DISSERTATION

INTEGRATING p300 FUNCTIONS IN HTLV-1 TRANSCRIPTION INITIATION

Submitted by

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ABSTRACT

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The HTLV-1 provirus overcomes a repressive chromatin environment for efficient transcription of its genome. This is accomplished by the robust recruitment of the coactivator protein, p300, to the viral enhancer sites through interactions with DNA bound pCREB and the viral transactivating protein, Tax. Recruitment of p300 to the HTLV-1 promoter results in histone acetylation and nucleosome depletion from the promoter region in the presence of the histone chaperone, Nap1.

To study the histone acetylation requirements for Nap1-dependent nucleosome disassembly, we utilized immobilized in vitro assembled chromatin templates containing site specific K→R mutations within the N-terminal tails of the histones. Through these studies, we identified histone H3, lysine 14 as the functionally relevant acetylation site for Nap1-dependent nucleosome disassembly. Additionally, we found a significant correlation between nucleosome disassembly from the HTLV-1 promoter and acetylation-dependent transcription activation. These studies suggest that nucleosome disassembly is a prerequisite for transcription activation, as nucleosome disassembly creates a nucleosome free region within the HTLV-1 promoter, allowing for the subsequent recruitment of Pol II and general transcription machinery for activation of transcription.

The identification of a single and specific acetyl-lysine residue led us to the hypothesis that the p300 acetyl-lysine binding domain (bromodomain) was involved in

HTLV-1 transcription activation through recognition of H3K14ac. To test this hypothesis, we utilized a p300 bromodomain deletion mutant and a CBP/p300 specific bromodomain inhibitor, (SGC-CBP30), to investigate the involvement of the p300 bromodomain in HTLV-1 transcription activation. Importantly, we found that the p300 bromodomain is not involved in the initial recruitment of the coactivator to the chromatin template as previously proposed, rather the bromodomain functions after recruitment to the promoter and following acetylation of the histone tails. These findings are consistent with a role for the p300 bromodomain in nucleosome disassembly and uncover a novel function for the bromodomain in gene activation.

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TABLE OF CONTENTS

CHAPTER 1
INTRODUCTION TO HTLV-1 TRANSCRIPTIONAL REGULATION

1.1a DISCOVERY OF HTLV-1

During the late 1970's, Japanese clinicians identified a cluster of leukemias in southwestern Japan termed adult T-cell leukemia (ATL), hypothesizing that the clustering was due to an infectious agent (2, 3). Between 1980 and 1982, researchers in the USA and Japan independently isolated a virus shown to be the causative agent of ATL (4-6). This was the first identification of a human oncogenic retrovirus. The virus was eventually named human T-cell leukemia virus (HTLV-1) and was different from previously known transforming retroviruses from other animal hosts in that HTLV-1 did not carry viral homologues of cellular proto-oncogenes (9). Rather, HTLV-1 encoded for accessory proteins, namely the viral encoded oncoprotein, Tax, which contributes to HTLV-1's transforming capabilities. In addition to causing ATL, HTLV-1 infection has also been demonstrated to cause a progressive neurological disorder termed HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (10, 11).

1.1b PREVALANCE AND TRANSMISSION OF HTLV-1 INFECTION

It is currently estimated that 5-10 million people are infected with HTLV-1 worldwide (13). Endemic regions include southwestern Japan, Africa, the Caribbean Islands, and South America.

Initial transmission of the virus requires cell-cell contact and is therefore transmitted from an infected individual to an uninfected individual through exposure to

contaminated blood products, through sexual contact, or from mother to child through breast feeding. CD4+ T cells are the primary infected cell type in vivo, although it has been demonstrated that CD8+ T cells, B lymphocytes, monocytes, dendritic cells, macrophages and astrocytes may also be infected with HTLV-1 in vivo (14-16). It has been reported that the glucose transporter 1 protein (GLUT1), the surface heparin proteoglycan -1 and the neuropilin-1 proteins are involved in binding and entry of the virus [reviewed in (17)], however, how these proteins are involved in tropism of the virus is not understood, as many cell types express these proteins on their cell surface.

1.1c HTLV-1 ASSOCIATED DISEASES

Most infected individuals remain asymptomatic carriers throughout their lifetime, however, 1-5% develop ATL (18) and up to 4% develop HAM/TSP (19). A large part of current HTLV-1 research efforts are focused on the molecular basis of HTLV-1 dependent malignant transformation of T cells and why only a small percentage of infected individuals develop the leukemia.

It is well established that HTLV-1 is the etiological agent of ATL. HTLV-1 infection was first linked to adult T-cell leukemia in 1980 when a retrovirus was isolated from a cell line derived from a patient mistakenly diagnosed with cutaneous T-cell lymphoma (later to be recognized as ATL) (4). Shortly thereafter, a retrovirus was successfully isolated from cells derived from ATL patients (5). Adding to the evidence that HTLV-1 causes ATL, leukemic cells taken directly from numerous ATL patients tested positive for the integrated HTLV-1 provirus (20). To date, the monoclonal integration of the HTLV-1 provirus has been detected in all cases of ATL examined (21).

A diagnostic feature of ATL is the presence of morphologically abnormal lymphocytes in the peripheral blood. Leukemic cells have a characteristic "flower" shape due to a multilobulated nucleus and very little cytoplasm. Accompanying abnormal lymphocyte morphology, patients with ATL might also develop hypercalcemia, as well as skin lesions from infiltration of leukemic cells into the epidermis. ATL patients are also at an increased risk of opportunistic infections due to weakening of the immune system.

There are four subtypes of ATL: smoldering, chronic, lymphoma-type and acute. The latter two subtypes are aggressive, as it has been recently reported that median survival time from diagnosis is approximately 13 months, despite modern ATL treatments that include multi-drug chemotherapy, a combination of azidothymidine (AZT) and IFN-α therapy, and allogeneic hematopoietic stem cell transplantation (22, 23).

The mechanism by which HTLV-1 infection leads to malignant transformation of T cells is still not completely understood. However, the main theory postulates that the virally encoded regulatory protein, Tax (discussed below), mediates disruption of normal cellular functions such as DNA repair, cell cycle, and apoptosis, leading to cell proliferation and accumulation of genetic mutations. However, ATL cells express very little Tax protein and the clinical latency is long; typically 40-60 years after infection with the virus. A current hypothesis accommodating these two observations proposes cycles of transient Tax expression, allowing for infected cells not expressing Tax to i) replicate by clonal expansion ii) evade the immune system iii) and slowly accumulate mutations, thus increasing the likelihood of malignant transformation (24).

As mentioned, another disease caused by HTLV-1 infection is HAM/TSP and was first described in 1986 (11). HAM/TSP is a progressive, chronic and incapacitating neuroinflammatory disorder. The disease manifests itself as muscle weakness, stiffness and spasms in the lower extremities, similar to the symptoms of multiple sclerosis. HAM/TSP patients also frequently become incontinent and incur sensory disturbance as a result of loss of nervous function.

The myelopathy results from chronic inflammation and subsequent demyelination of the spinal cord. Although the exact mechanism by which HTLV-1 infection induces disease is unknown, the current understanding is that overactivated, infected CD4+ T cells migrate across the blood-brain barrier from the peripheral blood to the central nervous system and begin to express Tax and secrete proinflammatory cytokines. The cytokines stimulate the production of chemokines that recruit more HTLV-1 infected CD4+ T cells and HTLV-1 specific CD8+ T cells to the site of inflammation, thereby ultimately causing damage to the nearby nervous tissue [reviewed in (25)].

1.1d   THE HTLV-1 GENOME AND VIRAL LIFE CYCLE

HTLV-1 is a member of the *Retroviridae* family of viruses, specifically of the *Deltaretrovirus* genus. In addition to HTLV-1, *Deltaretrovirus* genus members include Simian T-cell leukemia virus, Bovine leukemia virus and HTLV-2, 3 and 4 (26).

The HTLV-1 viral life cycle can be divided into two phases: the establishment phase, which primarily depends on viral proteins carried with the virion and the expression/replication phase, which primarily uses host cell machinery (Figure 1.1).
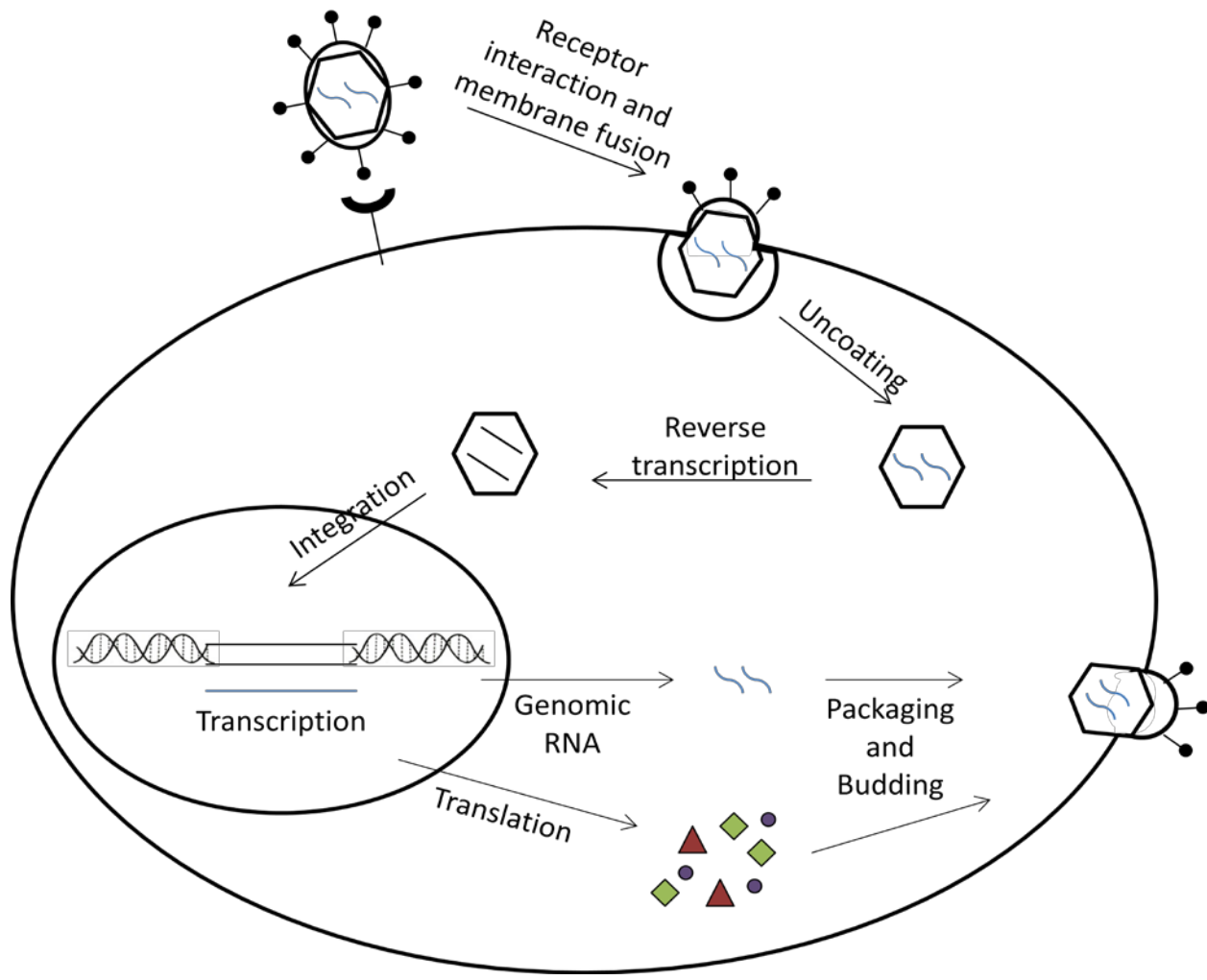
Figure 1.1. **The Life cycle of HTLV-1.** Adapted from Dudley et al. 2011

During the establishment phase, the virus gains entry into the host cell via interaction of its surface glycoprotein (gp46) and transmembrane protein (gp21) with a cellular receptor. This interaction results in membrane fusion and subsequent uncoating of the virus. Contained in the HTLV-1 virion core are two copies of the (+) sense single stranded RNA (+ssRNA) genome, as well as viral proteins required to carry out reverse transcription and provirus integration once the viral genome has entered the host cell cytoplasm (27). Viral reverse transcriptase uses two enzymatic activities to produce a DNA provirus from the ssRNA genome: RNA dependent DNA polymerase activity and RNase H activity. Upon reverse transcription, the double stranded DNA molecule translocates to the nucleus where it is randomly integrated into the host cell genome by the viral integrase. Importantly, the U3 region of the LTR contains the HTLV-1 promoter that is critical for driving expression of the HTLV-1 provirus.

The protein encoding region of HTLV-1 is found between the LTRs. The *gag* gene encodes for structural proteins including capsid (CA), nucleocapsid (NA) and matrix (MA) proteins. The *pro* gene encodes for a protease that is responsible for processing the gag/pro/pol polyprotein. Reverse transcriptase (RT) and integrase (IN) are encoded for by the *pol* gene. The *env* gene encodes for envelope glycoproteins that are involved in receptor recognition during viral entry. HTLV-1 is considered a complex retrovirus and as such encodes for unique regulatory and accessory proteins in the pX region near the 3' end of the provirus in addition to the prototypical *env*, *prot*, *pol* and *gag* gene products just mentioned. Regulatory proteins include Tax, which is responsible for transactivation of the provirus as well as deregulation of numerous other cellular functions, and Rex, which is involved in the nuclear export of unspliced mRNAs

encoding gag/pol/env (28, 29). Accessory proteins, p12, p13 and p30 are involved in various mechanisms that fine tune expression of the provirus and regulate cell growth, allowing for replication and viral persistence [reviewed in (30)]. In addition, an antisense transcript is made from the 3' LTR and codes for a regulatory protein called HTLV-1 basic leucine zipper factor (HBZ). HBZ promotes proliferation of T cells while downregulating expression of the provirus, again regulating viral replication and persistence (31) (Figure 1.2).

