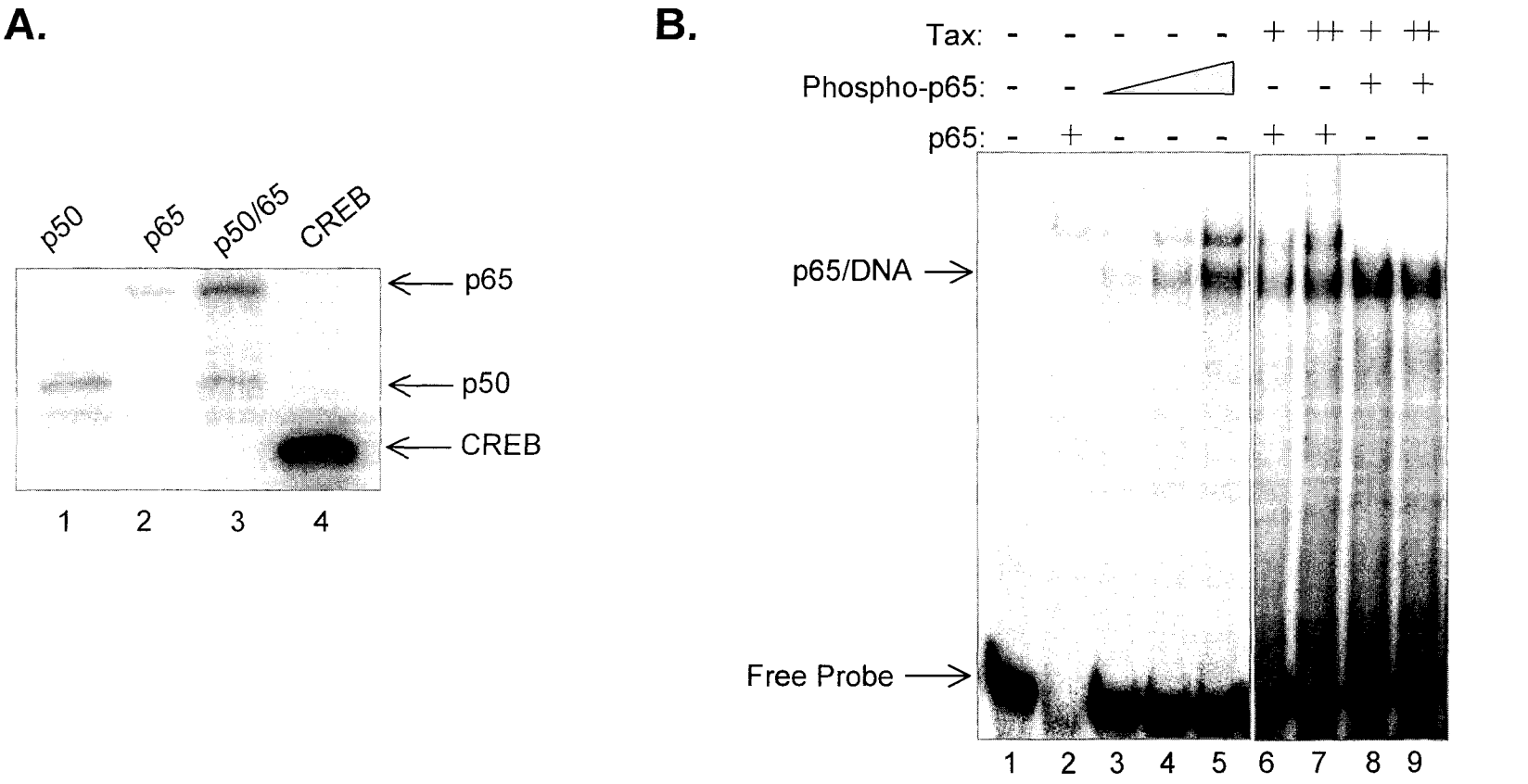

Figure 5.4 **Phosphorylation of NF-κB proteins does not affect Tax recruitment.** (A) Phosphorylation of p50 (10 pmol), p65 (10 pmol), and the p50/p65 heterodimer (10 pmol) with PKA and γ-^{32}P-ATP. Phosphorylation of CREB (10 pmol) was used as a control. Samples were resolved by 10% SDS-PAGE. (B) p65 (60 ng), and phospho-p65 (30, 60, 120 ng), were incubated with two amounts of Tax (50, 100 ng) and NF-κB consensus site probe. Protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The position of the protein-DNA complex with the NF-κB consensus site probe is indicated.

subunit of PKA, relative to phosphorylation of purified recombinant CREB. To test the function of phospho-NF-κB, we bulk phosphorylated both p50 and p65 separately or as a heterodimer with PKA. We then compared the ability of Tax to interact with the phosphorylated forms of p50 or p65 as compared with unmodified p50 and p65 in an EMSA as shown in figure 5.4 B. Phospho-p65 (lanes 3-5) was titrated onto an NF-κB oligonucleotide, and its binding was compared to wild-type p65 (lane 2). The ability of Tax to affect the DNA binding abilities of p65 was assayed in lanes 6-9. Purified recombinant Tax protein was titrated with a constant amount of p65 (lanes 6 and 7), and phospho-p65 (lanes 8 and 9). No detectable change in binding affinity was observed upon addition of Tax. It must be noted, however, that the p65 was not efficiently phosphorylated, possibly affecting the outcome of the experiment.