During the expression and replication phase, the host cell transcription machinery navigates the chromatin environment to express the HTLV-1 provirus in the form of a cellular messenger RNA. The messenger RNA is either spliced and subsequently translated to create viral gene products necessary for virion production and release, or remains unspliced, serving as the genome for progeny viruses. Once gene products are made, they are packaged into virions at the cell surface where virions either bud from the host cell membrane or are transferred from an infected cell to an uninfected cell through the formation of a viral synapse.

1.2 TAX-MEDIATED TRANSCRIPTION ACTIVATION OF THE HTLV-1 PROVIRUS

Upon initial infection and during periods of reactivation, HTLV-1 must overcome the repressive nature of its chromatin environment to express viral genes that are needed for viral persistence. HTLV-1 accomplishes this through the use of a series of Tax-responsive enhancer elements located within the U3 region of the LTRs of the provirus. These enhancer elements serve as binding sites for the recruitment of viral and non-viral proteins that subsequently modulate the chromatin structure, allowing for
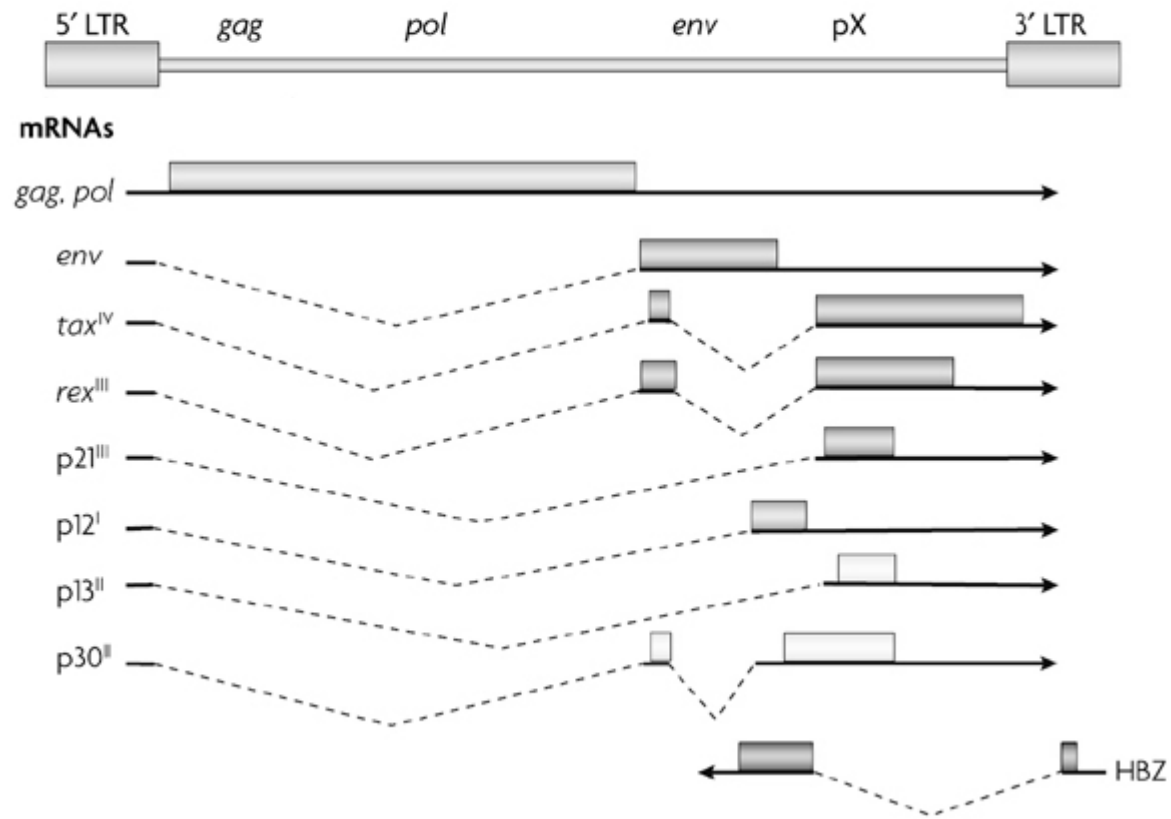
Figure 1.2. **Schematic of the HTLV-1 genome.** Open reading frames corresponding to their respective unspliced and spliced mRNAs and encoded proteins are shown. Modified from (8).

initiation of HTLV-1 transcription. Understanding Tax-mediated transcription activation of the HTLV-1 provirus provides insight into inhibiting viral replication, and serves as an outstanding model system for studying general cellular mechanisms of gene activation.

1.2a CREB/TAX PROTEIN-PROTEIN AND PROTEIN-DNA INTERACTIONS AT THE HTLV-1 PROMOTER ARE REQUIRED FOR TRANSCRIPTION ACTIVATION

Found within the U3 region of the 5' and 3' LTRs of the HTLV-1 provirus is a series of Tax-responsive enhancer elements (32-34). These enhancer elements, termed viral CREs (vCREs), are off-consensus cellular cAMP response elements (CRE) flanked by short sequences of GC-rich DNA. There are three vCREs located at approximately -100, -200 and -250 bp upstream from the transcription start site (tss) (33). The vCREs serve as binding sites for the viral transactivator protein, Tax, as well as for cellular transcription factors, namely the ATF/CREB family members (35-39) (Figure 1.3A). The vCREs differ from cellular CREs by 0-2 base pairs within one of the half sites of the vCRE, thereby resulting in 10-fold reduced affinity for CREB. It has been demonstrated that Tax enhances CREB binding to the vCREs not only through protein-protein interactions that promote CREB dimerization through its bZIP domain, but also through an interaction with the minor groove of the GC-rich flanking sequence of the vCREs (35, 40-46). Most importantly, the stable Tax/CREB/vCRE complex is required for transcription from the HTLV-1 promoter (35, 47). Furthermore, phosphorylation of CREB at Ser[133] significantly enhances recruitment of the coactivator CREB binding protein (CBP)/p300 (48, 49) (Figure 1.3B).
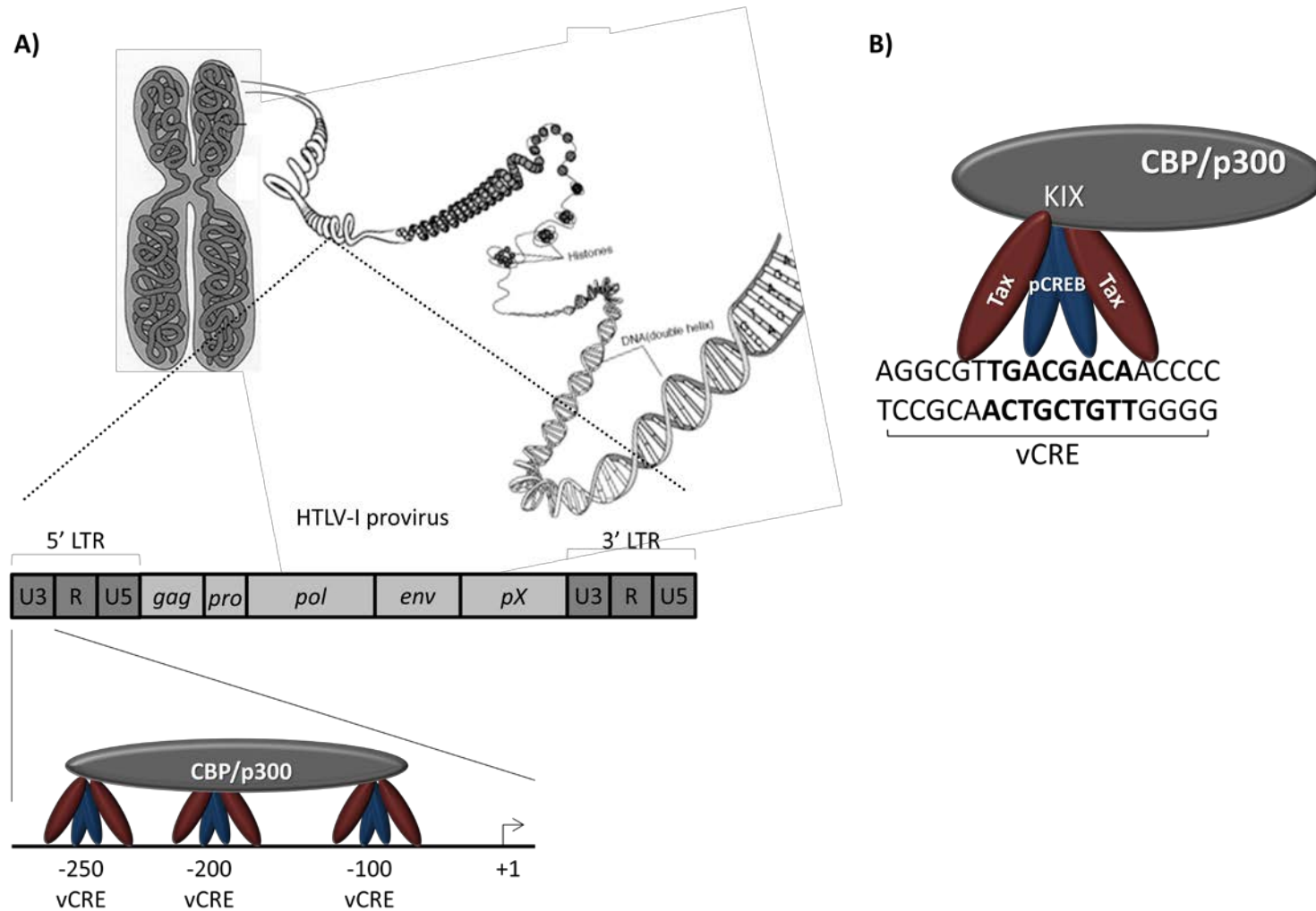
Figure 1.3. **The HTLV-1 enhancer elements.** A) Location of the viral enhancer elements within the integrated HTLV-1 provirus. Adapted from Nyborg et al. 2009 (7). B) Illustration of the protein-protein and protein DNA interactions at a single representative vCRE.

1.2b CBP/P300 COACTIVATOR RECRUITMENT

CBP and p300 were first identified as coactivator proteins independently from each other through interactions with their binding partners, CREB and viral oncoprotein E1A, respectively (50-53). Soon after their characterization, it was realized that CBP and p300 share high amino acid sequence homology and therefore, functional domains (51). CBP/p300 are large, multidomain cellular coactivator proteins that are involved in several cellular processes, including cell growth, development, differentiation and transformation (54). They are frequently considered functionally homologous in the literature, although subtle differences do arise during particular cellular processes (55, 56).

The coactivator proteins bind numerous cellular transcription factors to promote gene activation, serving as adaptor molecules that simultaneously interact with and recruit components of the basal transcription machinery (57). It is not surprising then that the CBP/p300 interactome includes over 400 different proteins (58). In addition to transcription factor binding and recruitment of basal transcription machinery, CBP and p300 function as coactivators through their intrinsic acetyltransferase activity, capable of acetylating histone proteins as well as non-histone proteins (59-62).

The major defined CBP/p300 domains involved in protein binding are the cysteine-histidine rich regions, CH1 and CH3, the KIX domain, and the interferon binding domain (iBiD) (54). Acetyltransferase activity is located within the core domain, which is comprised of a bromodomain, RING domain, PHD domain and HAT domain. The bromodomain specifically recognizes acetylated lysine residues and is involved in

coactivator targeting (63, 64), while the RING domain negatively regulates p300 HAT activity, possibly by sterically occluding the HAT active site (65) (Figure 1.4).

Tax interaction with CBP/p300 occurs through multiple domains on the coactivators. First, Tax interacts with a carboxy-terminal region of CBP through an amphipathic helix of Tax (aa 312-319). Interestingly, this region of CBP, found within the iBiD binding domain, is the steroid receptor coactivator (SRC-1) interaction domain. Tax competes with SRC-1 for binding with CBP and importantly, point mutagenesis within the corresponding Tax and CBP interacting domains decreased Tax activation of the HTLV-1 promoter *in vitro* and *in vivo* (66). Second, it has been well demonstrated that Tax directly interacts with CBP through an interaction with the KIX domain (67-70). The minimal KIX domain required for interaction with Tax is aa 588-683, which is a similar region to that required for pCREB interaction. Since both pCREB and Tax bind KIX simultaneously, this observation suggests that Tax and pCREB bind the hydrophobic core of the KIX domain, albeit at different surfaces (70, 71). Notably, disruption of the Tax/KIX or pCREB/KIX interaction inhibits Tax transcriptional activation of HTLV-1 *in vitro* and *in vivo* (72). Lastly, the CH1 domain of CBP has also been implicated in an interaction with Tax, where Tax and p73 binding is mutually exclusive (73). The functional consequence of robust recruitment of CBP/p300 to the HTLV-1 promoter and its role in mediating chromatin remodeling at the HTLV-1 promoter for subsequent transcription activation is discussed in further detail in section 1.4.
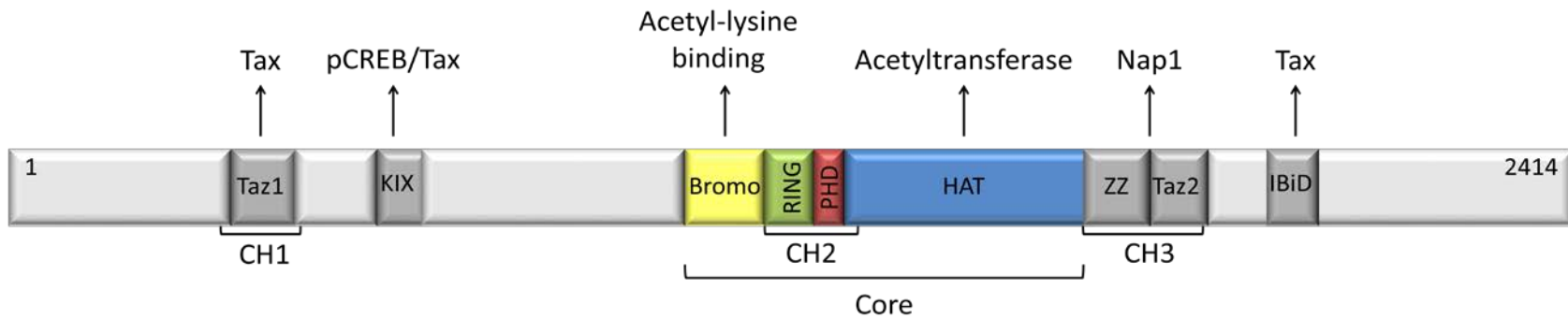
Figure 1.4. **p300 domains.** Domains known to interact with Tax and pCREB are indicated. Also indicated is the p300 core domain consisting of the bromodomain and HAT domain.

1.3   OVERCOMING REPRESSIVE CHROMATIN STRUCTURE DURING TRANSCRIPTION ACTIVATION THROUGH HISTONE ACETYLATION

DNA exists in the nucleus of a cell as a nucleoprotein complex, called chromatin, which is inhibitory to many processes requiring access to the genetic material. Therefore, cellular mechanisms exist to modulate chromatin structure and gain access to the DNA. Mechanisms involved in chromatin structure regulation in the context of transcription activation and histone acetylation are discussed below.

1.3a   CHROMATIN STRUCTURE REGULATION

The most basic unit of chromatin is the nucleosome, which is comprised of approximately 146 bp of DNA wrapped 1.65 times around an octamer of the core histone proteins, H2A, H2B, H3 and H4 (74, 75). Two H2A/H2B dimers and a H3/H4 tetramer comprise the octameric protein component of the nucleosome. The histone proteins have an unstructured N-terminal "tail" and a structured histone fold domain. The fold domains are held within the nucleosome and make up its core, while the highly basic tails extend past the gyres of the wrapped DNA.

Chromatin is composed of arrays of nucleosomes that are capable of taking on various structural forms (Figure 1.5). Chromatin folding and compaction is mediated by intra-nucleosomal contacts of the N-terminal histone tails with neighboring nucleosomes and linker DNA, as well as by linker histone H1 binding [reviewed in (76)]. These interactions lead to more compact and folded chromatin states (i.e. more repressive), going from an extended, 10 nm "beads on a string" structure to a compacted 30 nm fiber, as evidenced by electron micrograph images of chromatin from chicken
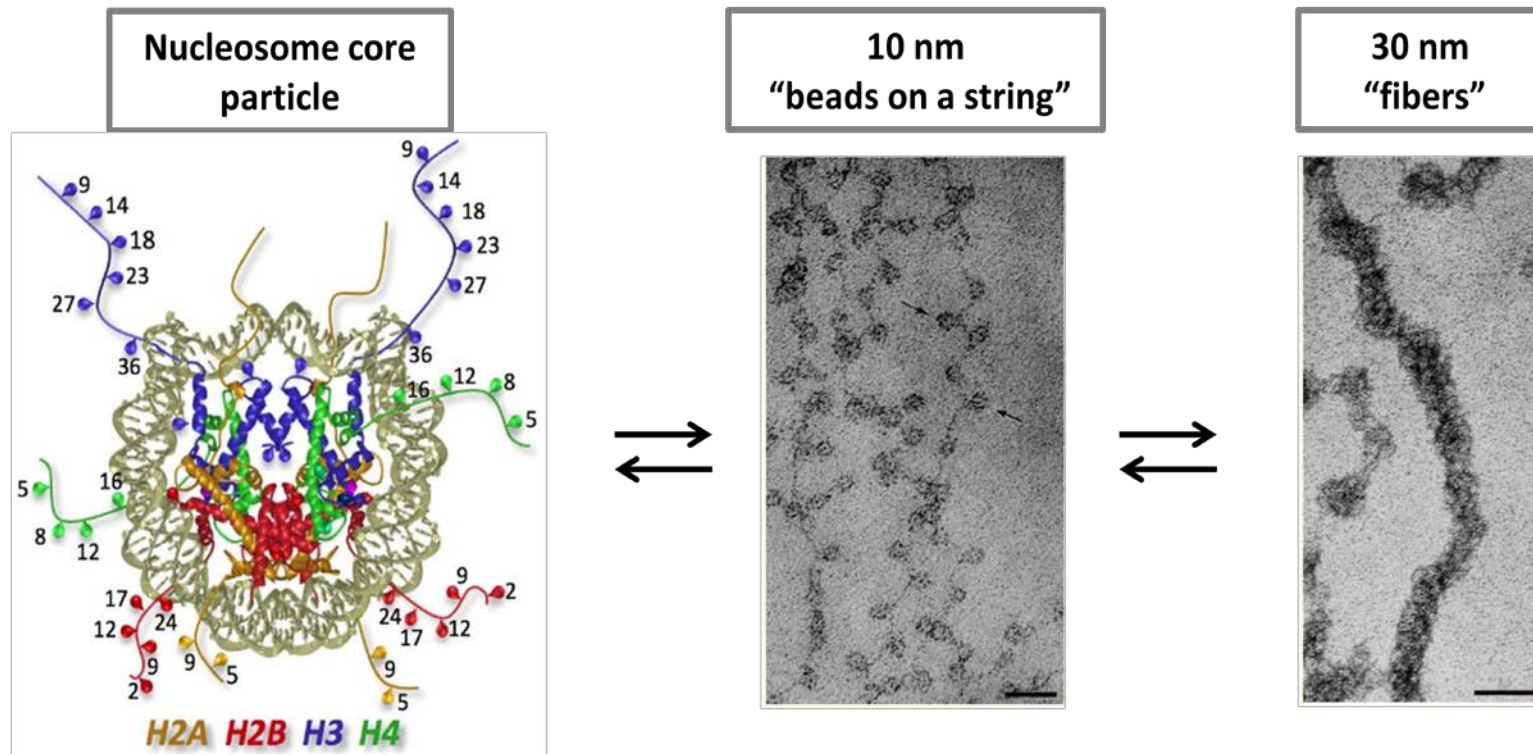
Figure 1.5. **Chromatin structure hierarchy.** Nuclosomes are the most basic unit of chromatin. Chromatin consists of nucleosome arrays where histone tails interact with linker DNA and neighboring nucleosomes to form higher ordered chromatin structures as seen in the electron micrographs taken from Olins et al. 2003 (7). The nucleosome core particle was taken from Hansen et al. 2010 (12).

erythrocytes at low and moderate ionic strength (7, 77). It should be noted that the exact structure of 30 nm and larger chromatin fibers in vivo is unknown and that their existence in vivo remains controversial [reviewed in (78, 79)].

It is well established that chromatin is generally repressive to transcription (80-84). Therefore, eukaryotic cells have developed mechanisms for the decompaction and movement of nucleosomes at promoter regions allowing for the binding of transcriptional activators and transcription machinery and subsequent gene activation. Thus, the dynamic regulation of chromatin structure is essential to controlling which genes are actively transcribed.

Chromatin structure and fluidity is regulated by numerous mechanisms; however, three major modes of regulation are generally recognized: **i)** Chromatin remodeling complexes use the energy from ATP hydrolysis to alter nucleosome occupancy and positioning and thus chromatin structure (85). **ii)** Histone chaperones exchange canonical histones with histone variants. Upon incorporation into nucleosomes, these variants may create less stable octamers and facilitate in the removal of nucleosomes from promoter regions, activating transcription (86, 87). **iii)** Perhaps the most studied, but least understood due to complexity and an overwhelming number of combinatorial scenarios, is regulation through covalent post-translational modifications of the histone tails (88). These include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination and proline isomerization (89). These modifications occur in various combinations to keep DNA either compact or open via the loss, sliding, or exchange of a nucleosome (90-92). An important consequence of

16

post-translational modification of the histone tails is that the N-terminal histone tails themselves become important regulators of chromatin dynamics.

1.3b   CHROMATIN STRUCTURE REGULATION THROUGH HISTONE ACETYLATION

As just mentioned, N-terminal histone tails are sites of numerous post-translational modifications (PTMs) that affect compaction of the chromatin fiber. Of these modifications, acetylation was the first identified and is perhaps the best studied and most directly associated with gene activation (93). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the enzymes responsible for the addition and removal of an acetyl moiety to the ε-amino group of lysine residues. Together, HATs and HDACs help regulate chromatin dynamics and therefore, gene regulation.

The addition of an acetyl group onto a lysine residue sidechain acts in two ways; through charge neutralization of the positively charged lysine residue and through creation of a new binding site for an additional protein. At physiological pH, the positively charged lysine residue is neutralized upon acetylation, leading to the disruption of histone tail contacts with linker DNA and acidic patches on other nucleosomal histones (94). Studies have shown that chromatin decompaction and transcription activation occur as a result of both global and residue specific tail acetylation. For example, the H4 tail is acetylated at lysines 5, 8, 12 and 16. Acetylation of lysines 5, 8 and 12 appear to contribute to the cumulative nature of the charge neutralization model, whereas K16 acetylation appears to possess a unique function in transcription (95). This is further corroborated in vitro where specific acetylation of

H4K16 reduces 30 nm fiber compaction (94, 96). Importantly, while acetylation of histone tails results in decompaction of the chromatin fiber, it does not result in the destabilization of a nucleosome, per se (83).

The addition of an acetyl group can also create an epitope for the binding/recruitment of other protein to chromatin. In particular, proteins containing bromodomains, which recognize acetylated lysines, are directly recruited and act on the chromatin fiber (97). Indeed, many chromatin modifying complexes, including HATs and ATP-dependent remodelers, contain at least one bromodomain, further contributing to the specificity of their substrate binding (98). As a consequence, upon substrate binding through acetyl-lysine recognition, these complexes can act on the chromatin through ATP-dependent nucleosome remodeling, histone exchange, or further post-translational modification.

Histone tail acetylation has long been correlated with actively transcribed genes. Recently, chromatin immunoprecipitation (ChIP) assays in yeast and higher eukaryotes have shown genome-wide that H3 and H4 acetylation corresponds to active gene regions (99-102). Additionally, ChIP-seq data has demonstrated that CBP/p300 occupancy at promoter regions positively correlates with DNase1 hypersensitive sites (103). However, the relative contribution of charge neutralization versus targeted histone tail acetylation for gene activation is unknown. Thus, while histone acetylation is known to favor a more relaxed chromatin state, and is associated with an increase in transcription, the precise mechanism by which acetylation promotes a transcriptionally permissive state is less clear.

1.4   ACETYLATION-DEPENDENT CHROMATIN REMODELING OF THE HTLV-1 PROMOTER FACILITATES TRANSCRIPTION ACTIVATION

There have been numerous studies focused on the mechanism of Tax transactivation of the HTLV-1 provirus in a chromatin environment. Summarized below are the studies that contributed to the foundation of the research presented herein.

In vitro studies utilizing recombinant chromatin templates have demonstrated that the pCREB/Tax/vCRE recruited HAT activity of p300 through the p300-KIX domain is required for transcription activation of the HTLV-1 gene (72, 104, 105). Additionally, chromatin templates lacking N-terminal histone tails have reduced dependence on Ac-CoA and p300 for transcription activation, suggesting that the N-terminal histone tails are the main targets of p300 HAT activity (104).

Tax-dependent p300 recruitment to an integrated HTLV-1 promoter driving a luciferase gene was correlated with *decreased* acetylated H3 at the promoter and coding region of the gene. This loss in acetylated histones was found to be due to a loss of histones at the HTLV-1 promoter and not a decrease in acetylation itself. Adding to the evidence that nucleosomes were depleted from the HTLV-1 promoter upon Tax activation, chromatin isolated from cells transiently transfected with Tax showed increased sensitivity to micrococcal nuclease digestion than chromatin isolated from cells not expressing Tax. The above observations positively correlated with an increase in Pol II recruitment and strong activation of transcription. These data suggest a mechanism by which Tax regulates the removal of histones from the promoter for subsequent Pol II recruitment and gene activation (106).

To study the loss of nucleosome occupancy at the HTLV-1 promoter *in vitro*, an immobilized template assay was developed using the full HTLV-1 viral promoter linked to a streptavidin coated magnetic bead via a 5' biotin moiety. The immobilized promoter was then assembled into chromatin using the method of salt deposition. *In vitro* nucleosome disassembly was found to be dependent upon binding of Tax, together with the cellular transcription factor pCREB to the vCREs, and their subsequent recruitment of the cellular coactivator and HAT, p300. In the presence of Ac-CoA, acceptor DNA and the nucleosome assembly protein, Nap1, the nucleosome octamer was disassembled from the promoter. Importantly, nucleosome disassembly was independent of transcription and ATP-dependent chromatin remodeling. Nap1 is a highly conserved histone chaperone that transports histones H2A/H2B from the cytoplasm to the nucleus and is predominately thought to have a role in nucleosome assembly. This study, however, presents a novel functional role of Nap1 in acetylation-dependent nucleosome disassembly (107).

Together, these studies demonstrated that HTLV-1 enhancer elements recruit cellular transcription factor pCREB, and that the DNA-bound pCREB/Tax complex strongly recruits cellular coactivator proteins CBP/p300 to initiate HTLV-1 transcription activation. Transcription activation requires the HAT activity of CBP/p300 that is tethered to the vCRE through interactions with pCREB and Tax within the p300 KIX domain. In the presence of Nap1, nucleosomes are disassembled from the promoter in an Ac-CoA dependent mechanism, creating a more permissive substrate for the recruitment of general transcription machinery, resulting in subsequent transcription activation.