We were also interested in testing the effect of NF-κB acetylation on complex formation with Tax. Recent studies have shown that the transcriptional coactivator CBP/p300 acetylates p65 (39). These investigators suggested that acetylation aids in release of NF-κB from the cytoplasmic inhibitor and increases its transport into the nucleus (39). However, potential effects of acetylation on p65 transcription function remain to be elucidated. In vitro acetylation of NF-κB revealed that purified p300 weakly acetylated p50 (Fig. 5.5, lane 1), whereas p65 was acetylated (lane 2) comparably to p53 (a well established p300 substrate, lane 4). Similar to the studies described with phosphorylation, we bulk acetylated p65 with unlabeled acetyl CoA, and

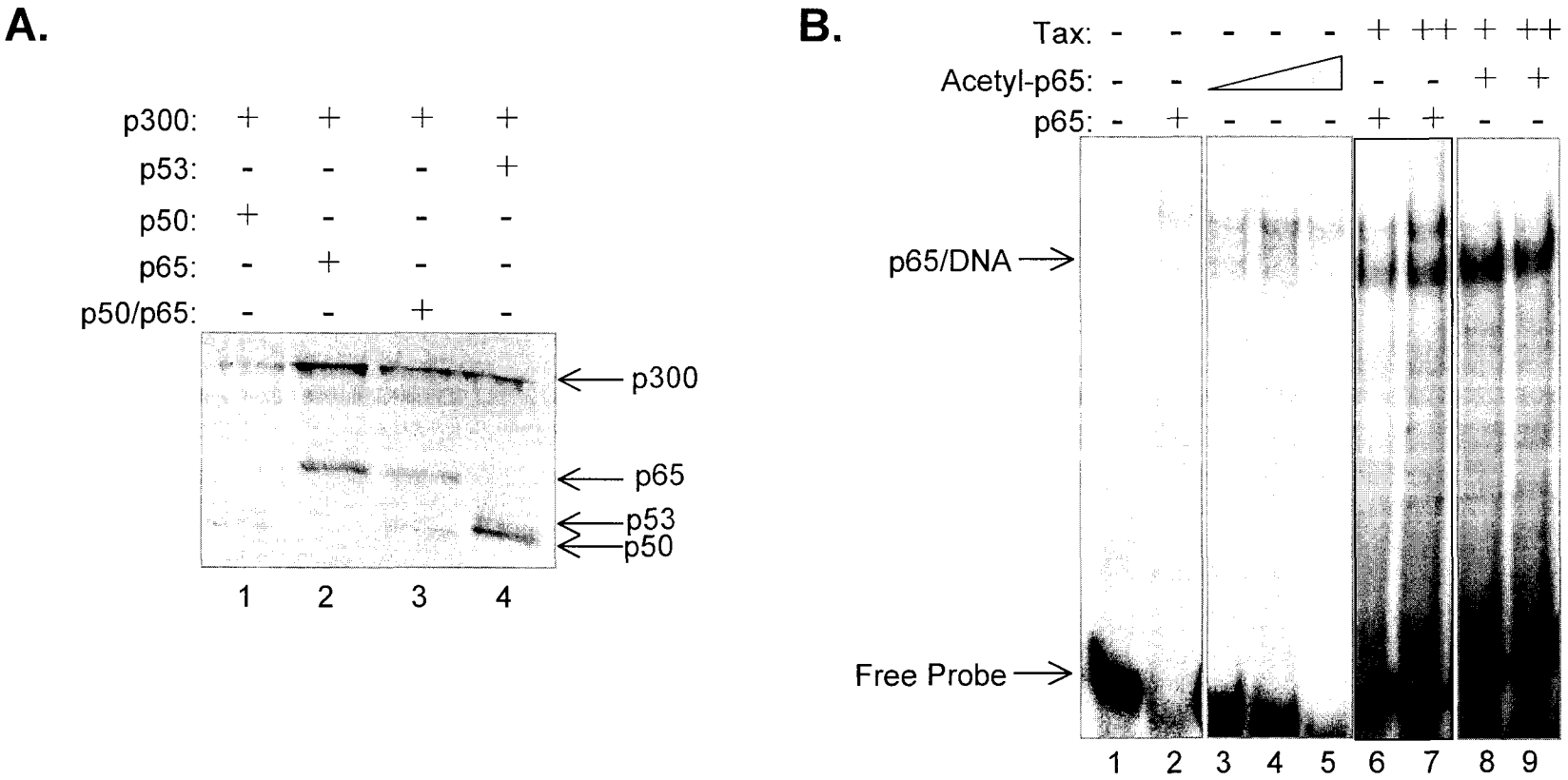

Figure 5.5 **Acetylation of NF-κB proteins does not affect Tax recruitment .** (A) Acetylation of p50 (200 ng), p65 (200 ng) and the p50/p65 heterodimer (200 ng) was done in the presence of p300 (150 ng) and ^{14}C-labeled acetyl CoA. p53 (200 ng) was acetylated as a positive control. Samples were resolved by 10% SDS-PAGE. (B) p65 (60 ng),and acetyl-p65 (30, 60, 120 ng), was incubated with increasing amounts of Tax (50, 100 ng) and NF-κB consensus site probe. Protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The position of the protein-DNA complex with the NF-κB consensus site probe is indicated.