Based on these observations, my thesis work sought out to identify the precise acetylation requirements for nucleosome disassembly and transcription activation. Using chromatin templates assembled from histones containing K→R mutations within the histone tails, coupled with nucleosome disassembly and in vitro transcription assays, we discovered that acetylation of only a single lysine residue on histone H3, lysine 14, was required for nucleosome disassembly and transcriptional activation (1). Furthermore, this study directly linked nucleosome disassembly with transcription activation, as chromatin templates permissive to nucleosome disassembly were permissive to transcription activation. Moreover, chromatin templates that carried a mutation in H3-K14 were refractory to nucleosome disassembly and transcription activation.

In considering potential mechanisms for these observations, we hypothesized that, following acetylation of H3-K14 by p300, the acetylated lysine-binding bromodomain of p300 recognizes the K14 acetylated H3 tail and leverages the nucleosome open, creating a "destabilized" nucleosome that is disassembled by the histone chaperone, Nap1. Significantly, we have found that a p300 carrying a deletion of the bromodomain is defective for transcription activation. Importantly, the transcription defect is not a consequence of a defect in promoter recruitment, or acetylation activity, as previously proposed (63). Rather, our research demonstrates that deletion of the bromodomain produces a block in transcriptional activation at a step following p300 recruitment to the promoter, and acetylation of the neighboring histone tails. These data support a possible role of the p300 bromodomain in facilitating the depletion of nucleosomes from the HTLV-1 promoter.

21

CHAPTER 2

NUCLEOSOME EVICTION AND ACTIVATED TRANSCRIPTION REQUIRE p300 ACETYLATION OF HISTONE H3 LYSINE 14[1]

2.1   SUMMARY

Histone posttranslational modifications and chromatin dynamics are inextricably linked to eukaryotic gene expression. Among the many modifications that have been characterized, histone tail acetylation is most strongly correlated with transcriptional activation. In *Metazoa*, promoters of transcriptionally active genes are generally devoid of physically repressive nucleosomes, consistent with the contemporaneous binding of the large RNA polymerase II transcription machinery. The histone acetyltransferase p300 is also detected at active gene promoters, flanked by regions of histone hyperacetylation. Although the correlation between histone tail acetylation and gene activation is firmly established, the mechanisms by which acetylation facilitates this fundamental biological process remain poorly understood. To explore the role of acetylation in nucleosome dynamics, we utilized an immobilized template carrying a natural promoter reconstituted with various combinations of wild-type and mutant histones. We find that the histone H3 N-terminal tail is indispensable for activator, p300,

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[1] Chapter two describes a study of the histone acetylation requirements for Nap1-dependent nucleosome disassembly and transcription activation. I performed the following experiments shown in this chapter: Fig. 2.4C, 2.6A and B, and Fig. 2.7A-C. This work has been published in the journal, *Proceedings of the National Academy of Sciences of the United States of America*, and is presented here exactly as it appeared in **Luebben, W.R., Sharma, N.S., Nyborg, J.K**. 2010. Nucleosome eviction and activated transcription require p300 acetylation of histone H3 lysine 14. Proc Natl Acad Sci U S A. 107(45): 19254–19259.

and acetyl-CoA-dependent nucleosome eviction mediated by the histone chaperone Nap1. Significantly, we identify H3 lysine 14 as the essential p300 acetylation substrate required for dissociation of the histone octamer from the promoter DNA. Together, a total of 11 unique mutant octamer sets corroborated these observations and revealed a striking correlation between nucleosome eviction and strong activator and acetyl-CoA-dependent transcriptional activation. These novel findings uncover an exclusive role for H3 lysine 14 acetylation in facilitating the ATP-independent and transcription-independent disassembly of promoter nucleosomes by Nap1. Furthermore, these studies directly couple nucleosome disassembly with strong, activator-dependent transcription.

2.2   INTRODUCTION

Chromatin facilitates the extraordinary compaction of genomic DNA in all eukaryotes. Nucleosomes, the basic repeating unit of chromatin, are composed of two copies each of the core histone proteins H2A, H2B, H3, and H4 with 147 bp of DNA wrapped around the histone octamer (74, 75). Nucleosomes present an intrinsic paradox for the cell: They must form stable contacts with the DNA to compact the genetic material, and they must allow local disassembly to expose the underlying DNA for the chromosomal transactions required for life. Eukaryotes have evolved multiple strategies to negotiate the inherently repressive chromatin to access their genetic material, including posttranslational modifications of the nucleosomal histones (108).

Histone modifications occur primarily on the N-terminal tails that extend beyond the nucleosome core particle. The first identified and most intensively studied histone

23

modification is acetylation (93, 109). Gene-associated histone H3 and H4 tail acetylation is one of the strongest predictors of transcriptional activity in eukaryotes (12, 109). In native chromatin, acetylation of specific lysine residues on the H3 and H4 N-terminal tails is nonrandom and highly ordered (110), a finding in agreement with the patterns of acetylation associated with multiple histone acetyltransferases (HATs) throughout eukaryotes (111-114). Histone tail acetylation has multiple effects on chromatin structure and fluidity. These include H4 acetylation-dependent relaxation of the chromatin fiber and recruitment of ATP-dependent chromatin-remodeling complexes via acetyl-lysine binding bromodomains (94, 115-119). However, the mechanism by which histone tail acetylation elicits chromatin reconfiguration and coupled transcriptional activation is unknown (12).

In recent years, numerous high profile mapping studies of nucleosomes and their modifications identified nucleosome-free regions (NFRs) at the promoters of transcriptionally active genes (86, 120-125). The core promoter must be devoid of nucleosomes to enable binding of the RNA polymerase holoenzyme complex. Several studies have correlated the formation of promoter NFRs with the acetylation of specific lysines on the H3 tail (121, 123, 124, 126). Furthermore, the binding of the essential coactivator and HAT, p300, correlates with NFRs and adjacent regions of H3 hyperacetylation (103, 121). These observations suggest an underlying functional connection between histone N-terminal tail acetylation and the disassembly of promoter-associated nucleosomes. However, the link between histone tail acetylation and NFR formation remains obscure.

Studies in our laboratory recently uncovered a biochemical pathway of activator and p300-dependent promoter nucleosome disassembly mediated by the histone chaperone Nap1 (107). Nucleosome eviction from our model retroviral promoter in vitro recapitulated activator-dependent nucleosome loss from this promoter in vivo (106). Nucleosome disassembly in vitro required purified activators, catalytically active p300, Nap1, and acetyl CoA (Ac-CoA). The four core histones served as the presumed p300 acetylation substrates. Notably, promoter nucleosome disassembly was independent of ATP, chromatin-remodeling factors, and transcription per se, but required acceptor DNA and Nap1 (107). These observations highlight a unique function for Nap1 in acetylation-dependent octamer disassembly and expand our understanding of the multiple mechanisms utilized by eukaryotes to mobilize repressive nucleosomes during gene activation.

In the present study, we explore the role of histone acetylation by p300 during nucleosome disassembly and define the interconnectivities among histone acetylation, promoter nucleosome disassembly, and transcriptional activation. We utilized an in vitro immobilized template assay to investigate activator and Ac-CoA-dependent disassembly of nucleosomes from a native promoter. We compared chromatin templates composed of a mixture of wild-type histones and histones carrying lysine to arginine (K → R) N-terminal tail mutations. This analysis identified the histone H3 tail as the essential, functionally relevant acetylation target in nucleosome eviction by Nap1. Chromatin templates bearing these point mutations were also tested in parallel in vitro transcription assays. We observed a significant correlation between activator/Ac-CoA-dependent nucleosome eviction and activator/Ac-CoA-dependent transcriptional

activation, establishing a critical functional link between H3 tail acetylation, NFR

formation, and potent activation of transcription. We next introduced specific K → R

point mutations in the H3 tail and tested chromatin bearing these mutations in both

nucleosome eviction and transcriptional activation. Lysine 14 (H3 K14) emerged as the

essential functional target of p300 acetylation. Remarkably, a single point mutation

throughout the entire nucleosome, H3 K14 → R, was sufficient to block acetylation-

dependent nucleosome disassembly. Likewise, chromatin reconstituted with the

reciprocal octamer (WT H3 K14, with K → R tail mutations throughout the remainder of

the nucleosome), supported acetylation-dependent nucleosome eviction comparable to

that of wild-type chromatin. These data identify H3 K14 acetylation as the pivotal event

in Nap1-mediated promoter nucleosome disassembly. Together, these unique findings

provide a molecular mechanism linking histone acetylation with gene activation and thus

significantly advance our understanding of the biological outcome of this critical

epigenetic modification.

2.3   RESULTS

2.3a   p300 TARGETS THE HISTONE AMINO-TERMINAL TAILS FOR NUCLEOSOME DISASSEMBLY

To identify the specific histone targets of acetylation by p300, we used a

chromatin-based immobilized template assay comprised of the model human T-cell

leukemia virus (HTLV-1) promoter linked to a G-less cassette. This system has been

previously described (107) and is illustrated in Figure 2.1. The natural HTLV-1 promoter
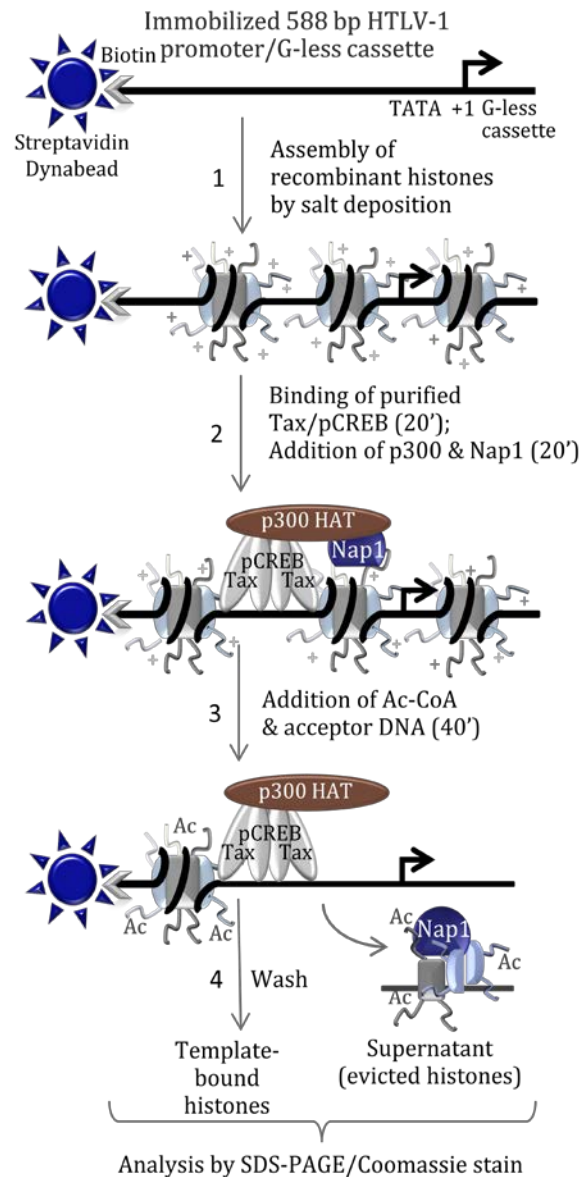
Figure 2.1. **Immobilized template/nucleosome disassembly assay.** The schematic shows the 588-bp immobilized template carrying the natural T-cell leukemia virus (HTLV-1) model promoter linked to a downstream G-less cassette. The numbers denote specific steps in the reaction. The HTLV-1 promoter carries three viral cAMP response elements, or vCREs, which serve as binding sites for the Tax/pCREB complex. These elements are located at approximately -100, -200 and -250 bp upstream of the transcription start site. Chromatin is reconstituted on the 588-bp immobilized promoter template by the method of salt deposition, at a histone:DNA ratio that produces an average density of three nucleosomes/template (step 1). The nucleosomes assemble randomly on the template, as the native HTLV-1 promoter does not carry intrinsic nucleosome positioning sequences. Following chromatin assembly, highly purified Tax and pCREB are incubated with the template, followed by the addition of purified, full-length p300 and human Nap1 (step 2). The DNA-bound Tax/pCREB complex serves as a high-affinity binding site for p300, which is recruited via interaction between the activators and the KIX domain of p300. The addition of acetyl CoA and acceptor DNA initiates nucleosome disassembly (step 3). At the completion of the reaction, the immobilized promoter is magnetically isolated, the supernatant removed, and the template washed and resuspended in SDS sample dyes (step 4). Both the template-bound and supernatant fractions are analyzed by SDS-PAGE and the histones visualized by Coomassie staining. For the in vitro transcription assays, the same protocol is followed through step 2, except that nuclear extract (+/- Ac-CoA) is added in place of Nap1 and p300 (which are supplied by the human T-cell nuclear extract). Following preinitiation complex formation, nucleoside triphosphates are added to initiate RNA synthesis and the transcripts are analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

27

carries three highly conserved 21-bp enhancer elements, called viral CREs (vCREs), located at -100, -200, and -250 bp upstream of the transcriptional start site (see ref. (127)). The vCREs serve as binding sites for the virally encoded transcriptional activator protein Tax, which binds together with the phosphorylated form of the cellular transcription factor cAMP response element binding protein, or CREB (pCREB). Together, the Tax/pCREB complex recruits p300 to promote strong HTLV-1 transcriptional activation in vivo and in vitro (127).

To begin characterization of acetylation-dependent nucleosome disassembly, we first performed a HAT assay to examine the pattern of p300 acetylation on both the template-bound histones and the histones liberated into the supernatant fraction (evicted). The 588-bp HTLV-1 promoter fragment was assembled into chromatin using highly purified, recombinant *Xenopus* histones by the method of salt deposition (Fig. 2.2B). Highly purified Tax, pCREB, p300, and human Nap1L1 (hNap1) (see Fig. 2.2A) were preincubated with the chromatin template, and nucleosome eviction was assayed in the presence of [^{14}C] Ac-CoA. Fig. 2.3A shows that all four core histones were released from the immobilized promoter template in the presence of [^{14}C] Ac-CoA and that the evicted histones were highly acetylated. This observation is further corroborated in an eviction time course shown in Fig. 2.4A.

Because the histone N-terminal tails are the primary targets of p300 (112), we next sought to determine if histone tail acetylation was required for nucleosome eviction. We prepared histone octamers carrying K → R point mutations at the known in vivo targets of acetylation on all four histones (110) (Fig. 2.3B). The K → R mutations retain the positive charge at each position, but are no longer substrates for acetylation.
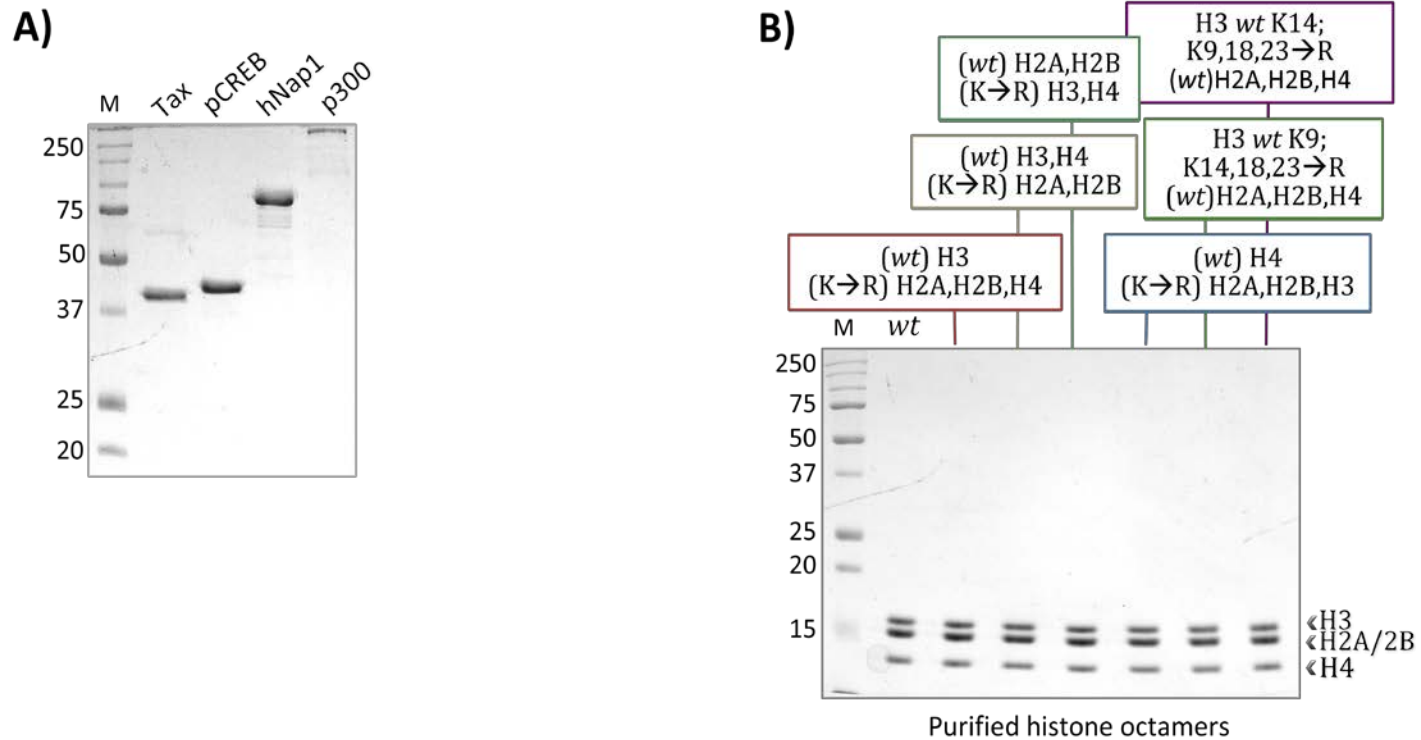
Figure 2.2. (A) Purified activators, p300, Nap1 and histone octamers used in the assays presented herein. Highly purified Tax, pCREB, p300 and human Nap1 (hNap1) were separated on a 12% SDS polyacrylamide gel and visualized by Coomassie staining. (B) Octamers composed of the various combinations of wild-type and mutant core histones were analyzed on a 12% SDS-polyacrylamide gel to confirm the purity and consistency of each octamer preparation. The individual mixed octamer sets were assembled into chromatin in the immobilized HTLV-1 promoter template and analyzed for their ability to support activator/Ac-CoA-dependent nucleosome disassembly, as indicated in each experiment.
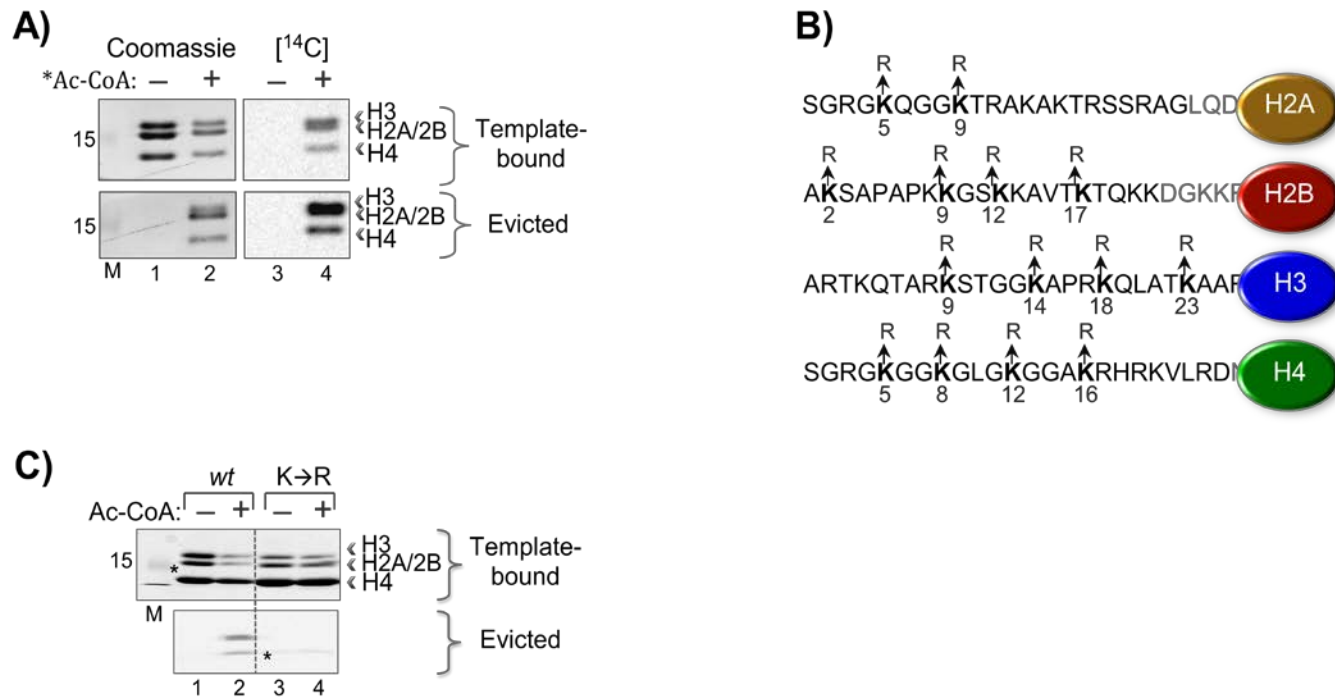
Figure 2.3. **Histone amino-terminal tail acetylation is required for nucleosome disassembly.** (A) HAT assay reveals that H3 and H4 are highly acetylated in the evicted (supernatant) fraction. The nucleosome eviction assay was performed as shown schematically in Fig. 2.1. Briefly, the promoter template was assembled with wild-type core histones, and nucleosome eviction was analyzed following the addition of highly purified Tax, pCREB, p300, hNap1, and acceptor DNA; in the absence or presence of [^{14}C] Ac-CoA. The purified proteins used in these assays are shown in Fig. 2.2A. The same gel was analyzed by Coomassie staining and PhosphorImage analysis. Molecular weight markers (M) are indicated. (B) Schematic of the four core histone N-terminal tails showing the positions of the lysine to arginine (K → R) point mutations (43). (C) Lysines in the N-terminal core histone tails are required for Ac-CoA-dependent nucleosome eviction. Chromatin was assembled with either wild-type or K → R histone octamers. Nucleosome disassembly was assayed as described in *A* and Fig. 2.1 (using nonradiolabeled Ac-CoA). Dashed lines demarcate the lanes. Asterisk denotes contaminating streptavidin (from the Dynabeads®) that comigrates with H4.

30
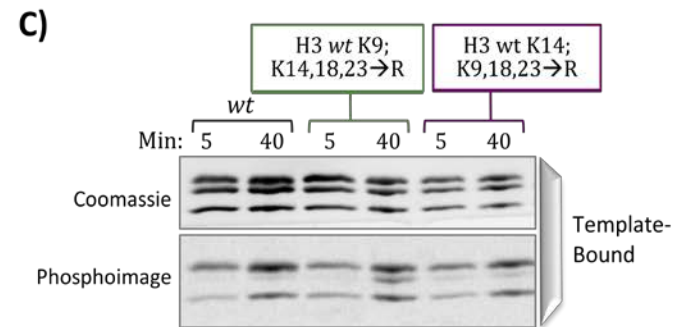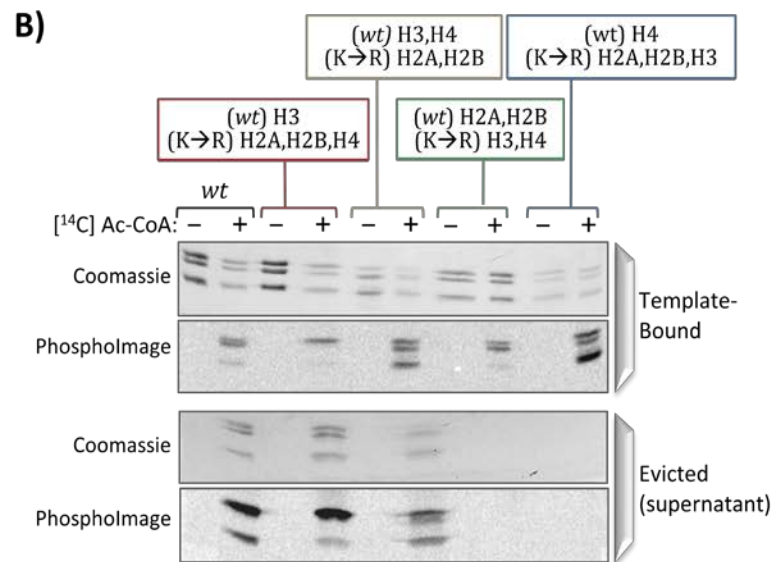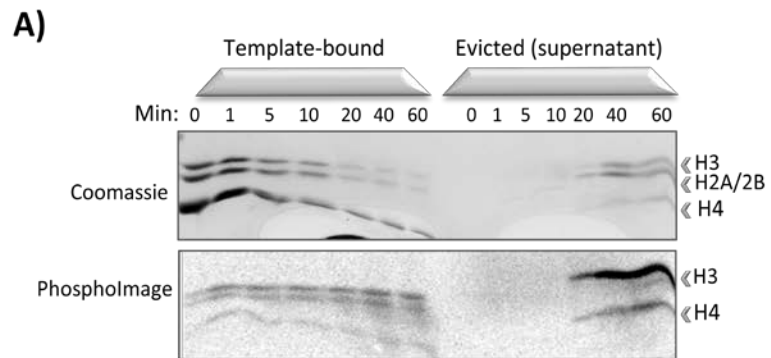
Figure 2.4. **Histone acetyltransferase assays.** (A) Time course HAT assay indicates that H3 and H4 are the primary p300 acetylation targets in the nucleosome eviction assay. Experiment performed as described in Fig. 2.3A and Fig. 2.1 using wild-type core histones and 14C-labeled acetyl-CoA in place of unlabeled acetyl CoA. The template-bound and evicted histones were separated by 12% SDS-PAGE and visualized by Coomassie staining. The gel was dried and histone acetylation was visualized by PhosphorImager analysis. Minutes following [14C] Ac-CoA addition are indicated. (B) To accompany the nucleosome disassebmly experiment shown in Fig. 2.5A, a HAT assay was performed to analyze the p300 histone acetylation patterns specific to each "mixed octamer" set, carrying various K→R point mutations. The nucleosome disassembly assay was performed as decribed for the experiment shown in Fig. 2.5A, except [14C] Ac-CoA was used in place of unlabeled Ac-CoA. (C) To accompany the nucleosome eviction assay shown in Fig. 2.6B, we performed a HAT assay to confirm p300 acetylation activity on chromatin assembled with the H3 K→R tail mutants. The HAT assay was performed on the entire reactions at 5 and 40 min following [14C] Ac-CoA addition.