tested the effect of the modification on interaction with Tax. Testing the DNA binding properties of the acetylated proteins in an EMSA revealed no difference in binding as compared to unmodified p65. Acetyl-p65 (Fig. 5.5, lanes 3-5) was titrated onto an NF-κB oligonucleotide, and its binding was compared to unmodified p65 (lane 2). The ability of Tax to affect the DNA binding abilities of p65 was also assayed (Fig. 5.5). Purified recombinant Tax protein was titrated with a constant amount of p65, and acetyl-p65 (lanes 6-9). No detectable change in binding was observed upon addition of Tax.

5.4e Transcriptional activation of NF-κB

We next were interested in testing the function of NF-κB as a transcriptional activator in vitro. First, we engineered a NF-κB responsive G-less transcription template. Three tandem copies of a NF-κB oligonucleotide was cloned upstream of the first 52 bases of the transcription start site of the HTLV-1 promoter, driving the expression of a 190 nucleotide guanine-less transcript. A schematic of the template is shown in figure 5.6 A. We tested recombinant purified p50, p60, and the p50/p65 heterodimers for their transcriptional activity in vitro on this transcription template. An in vitro transcription assay on nucleosome-free DNA is shown in Figure 5.6 B. Titration of p65 produced a 4 and 6 fold activation of transcription, respectively (lanes 2,3). Addition of purified recombinant Tax had no effect on this promoter, as expected (lanes 4, 5). Furthermore, addition of Tax and p65 together produced no further activation over p65 transcription levels

A.

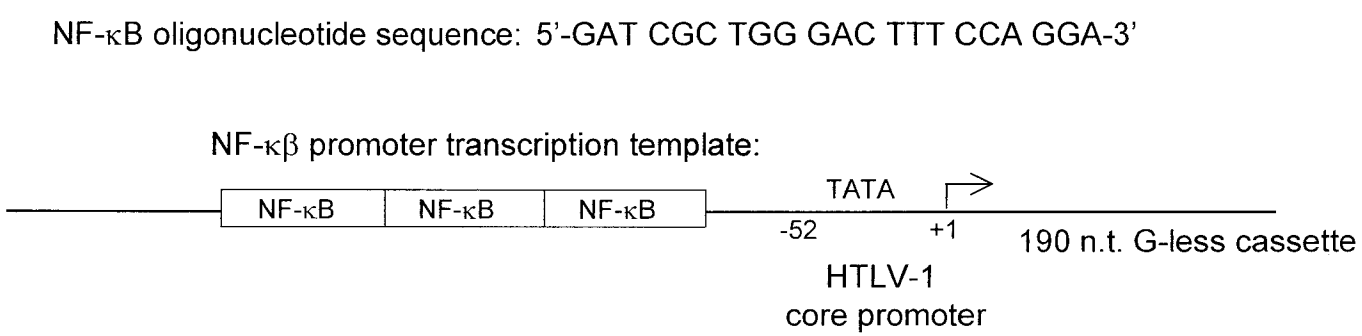

Figure 5.6 **Transcriptional activation in vitro by NF-κB and Tax.** (A) Schematic representation of NF-κB G-less transcription template. Core HTLV-1 promoter drives the expression of 190 nucleotide transcript.