31

Octamers composed of either wild-type histones, or histones carrying the K → R point

mutations, were assembled in parallel into chromatin on our model promoter template,

incubated with purified Tax, pCREB, p300, and Nap1, and assayed for nucleosome

eviction in the absence or presence of Ac-CoA. Fig. 2.3C shows that chromatin carrying

the K → R tail mutations does not support Ac-CoA-dependent nucleosome disassembly

(lanes 3 and 4), indicating that the histone N-terminal tails serve as the functionally

relevant p300 acetylation targets. From the data presented in Fig. 2.3., we conclude that

p300 acetylation of lysine residues present in one or more of the four core histone tails

is required for Nap1-mediated disassembly of promoter-associated nucleosome

2.3b   H3 N-TERMINAL TAIL LYSINES ARE REQUIRED FOR NUCLEOSOME

EVICTION AND TRANSCRIPTIONAL ACTIVATION

To identify the specific core histone tail (or tails) that functionally support

nucleosome disassembly, we prepared four unique sets of histone octamers (mixed

octamers) using combinations of wild-type histones and histones carrying the N-terminal

K → R mutations (Fig. 2.2B). Each of the mixed octamer sets were assembled into

chromatin on the immobilized HTLV-1 promoter and assayed for acetylation-dependent

nucleosome disassembly using purified components, exactly as described above.

Remarkably, these experiments revealed that the lysines present on the N-terminal tails

of H2A, H2B, and H4 were dispensable and that wild-type histone H3 alone was

sufficient for acetylation-dependent nucleosome disassembly (Fig. 2.5A). An eviction

HAT assay performed with these mixed octamers is shown in Fig. 2.4B. To further
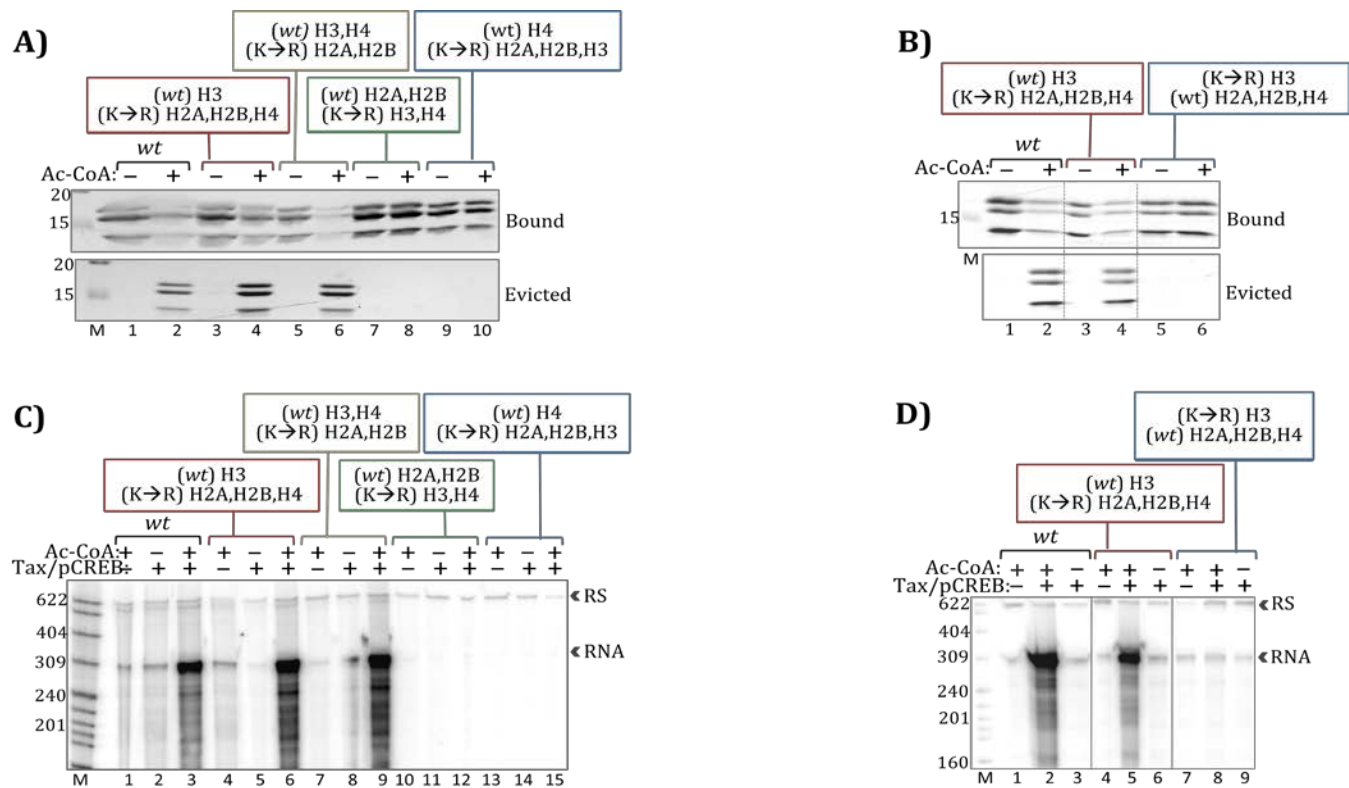
Figure 2.5. **The H3 amino-terminal tail is required for nucleosome disassembly and transcriptional activation.**
(A) Mixed octamers were prepared using combinations of wild-type histones and histones carrying K → R tail mutations (see Fig. 2.3*B*). The mixed octamers were assembled into chromatin and tested for their ability to support activator/Ac-CoA-dependent nucleosome disassembly, exactly as described in the Fig.2.3 *A* legend (and Fig. 2.1). (B) Direct comparison of chromatin templates carrying the wild-type H3 tail assembled together with K → R tail mutations in H2A, H2B, and H4, with the reciprocal combination of core histones. (C and D) Using the chromatin templates shown in *A* and *B*, in vitro transcription reactions were performed using T-cell nuclear extract in the absence or presence of activators (Tax/pCREB) and/or Ac-CoA. The transcript (RNA) and recovery standard (RS) are indicated. The chromatin samples in 2C (lanes 1–9) and 2D are slightly underassembled, enabling visualization of "basal" transcription. A more detailed description of the in vitro transcription assay is provided in the legend to Fig. 2.1.

33

confirm that the H3 N-terminal tail served as the functionally relevant p300 acetylation target, we compared nucleosome eviction on chromatin composed of K → R H3, assembled with wild-type H2A, H2B, H4, in parallel with the reciprocal chromatin, wild-type H3, assembled with K → R H2A, H2B, H4. Fig. 2.5B establishes that acetylation-dependent nucleosome eviction exclusively requires lysine residues present on the H3 N-terminal tail.

The disassembly of nucleosome octamers from our model promoter fragment must, by definition, reduce nucleosome density on the template. Because the fragment is 588 bp in length and assembles ~3 nucleosomes (107), it is likely that the core promoter is nucleosome-free on a significant fraction of the templates. We therefore hypothesized that chromatin templates permissive to nucleosome eviction should serve as excellent substrates for preinitiation complex formation and activated transcription.

Similarly, templates reconstituted with chromatin carrying K → R point mutations that block nucleosome eviction should also block activated transcription. As such, the chromatin templates used in Fig. 2.5A and B provided us with powerful tools to directly test whether acetylation-dependent nucleosome disassembly is a prerequisite for transcriptional activation. Of note, we previously established that transcription from our chromatin-assembled model HTLV-1 promoter is strongly dependent upon Tax/CREB, p300, and Ac-CoA (72, 104, 107). To test transcriptional activation from the same wild-type and K → R mutant chromatin templates described in Fig. 2.5 A and B above, we performed in vitro transcription assays in the absence or presence of activators (Tax/pCREB) and/or Ac-CoA. The experiments were carried out similar to the protocol shown in Fig. 2.1, except that following Tax/pCREB binding, nuclear extract was

incubated with the templates to enable preinitiation complex formation. The T-cell

nuclear extract supplies the RNA polymerase holoenzyme complex, p300, and

nucleosome disassembly activity indistinguishable from Nap1 (107). Remarkably, we

observe a precise correlation between activator/Ac-CoA-dependent nucleosome

eviction and strong activator/Ac-CoA-dependent transcriptional activation (Fig. 2.5).

Specifically, the three octamers carrying wild-type H3 supported both eviction and

transcription, whereas the three octamers carrying K → R mutations in the H3 tail failed

to support eviction and transcription (Fig. 2.5 C and D; summarized in Table 2.1).

Together, the findings presented in Fig. 2.5 and Table 2.1 demonstrate that the histone

H3 tail alone is sufficient for acetylation-dependent nucleosome disassembly and

transcriptional activation and implicate one (or more) of the H3 tail lysine residues as

the functionally relevant p300 acetylation target (e.g., K9, K14, K18, and/or K23).

Further, the precise correspondence between nucleosome eviction and transcriptional

activation directly links these two fundamental biological processes (Table 2.1).

2.3c   LYSINE 14 ON THE H3 N-TERMINAL TAIL IS NECESSARY AND SUFFICIENT FOR NUCLEOSOME EVICTION AND TRANSCRIPTIONAL ACTIVATION

Based on the data presented above, we next focused on identifying the specific

H3 N-terminal tail lysine(s) responsible for supporting acetylation-dependent

nucleosome eviction. We first prepared two H3 tail mutants carrying wild-type K9 (WT

K9) or wild-type K14 (WT K14), each against a background of K → R mutations at the

other three positions in the H3 tail (Fig. 2.6A). Octamers composed of H3 tail mutants,

together with wild-type core histones H2A, H2B, and H4 (Fig. 2.2B), were assembled
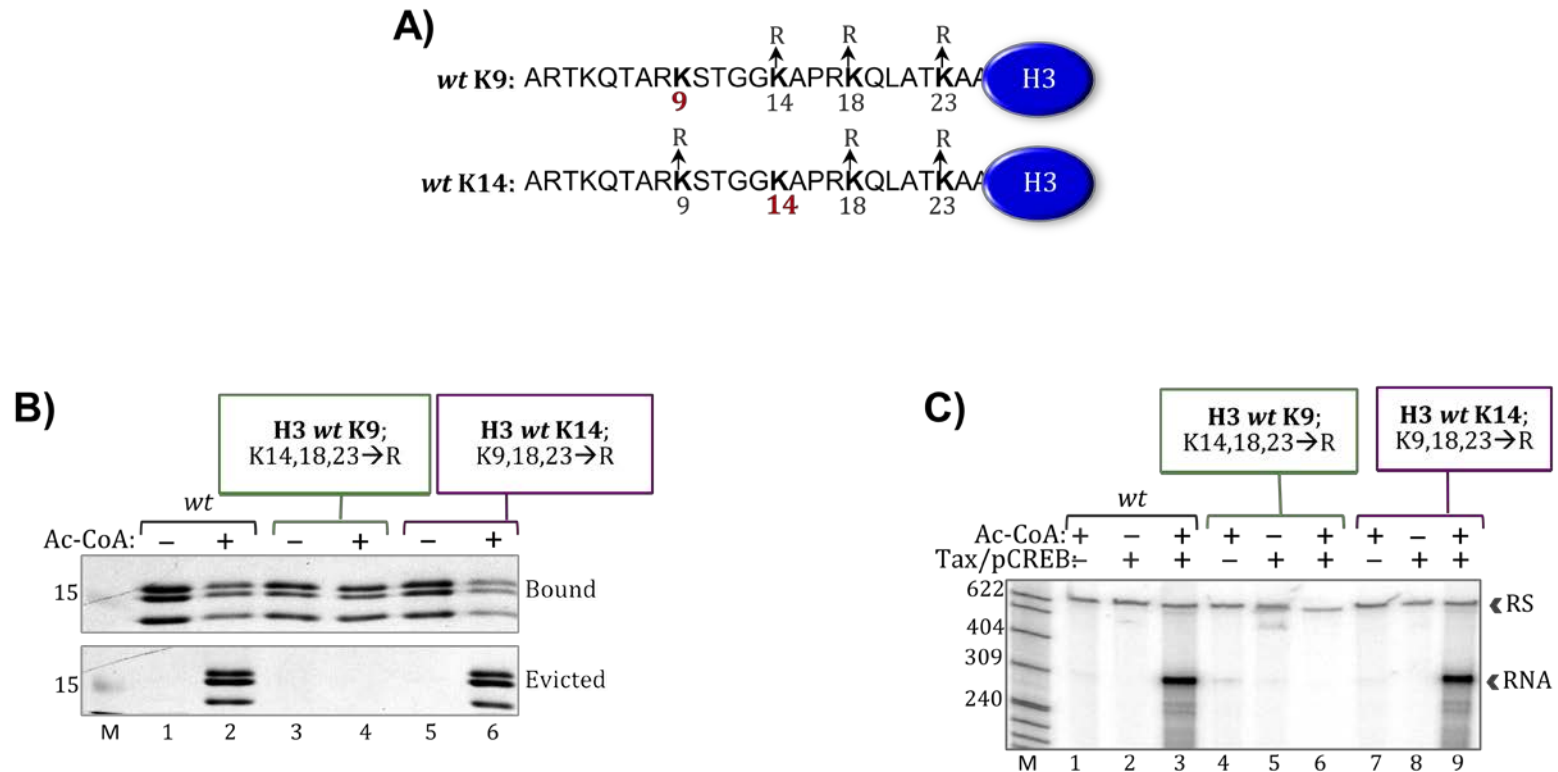
Figure 2.6. **Point mutations in the H3 tail implicate K14, and not K9, as the functional target of acetylation in activator-dependent nucleosome disassembly and transcriptional activation.** (A) Schematic of the H3 tail showing the K → R point mutations. (B and C) The histone H3 tail mutants were reconstituted with wild-type core histones H2A, H2B, and H4. The chromatin templates were assayed for nucleosome disassembly and transcriptional activation as described above.

into chromatin and tested for their ability to support activator-dependent and Ac-CoA-dependent nucleosome disassembly and transcriptional activation, as described in Fig. 2.5. As shown in Fig. 2.6 B and C, nucleosomes carrying wild-type H3 K9, with K $\rightarrow$ R mutations at positions 14, 18, and 23, failed to support nucleosome eviction or transcriptional activation. In marked contrast, nucleosomes carrying wild-type H3 K14, with K $\rightarrow$ R mutations at 9, 18, and 23, supported both eviction and transcription, comparable to that observed with wild-type chromatin. We verified K14 acetylation with this mutant by mass spectrometry. As a control, a HAT assay time course demonstrates that chromatin assembled with both of these mutants support p300 acetylation throughout the nucleosome (Fig. 2.4C). These results establish that acetylation of H3 K14 is sufficient for both Nap1-mediated nucleosome disassembly and activator-dependent in vitro transcription.