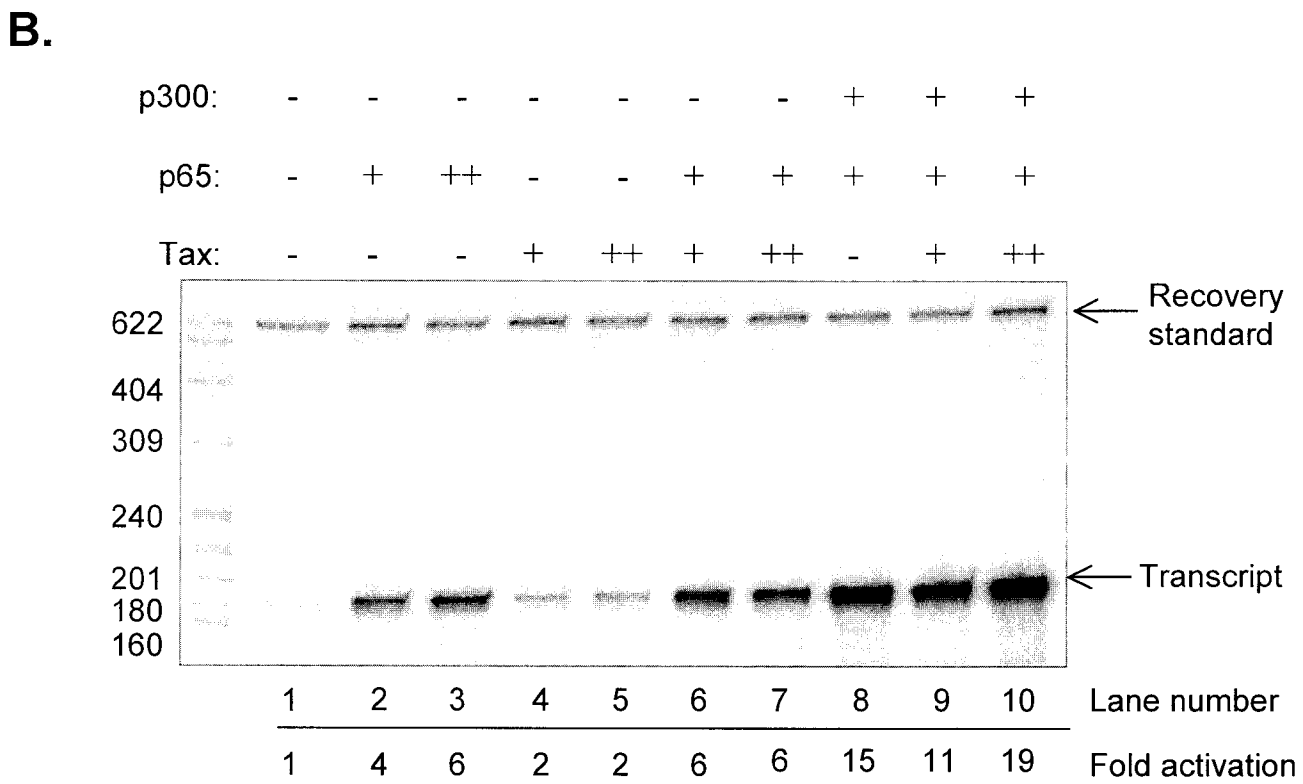

Figure 5.6 **NF-κB activation on nucleosomoe-free templates.** (B) The in vitro transcription assay was performed on NF-κB G-less template and CEM nuclear extracts in the absence (-) or presence (+) of purified, recombinant p65 (60 and 120 ng), Tax (50 and 100 ng) and p300 (150 ng), as indicated. All reactions were performed in the presence of acetyl-CoA. The positions of full-length 190-nt RNA transcript, labeled DNA recovery standard, and labeled DNA molecular size markers (in nucleotides) are indicated.

C.