To further establish the functional relevance of K14 acetylation, we introduced a single K $\rightarrow$ R point mutation at this position in the H3 tail and reconstituted chromatin templates with WT H2A, H2B, and H4 (Fig. 2.7A). Fig. 2.7B shows that this single K14 $\rightarrow$ R point mutation within the nucleosome blocks acetylation-dependent nucleosome eviction. We next prepared chromatin carrying K $\rightarrow$ R point mutations at every major N-terminal tail lysine residue in the nucleosome, *except* position H3 K14. Fig. 2.7C shows that wild-type H3 K14, present as the only major acetylation site, supports disassembly of the nucleosome octamer from the promoter DNA. These experiments establish that H3 K14 is both necessary and sufficient for activator, p300, and Ac-CoA-dependent promoter nucleosome disassembly by Nap1.
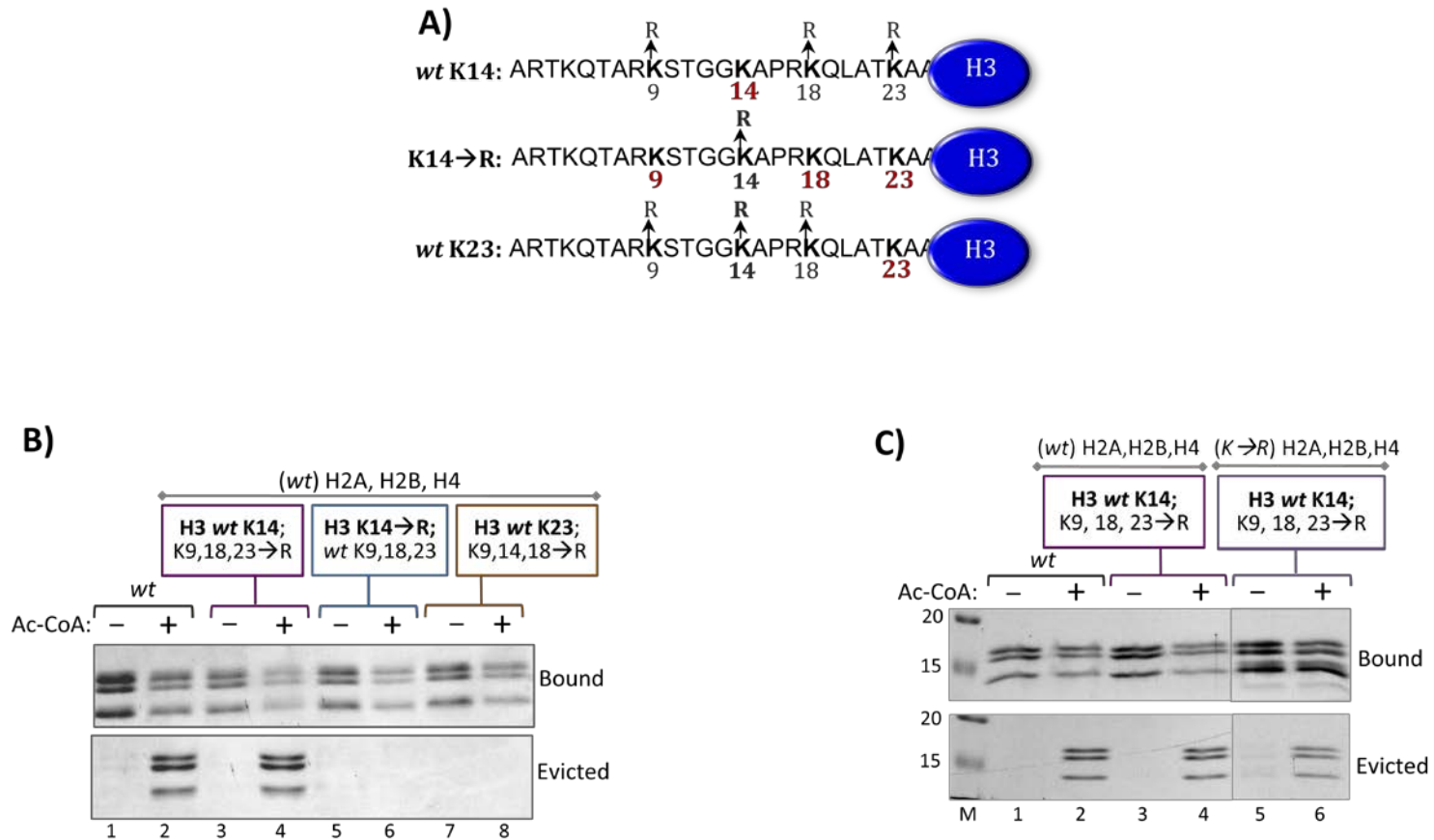
Figure 2.7. **H3 K14 is necessary and sufficient for acetylation-dependent nucleosome disassembly.** (A) Schematic of the H3 tail showing the K → R point mutations. Relevant wild-type lysines are shown in red. (B) Each of the indicated H3 tail mutant was reconstituted with wild-type H2A, H2B, and H4 and tested in activator/Ac-CoA-dependent nucleosome eviction assays. (C) The "WT K14" histone H3 tail mutant (K9, 18, 23 → R) was reconstituted with core histones carrying K → R mutations throughout the H2A, H2B, and H4 N-terminal tails (see Fig. 2.2B). Importantly, this chromatin (lanes 5 and 6) carries H3 K14 as the *only major site of acetylation* throughout the nucleosome. This chromatin was tested in parallel with H3 WT K14 reconstituted with wild type H2A, H2B, and H4 (from *4B*, lanes 3 and 4) in activator/Ac-CoA-dependent nucleosome eviction.

2.4   DISCUSSION

We provide compelling evidence that p300 acetylation of histone H3 lysine 14 is essential for activator-dependent promoter nucleosome disassembly by the histone chaperone Nap1. Histone H3 K14 is also required for robust activator and acetyl-CoA-dependent transcription in vitro. Remarkably, introduction of only a single arginine point mutation at position H3 K14 was sufficient to block promoter nucleosome disassembly, demonstrating that acetylation at any other lysine throughout the nucleosome cannot support eviction. In the reciprocal experiment, chromatin carrying wild-type H3 K14, with K $\rightarrow$ R tail mutations at every major acetylation target throughout the nucleosome, fully supported acetylation-dependent nucleosome eviction. These data convincingly demonstrate that H3 K14 is a highly specific, nonredundant acetylation substrate that serves an essential role in nucleosome disassembly. As summarized in Table 2.1, a total of 13 unique octamer sets were reconstituted into chromatin on our model promoter template and tested for their ability to support activator (Tax/pCREB) and Ac-CoA-dependent nucleosome disassembly by Nap1. Eight of these chromatin templates were tested in parallel in activator and Ac-CoA-dependent in vitro transcription assays. Together, these studies revealed a compelling correlation between acetylation-dependent nucleosome eviction and activator-dependent transcriptional activation and establish H3 K14 as the single functionally relevant p300 target required for Nap1-mediated nucleosome eviction. These previously undescribed findings provide evidence demonstrating a direct role for the histone chaperone Nap1 in the disassembly of the nucleosome octamer following a highly specific acetylation event

Table 2.1. **Summary of the histone tail mutants, and their effect on acetylation-dependent nucleosome disassembly and transcriptional activation**

Octamer	Eviction?	Transcription?
(wt) H2A, H2B, H3, and H4	Yes	Yes
K→R H2A, H2B, H3, and H4	No	n.d.
K→Q H2A, H2B, H3, and H4	No	n.d.
Mixed Histone Octamers		
(K→R) H3, H4 / (*wt*) H2A, H2B	No	No
(K→R) H2A, H2B / (*wt*) H3, H4	Yes	Yes
(K→R) H2A, H2B, H3 / (*wt*) H4	No	No
(K→R) H2A, H2B, H4 / (*wt*) H3	Yes	Yes
(K→R) H3 / (*wt*) H2A, H2B, H4	No	No
Histone H3 Tail Mutations		
wt **K9**; K14,18,23→R (*wt*) H2A, H2B, and H4	No	No
wt **K14**; K9, 18, 23→R (*wt*) H2A, H2B, and H4	Yes	Yes
wt **K14**; K9,18,23→R (*K→R*) H2A, H2B, and H4 tails	Yes	n.d.
K14→R; *wt* K9, 18, 23 (*wt*) H2A, H2B, and H4	No	n.d.
wt **K23**; K9, 14, 18→R (*wt*) H2A, H2B, and H4	No	n.d.

n.d. Not determined.

All eviction results are activator (Tax/pCREB), p300, Nap1, acceptor DNA, and Ac-CoA dependent. Eviction reactions were scored based on the detection of histone proteins in the supernatant fraction, as described in the Methods section. The in vitro transcription reactions were scored based on the strong activation observed in the presence of Tax/pCREB and Ac-CoA. These data summarize the results presented herein, and performed at least three times with highly reproducible results.

(for review, see ref. (128)). Further, these studies demonstrate a direct functional linkage between site-specific histone acetylation, promoter nucleosome disassembly by Nap1, and gene activation. A model incorporating these sequential steps in the conversion of a silent gene to a transcriptionally active gene is shown in Fig. 2.8.

Identifying an exclusive role for the H3 tail in acetylation-dependent nucleosome eviction was unexpected, as both the H3 and H4 tails are well-established acetylation targets in nucleosome dynamics and gene activation (12, 109, 128). The H4 tail has been shown to mediate internucleosomal interactions and stabilize higher-order chromatin structures (94, 96, 129, 130). Specifically, acetylation of H4 K16 facilitates relaxation of the chromatin fiber, creating a template more permissive to gene activation (94, 116, 129). The natural promoter fragment used in our assays accommodates up to three randomly positioned nucleosomes, and immobilization of this fragment on the streptavidin-coated magnetic bead enforces a physical distance between the templates. As such, the chromatin used in our model system is unlikely to undergo extensive intra- and/or interfiber interactions, and the requirement for H4 tail acetylation is therefore bypassed. Taken together, our findings reveal the functional uncoupling of these two critical, yet mechanistically distinct, histone acetylation pathways. Importantly, the segregation of these two pathways enabled identification of H3 K14 as the precise acetylation target required for disassembly of the histone octamer from the DNA.

Based on chromatin modification studies that span decades, two distinct, yet mutually compatible models have emerged to consider the outcome of histone acetylation on chromatin dynamics (for review, see ref. (128)). In the first model, histone tail acetylation induces changes in the physical properties of the nucleosome that

41
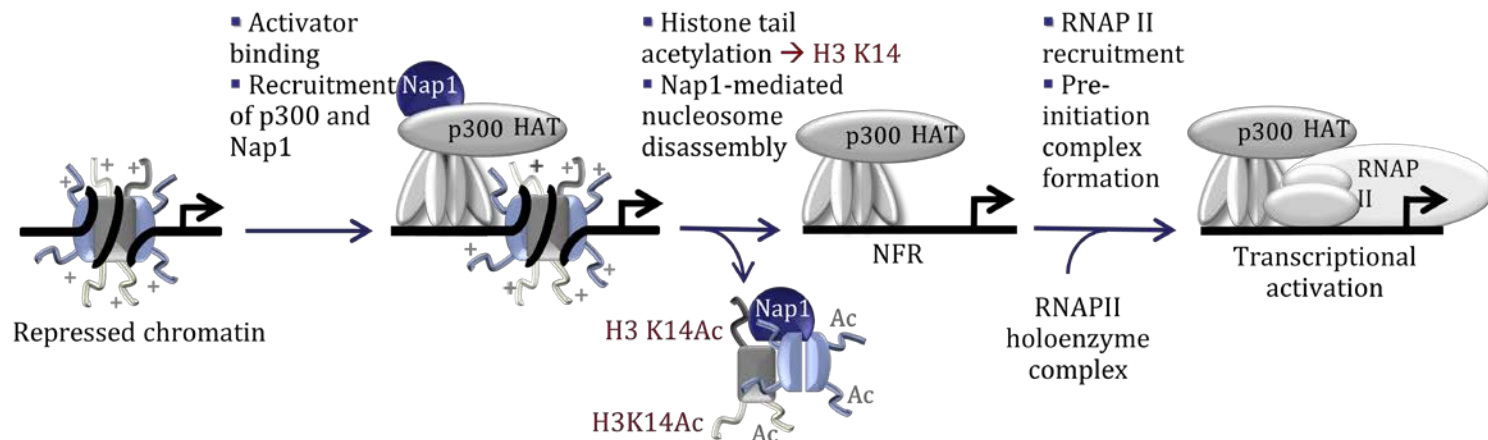
Figure 2.8. **Model showing the sequential events in acetylation-dependent nucleosome disassembly.** p300 is recruited to the promoter via interaction with activators, whereupon it acetylates the histone tails of the promoter-associated nucleosomes. Following H3 K14 acetylation, Nap1, via interaction with p300 (37, 38) mediates disassembly of the nucleosome octamer from the promoter DNA, producing a nucleosome-free region (NFR) that is permissive to the assembly of the general transcription machinery.

reduce inter- or intranucleosomal contacts, producing a more relaxed chromatin fiber. In the second model, acetylated lysines serve as recognition elements to mediate recruitment of chromatin modifying proteins via bromodomains. Our studies described herein are inconsistent with the first model, as the acetylation of a single lysine—at a very specific position within the nucleosome—largely negates a role for charge neutralization and/or reduced nucleosome stability. The specific identification of H3 K14, however, resonates with numerous previous studies that may provide clues to the mechanism of disassembly. Analysis of the acetylation patterns of native histones revealed that H3 K14 acetylation is very specific and ordered and is one of the lysine positions found to be highly acetylated in vivo (110). Consistent with these observations are the ubiquitous findings that H3 lysine 14 is the primary or major acetylation target of multiple HATs. These HATs are expressed throughout eukaryotes and include purified Gcn5, the Spt-Ada-Gcn5 acetyltransferase (SAGA), Ada2, and NuA3 complexes, CBP/p300-associated factor (PCAF), CREB binding protein (CBP), and p300 (111-114). The highly specific targeting of H3 K14 by multiple HATs provides strong evidence for a significant biological outcome associated with acetylation at this position. Of note, the ATP-dependent, bromodomain-containing, yeast chromatin-remodeling complex RSC (chromatin structure remodeling complex) has been shown to specifically bind H3 K14Ac, but not H3 K9Ac (or H3 K14 → Q) (117). In our system, however, the p300 bromodomain does not participate in recruitment of the coactivator to the promoter, because the chromatin template is assembled with unacetylated histones, and p300 is brought to the promoter via KIX domain interaction with DNA-bound Tax/pCREB (48, 127). Furthermore, we show herein that nucleosomes carrying K → R mutations

43

throughout the four core histone tails—except H3 K14—support eviction (see Fig. 2.7C). Therefore, additional acetyl-lysine interactions are unavailable to the p300 bromodomain during K14 acetylation. We cannot exclude, however, a role for a bromodomain/K14Ac interaction during disassembly of the octamer by Nap1. In support of this model, previous studies have shown an interaction between Nap1 and CBP/p300 in vivo and in vitro (131, 132). Determining the detailed mechanism by which the Nap1/p300 complex facilitates eviction of the H3 K14 acetylated nucleosomes is of significant interest.