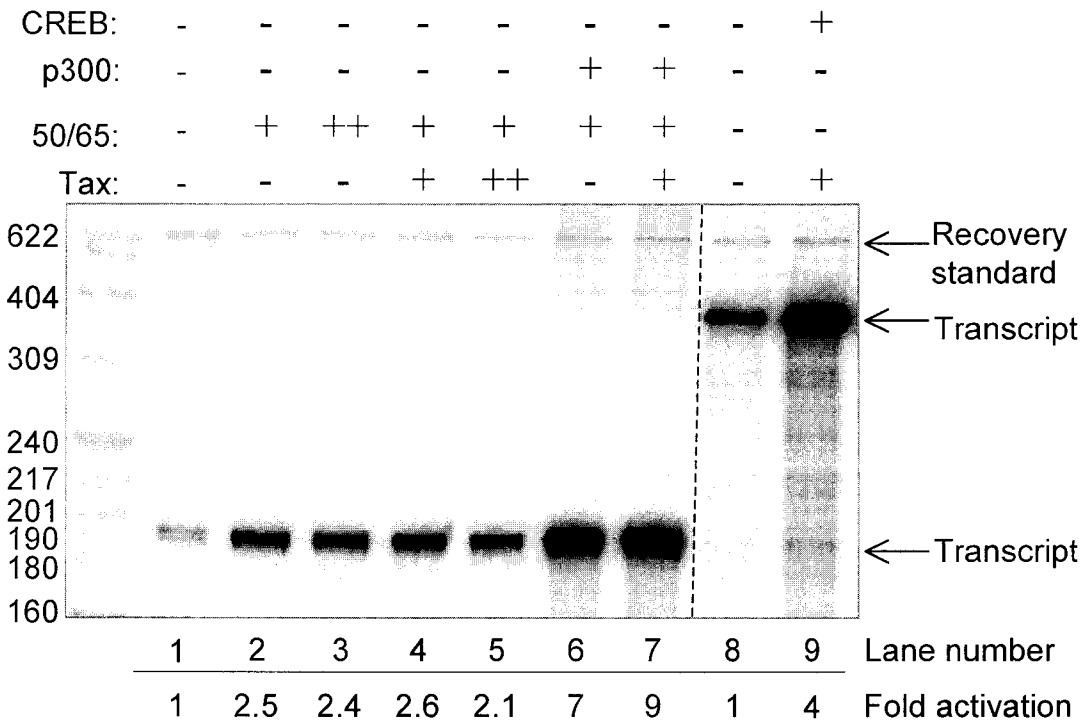

Figure 5.6 **NF-κB activation on unassembled DNA templates.** (C) The in vitro transcription assay was performed on NF-κB G-less, or 4TxRE G-less, and CEM nuclear extracts in the absence (-) or presence (+) of purified, recombinant p50/p65 (60 and 120 ng), Tax (35, and 75 ng), CREB (50 ng) and p300 (150 ng), as indicated. All reactions were performed in the presence of acetyl-CoA. The positions of full-length 190-nt and 380-nt RNA transcripts, labeled DNA recovery standard, and labeled DNA molecular size markers (in nucleotides) are indicated.

alone (compare lanes 3 and 7). Addition of p300 stimulated p65 transcription (lanes 3, 8). However, addition of Tax to p65 and p300 failed to show a further increase in transcription levels (compare lanes 8, 9, and 10) further suggesting that Tax and p65 do not cooperate in transcriptional regulation of this promoter template in vitro.

The p50/p65 heterodimer was also tested for its ability to stimulate transcription in the presence of Tax using the in vitro transcription assay. Addition of the heterodimer produced a 2.5-fold activation over basal transcription (lanes 2, 3). However, addition of two different amounts of Tax failed to produce an affect on NF-κB-mediated transcriptional activation (lanes 4, 5). Again, p300 increased the transcription levels of p50/p65 7-fold, but addition of Tax produced no effect on trancsription of the heterodimer or p300 alone. To control for the transcriptional activity of the Tax protein, the purified protein was tested on a Tax-responsive promoter (lanes 8-10). This 4TxRE transcription template contains the third viral CRE reiterated four times in tandem upstream of the first 52 base pairs of the HTLV-1 promoter. Together, these experiments suggest that Tax and NF-κB, at least in this system, do not promote transcriptional activation in a synergistic manner.

5.4f NF-kB-mediated transcriptional activation in a chromatin context is not responsive to Tax.

A recent study has shown that NF-κB activates transcription in vitro from chromatin-assembled templates (218). We hypothesized that studying

the transcriptional properties of NF-κB in a chromatin context may more accurately mimic the in vivo environment of the proteins. Chromatin assembly of the NF-κB G-less template was performed using the recombinant *Drosophila* assembly proteins Acf1/ISWI, GST-yNAP-1, and purified *Drosophila* core histones, as previously described (76, 77, 113). These assembly proteins are sufficient for the ATP-dependent formation of evenly spaced nucleosomal arrays. The empirical ratio of core histones to DNA was optimized for complete chromatin assembly (Figure 5.7 A). We performed in vitro transcription assays on the chromatin templates using a nuclear extract from CEM cells (a human T-lymphocyte cell line) as a source of RNA polymerase and general transcription factors. All experiments were performed in the presence of acetyl CoA. An in vitro transcription assay on chromatin assembled NF-κB G-less transcription templates is shown in Figure 5.7 B. The Tax-responsive 4TxRE transcription template was also included as a control (lanes 1 to 4). Reactions using non-nucleosomal DNA are shown (lanes 1, 2, and 5). The activity of purified recombinant Tax and purified recombinant CREB was assayed as a control (lanes 2 and 4). Basal transcription in the absence of activators is also shown (lanes 3 and 6). This experiment shows the results of adding the NF-κB activator before or after chromatin assembly, and preincubation of the p50/p65 heterodimer with DNA before chromatin assembly greatly increased the amount of activation. The addition of Tax did not enhance the transcriptional activation of NF-κB in all conditions tested (lanes 8, 9, 11, and 12).

A.

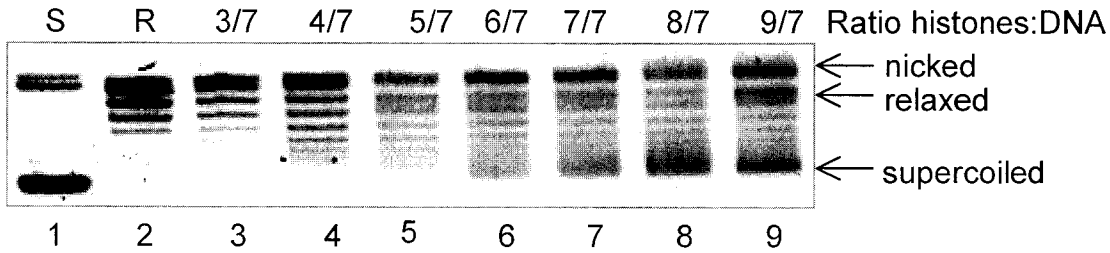

Figure 5.7 **NF-κB mediated transcriptional activation in a chromatin context is not responsive to Tax.** (A) One-dimensional DNA topological assay showing the NF-κB G-less transcription template assembled with *Drosophila* core histones in the presence of dAcf1/ISWI and GST-yNAP-1. The DNA topoisomers 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.

B.

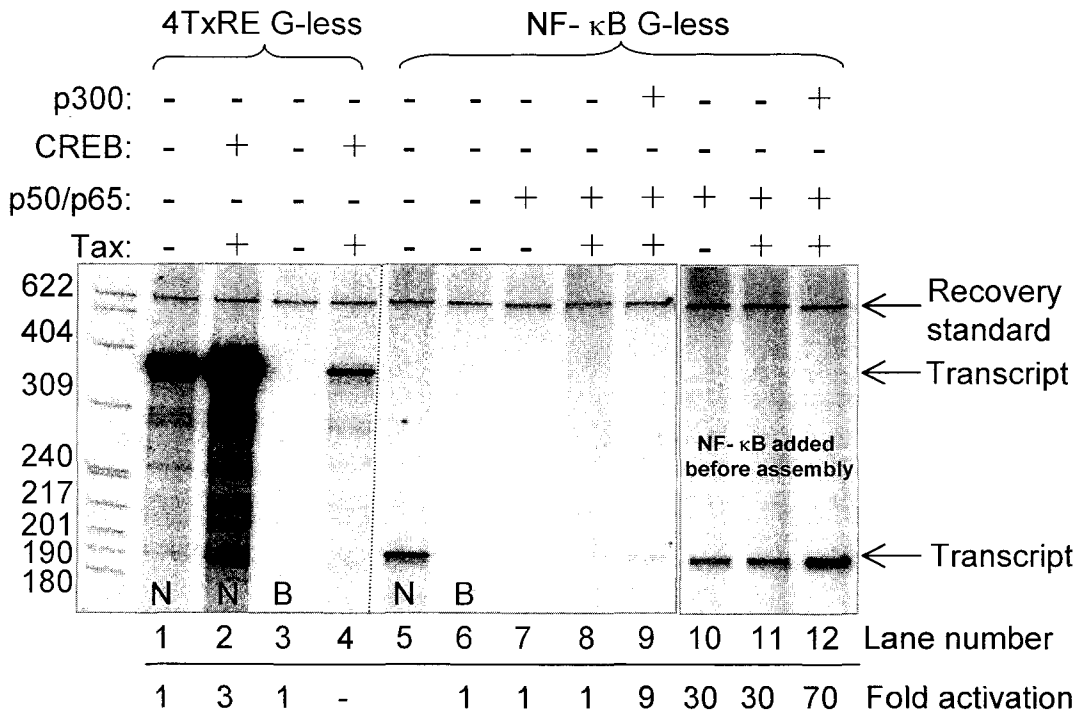

Figure 5.7 **NF-κB mediated transcriptional activation in a chromatin context is not responsive to Tax.** (B) Transcriptional activation on NF-κB/G-less, and 4TxRE G-less chromatin templates was analyzed in the presence (+), or absence (-) of p50/p65 (150 ng), Tax (100 ng), CREB (100 ng), and p300 (150 ng). The positions of molecular weight size markers, recovery standard, and full-length G-less transcripts are indicated. Basal transcription levels, although not visible in this exposure, were measurable and upon subtraction of the background signal generated a non-zero number that was set equal to 1 in terms of relative transcription. N=naked DNA, B=basal transcription.

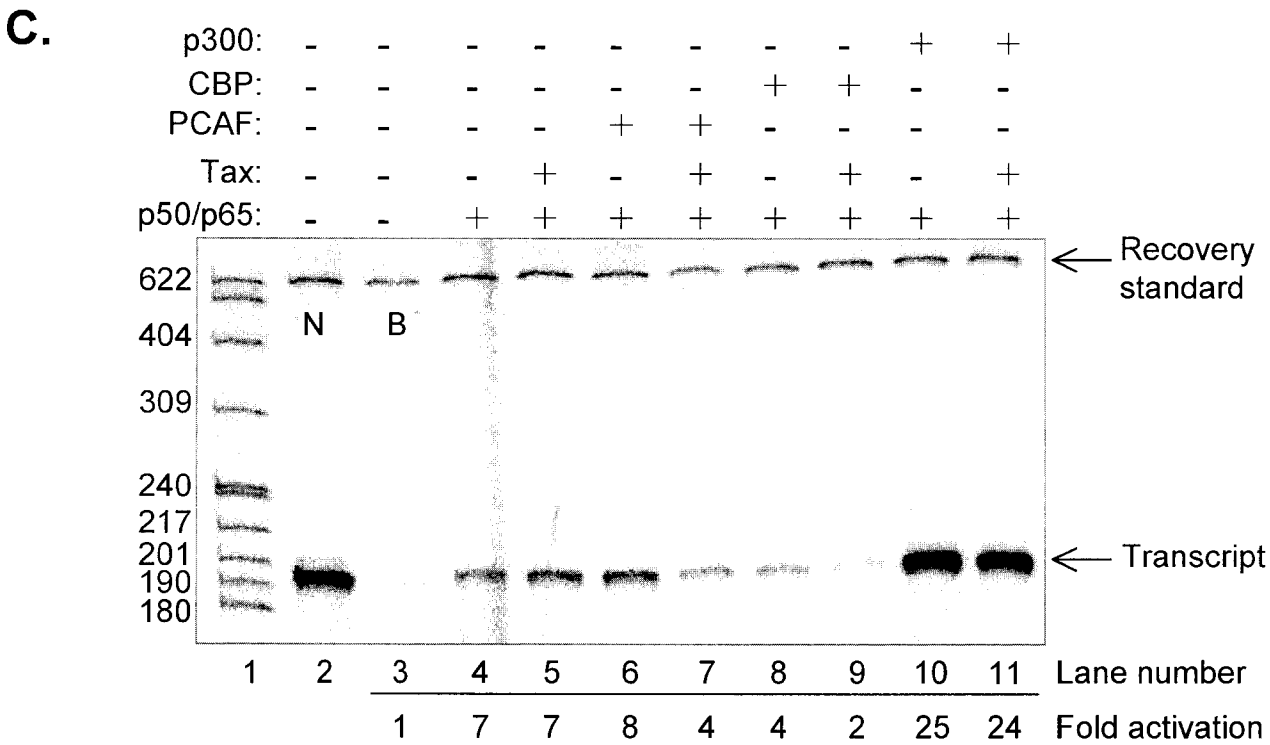

Figure 5.7 **NF-κB mediated transcriptional activation is not responsive to addition of p300, CBP, PCAF, or Tax.** (C) Transcriptional activation on NF-κB/G-less chromatin templates was analyzed in the presence (+), or absence (-) of p50/p65 (150 ng), Tax (150 ng), p300 (120 ng), CBP (120 ng), and P/CAF (120 ng). The positions of molecular weight size markers, recovery standard, and full-length G-less transcripts are indicated. Basal transcription levels, although not visible in this exposure, were measurable and upon subtraction of the background signal generated a non-zero number that was set equal to 1 in terms of relative transcription. N=naked DNA, B=basal transcription.

Addition of p300 activated transcription over levels of p50/p65 alone (lanes 9 and 12).

To further test the effect Tax has on NF-κB mediated transcription, we tested the transcription function of NF-κB with a variety of coactivators. p300 has a paralogue, called CBP, and these two proteins are conserved from *C. elegans* to *Homo sapiens*, and mediate many aspects of gene expression in metazoans (reviewed in 81, 82, 87, 281). We also wanted to test a third coactivator, p300/CBP associated factor (PCAF), a 832 amino acid cellular coactivator that was first identified in 1996 based on its sequence similarity with the yeast protein GCN5 (297). Since NF-κB has been shown to utilize other cellular coactivators, we wanted to test their function in relation to Tax (250). An in vitro transcription assay on chromatin-assembled DNA templates testing these three coactivators is shown in figure 5.7 C. Again, under these conditions, Tax had no effect on NF-κB mediated transcription. PCAF showed very little enhancement in NF-κB mediated transcription (compare lanes 4 and 6), and the addition of Tax may have actually decreased the amount of transcription seen (lane 7). CBP showed very little activation as compared to the p50/p65 heterodimer alone (compare lanes 4 to 8), while p300 showed a strong coactivation of NF-κB. However, we still failed to see an effect of Tax on transcription.

5.5 Discussion

The ability of Tax to promote the nuclear localization of the NF-κB proteins has been extensively studied and is widely held as the primary mechanism of NF-κB transcriptional activation by Tax. However, since it has been shown that Tax colocalizes in nuclear structures with the NF-κB proteins, and these transcriptionally active nuclear bodies also contained the coactivators CBP/p300 (23), we hypothesized that Tax may contribute to the NF-κB-dependent transcription through a direct mechanism in the nucleus. Furthermore, Tax has been shown to increase the DNA binding activity of NF-κB in vitro (10, 222), similar to that observed with Tax and CREB on a viral CRE. Previous studies have also shown that Tax binds directly to p65 (266). Since the cytoplasmic amounts of Tax have such a profound affect on the total protein levels of NF-κB in the nucleus, we hypothesized that Tax would also have a direct role in regulating the gene expression of NF-κB responsive promoters directly.

We were able to duplicate early studies showing that Tax physically interacts with p65, indicating that our proteins used in the in vitro assays are functional (152, 266). However, none of our in vitro assays showed any effects of Tax on NF-κB-mediated transcriptional activation. DNA binding assays and in vitro transcriptions in the presence of multiple coactivators failed to produce a stimulatory or repressive affect of Tax on the NF-κB proteins p50 and p65. Post-translational modifications including acetylation

and phosphorylation of p50 and p65 also failed to produce any effect from adding exogenous Tax to our functional assays.

One explanation for these results could lie in the synthetic in vitro system engineered for this project. Studying the effects of Tax on the function of NF-κB may require a more physiologically relevant system. A biologically relevant promoter such as HIV could be tested in the place of the NF-κB responsive promoter used in this study. Further experiments studying the activity of post-translationally modified NF-κB in in vitro transcriptions in the context of chromatin could also be performed. Unfortunately, this study failed to establish a role for Tax in the transcriptional function of NFκB on the promoter.

5.6 Acknowledgements

We would like to thank Dimitri Thanos for gifts of expression plasmids for p65 and p50. We would also like to thank Warner Greene for the gift of the expression plasmid for GST-p65.

Chapter 6

Summary and Future Directions

The ability of Tax to deregulate p53 function is complex and likely involves many processes. We have identified a possible mechanism for the ability of Tax to deregulate cellular function. To regulate transcription, p53 and Tax both utilize the cellular coactivators CBP/p300. Although competition for the cellular coactivators CBP/p300 could explain many of the effects caused by Tax, it is likely not the only way that Tax downregulates p53 function. Investigating other pathways that Tax could inhibit cell death and apoptosis may shed more light on the question. These include studying the effects of Tax on proteins like GSK-3β.

Studying basal-level transcription is important for understanding how HTLV-1 evades the immune response, and how it can remain in a semi-latent state for so many years before causing malignancy. Sp1 has previously been shown to bind at two distinct locations on the HTLV-1 promoter to activate transcription. We demonstrate that Sp1 binds with the highest affinity to the Sp1 site located between the second and third viral CREs. Sp1 is detected at the chromosomally integrated HTLV-1 promoter in living cells in both the absence and presence of Tax. Sp1 activates transcription modestly in vitro and in vivo in the absence of Tax, and a double point mutation at the preferred Sp1 binding site strongly down-regulates basal level transcription. Sp1 likely plays a role in basal transcription, but more studies are needed to fully understand this role. Studying Sp1 in MT4 cell lines may be a way to study the basal HTLV-1 promoter. Investigating whether Sp1 is present on both the 5' and 3' LTR, and if it works in concert with another cellular

transcription factor such as Ets is important. Post-transcriptional modifications of Sp1 such as acetylation and glycosylation may reveal other functional properties of Sp1 on the HTLV-1 promoter. Finally, an EMSA of Sp1 on the mutant vSp1 oligo would be important to ensure the mutant reporter plasmid does not bind Sp1.

We designed and synthesized six Tax-directed pyrole-imidazole polyamides specifically designed to block Tax binding to DNA at the HTLV-1 promoter. We found that four of these polyamides disrupt binding of the Tax/CREB complex in vitro, and that these same molecules also inhibit Tax-mediated transcription in vitro. However, of these four Tax/CREB-specific polyamides, only one polyamide appears to be uniquely Tax specific. We show that polyamides can enter the nuclei of HTLV-1 infected T-cells, and two of the four polyamides down-regulate virion production in these cells. Since the in vivo results of the polyamide studies differed from the in vitro results, elucidating the differences is needed to fully understand the effects of polyamides on inhibiting transcription. Measuring the off-rate of the polyamides on chromatin-assembled templates may explain the inability of the polyamides to work in vivo in the presence of many proteins. Since we do not know if polyamides bind to chromatin-assembled templates, experiments such as Dnase I footprinting assays could be helpful in answering this question.

We have studied the mechanisms of Tax deregulation via the NF-κB proteins by focusing on the events that occur directly at the promoters of NF-

κB-responsive genes in an in vitro transcription system. We found no direct ability of Tax to deregulate the function of NF-κB at the NF-κB responsive promoter. Since the results of the NF-κB results were negative in the study presented here, further study of the ability of Tax to regulate NF-κB may be more successful on a physiologically relevant promoter such as HIV or IL-15. Chromatin immunoprecipitation assays of Tax on these promoters in vivo may be helpful before further studies are pursued. Finally, using recombinant proteins purified from baculovirus-infected SF9 cells may produce more transcriptionally active p65 and p50 proteins.

Chapter 7

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