In summary, the downstream effects of histone N-terminal tail acetylation have been known for decades, yet defining the precise mechanism(s) by which histone acetylation promotes gene expression has been elusive. We demonstrate here that p300 acetylation of H3 K14 is both necessary and sufficient for Nap1-mediated disassembly of the histone octamer from the promoter DNA, and concomitant activator and Ac-CoA-dependent transcription in vitro. These unique findings link a specific histone tail acetylation event, NFR formation, and transcriptional activation in vitro. As such, they provide a molecular framework to interpret specific histone acetylation events in the context of NFRs and gene activation in vivo. Although our studies establish a direct connection between p300 acetylation of H3 K14 and histone chaperone mediated nucleosome disassembly, it is likely that this mechanism works in concert with additional posttranslational histone modifications and chromatin effector proteins to fine-tune the transcriptional response of a gene appropriate for a specific biological outcome.

2.5 MATERIAL AND METHODS

2.5a PROTEIN EXPRESSION AND PURIFICATION

pCREB (untagged) (133), Tax-His$_{6\,(134)}$, human Nap1 (GST-hNap1L1) (135), and His$_6$-p300 were purified using the methods described in their accompanying references (see also ref. (107)). *Xenopus* histones carrying the indicated H3 point mutations were prepared by site-directed mutagenesis and confirmed by sequence analysis. Histone proteins were expressed and purified as previously described (136). All the purified proteins are shown in Fig 2.2A and B. The GST-hNap1 expression plasmid was a gift from M. Giacca (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy) and G. Steger (Institute of Virology, University of Cologne, Cologne Germany) (135), and the plasmids encoding the four core histone K → R point mutants (shown in Fig. 2.2B) were a gift from R. Roeder (Rockefeller University, New York, NY) (137).

2.5b CHROMATIN ASSEMBLY, NUCLEOSOME DISASSEMBLY, IN VITRO TRANSCRIPTION ASSAYS

A 588-bp fragment carrying the complete HTLV-1 promoter linked to a 290-bp G-less cassette was prepared by PCR, incorporating a biotin group at the upstream end of the fragment and immobilized on streptavidin-coated magnetic Dynabeads (see refs.(107) and (48)). The PCR fragment was assembled into chromatin by salt deposition, as previously described (107). Nucleosome disassembly (see schematic, Fig. 2.1) and in vitro transcription assays were performed as described

previously (48, 107). Although not indicated in each figure, all nucleosome eviction

reactions were performed with highly purified Tax, pCREB, p300, hNap1, and acceptor

DNA.

CHAPTER THREE

TROUBLESHOOTING NAP1-DEPENDENT NUCLEOSOME DISASSEMBLY AND Ac-CoA-DEPENDENT TRANSCRIPTION ACTIVATION[2]

3.1   RESULTS AND DISCUSSION

3.1a   NAP1-DEPENDENT NUCLEOSOME DISASSEMBLY TROUBLESHOOTING EFFORTS

The Nap1-dependent nucleosome disassembly assay is complicated due to the many components of the reaction including the chromatin substrate, recombinant proteins pCREB, Tax, p300 and Nap1, reaction buffer, Ac-CoA, and competitor DNA. Table 3.1 summarizes the troubleshooting efforts put forth by myself and my colleagues from April 2010-January 2013 to troubleshoot the Nap1-dependent nucleosome disassembly assay.

The biotinylated promoter fragment used in the nucleosome disassembly assay is made using PCR. The PCR reaction was traditionally purified using phenol/chloroform extraction followed by ethanol precipitation to rid of NTPs and polymerase. We hypothesized that the purification method being used to purify the 588 bp DNA was inhibiting Nap1-dependent nucleosome disassembly. This hypothesis was tested by purifying the biotinylated 588 bp DNA using (1) phenol/chloroform extraction, (2) FPLC purification or (3) a centrifugal filter device and testing each in a nucleosome

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[2] Chapter three summarizes the work performed between April 2010 and January 2013 in an effort to regain the use of the Nap1-dependent nucleosome disassembly assay and Ac-CoA-dependent in vitro transcription system. The work summarized in Tables 3.1 and 3.2 was performed by myself and multiple others in the laboratory. I performed all of the experiments shown in Figure 3.1, 3.2 and 3.3.

Table 3.1. **Summary of troubleshooting efforts in obtaining Nap1-dependent nucleosome disassembly from the HTLV-1 promoter**. Experiments were performed as described in (1), with the above variations.

COMPONENT	VARIATIONS UTILIZED
Immobilized DNA	Optimization of PCR, FPLC purification vs phenol/chloroform and EtOH precipication
Reaction buffer	Excluding EDTA and including zinc, titration of $MgCl_2$ and KCl, bought all new reagents
Chromatin Assembly	Modifying salt deposition method to include BSA and/or Nap1 during assembly, ACF/Nap1 method of assembly, titration of histone:DNA
Ac-CoA	Three different lots, [14C] Ac-CoA, titration
Acceptor DNA	146 bp DNA (Widom sequence), polydA:dT, pUC19, 588 bp DNA, titration of the above
pCREB	Four different protein preparations, titration of all the preparations
Tax	Three different protein preparations, titration of all the preparations
p300	Three different protein preparations, titration of all the preparations
Nap1	Five different preparations of GST-tagged hNap1, five different preparations of His-tagged dNap1, titration of all the preparations
Nuclear Extract	Two different nuclear extract preparations, inclusion of phosphatase inhibitor during nuclear extract incubation, performed incubation period at 4°C and 30°C, titration of nuclear extract

disassembly assay. We found that the method of DNA preparation did not influence the outcome of the experiment, as visualization of histones in the supernatant was unsuccessful (data not shown).

The buffer conditions used in previous nucleosome disassembly experiments were 50 mM Tris pH 7.9, 100 mM KCl, 6.25 mM $MgCl_2$, 1 mM EDTA, 20% glycerol (v/v) and 2 mM DTT. p300 is a zinc containing protein and requires zinc to be properly folded. We hypothesized that the EDTA in the reaction buffer was chelating zinc, resulting in misfolded p300, preventing proper formation of the quaternary complex and preventing disassembly of nucleosomes by Nap1. Therefore, a modified reaction buffer was prepared, containing 10 μM Zinc and no EDTA, and then subsequently used in a Nap1-dependent nucleosome disassembly assay. Again, we were unable to visualize histones in the supernatant (data not shown). Divalent cations (particularly Mg^{2+}) have been shown to promote folding of chromatin fibers. We hypothesized that if the $MgCl_2$ or KCl concentrations in the reaction butter were not optimal, it could affect the folding state of the chromatin (i.e. beads on a string or folded/oligomerized) and therefore affect the efficiency of the Nap1-dependent nucleosome disassembly assay. To test this hypothesis, reaction buffer was made, ranging from 0-8 mM $MgCl_2$ and 50-100 mM KCl and subsequently tested in a Nap1-dependent nucleosome disassembly assay. Again, we failed to visualize histones in the supernatant (data not shown). Lastly, to avoid the possibility of contamination, new reagents were bought and new solutions made and then tested in a Nap1-dependent nucleosome disassembly assay. Again, we did not observe histones in the supernatant (data not shown).

Both the salt deposition method of chromatin assembly and the ACF/Nap1 method of chromatin assembly were used in past studies to produce chromatin templates permissive to Nap1-dependent nucleosome disassembly (1, 107). However, more recently, chromatin assembled by both of these methods failed to disassemble nucleosomes from the promoter template in a Nap1-dependent nucleosome disassembly assay (data not shown).

Past experiments permissive to both nucleosome disassembly and transcription activation typically utilized a 4:7 or 5:7 histone:DNA (mass:mass) ratio. However, due to variability in DNA and protein quantitation it can be difficult to compare histone:DNA ratios anytime a new preparation of DNA or histone octamer is used. Therefore, the appropriate histone:DNA that should be used, at least in part for in vitro transcription assays, is empirically determined by titrating the histone:DNA ratios. As such, histone:DNA ratios were titrated from 3:7 – 14:7 to account for these variabilities. However, none of the histone:DNA ratios tested supported Nap1-dependent nucleosome disassembly (data not shown).

One possibility for the Nap1-dependent nucleosome disassembly assay not working was that protein preparations had "gone bad" and become inactive. To test this hypothesis, members of the Nyborg Laboratory, as well the Protein Expression and Purification Facility, purified several preparations of each recombinant protein, summarized in Table 3.1. Again, however, histones were not evicted from the promoter template when testing and titrating each protein preparation in Nap1-dependent nucleosome disassembly (data not shown)

In the past, A CEM (HTLV-1 negative T-cell line) nuclear extract had been frequently used as the source of p300 and Nap1 in the nucleosome disassembly assay. We hypothesized that if p300 or Nap1 recombinant protein preparations were no longer active or functional, then nuclear extract would supplement the required components. As such, several different nuclear extract preparations were tested in the nucleosome disassembly assay, including one that had supported nucleosome disassembly in past experiments. These experiments were unsuccessful. We then hypothesized that a phosphatase in the nuclear extract was dephosphorylating pCREB, preventing the recruitment of p300 and therefore preventing Nap1-dependent nucleosome disassembly from the promoter template. To test this hypothesis, phosphatase inhibitors were added when using the nuclear extract in a nucleosome disassembly assay. Additionally, incubation of the pCREB/Tax bound promoter template with nuclear extract was performed at 4°C rather than 30°C to prevent possible dephosporylation. Unfortunately, these modifications did not produce a positive result.

Nap1-dependent nucleosome disassembly from the HTLV-1 promoter is acetylation-dependent. Therefore, the quality of the Ac-CoA used in the reaction is extremely vital to the success of the experiment. As such, fresh preparations of Ac-CoA were prepared and tested in Nap1-dependent nucleosome disassembly. Again, histones were undetectable in the supernatant (data not shown).

Lastly, there is a requirement for acceptor DNA for Nap1-dependent nucleosome disassembly. Numerous types of DNA function as acceptor DNA, as long as the DNA is more than 146 bp in length, suggestive of a nucleosome forming on the acceptor DNA upon nucleosome disassembly from the promoter template (unpublished data, Neelam

Sharma). Therefore, a variety of DNAs were titrated and tested during Nap1-dependent nucleosome disassembly, including 146 bp ("601" Widom sequence) DNA, pUC19 plasmid DNA, the alternating copolymer poly(dA:dT) and a non-biotinylated 588 bp promoter template. Similar to the experiments already mentioned, the appearance of evicted histones in the supernatant was not detected (data not shown).

Unfortunately, we have been unsuccessful in determining why we no longer visualize nucleosome disassembly from the HTLV-1 promoter template. It is likely that the failure to observe nucleosome disassembly is a combination of technical problems and/or reagents that have yet to be resolved. However, because we observed a strong correlation between a chromatin template being permissive to nucleosome disassembly also being permissive to Ac-CoA dependent transcription activation, we simultaneously focused research efforts on optimization of Ac-CoA dependent in vitro transcription assays.

3.1b   Ac-CoA DEPENDENT IN VITRO TRANSCRIPTION TROUBLESHOOTING EFFORTS

Concomitant with Nap1-dependent nucleosome disassembly assays not working, in vitro transcription assays were no longer Ac-CoA dependent. As such, similar approaches used to troubleshoot Nap1-dependent nucleosome disassembly were taken to optimize Ac-CoA-dependent in vitro transcription conditions and are summarized in Table 3.2.

A key component of the in vitro transcription assay is the HTLV-1 chromatin template. Historically, the laboratory had used both linear and plasmid templates for

Table 3.2. **Summary of troubleshooting efforts in obtaining Ac-CoA dependent transcription activation from the HTLV-1 promoter.** Experiments were performed as described in (1), with the above described variations. Underlined and bolded indicates the variation was successful in identifying conditions that supported Ac-CoA dependent transcription.

COMPONENT	VARIATIONS UTILIZED
DNA template	Immobilized 588 bp DNA, 4xTRE plasmid, -306 HTLV-1 plasmid, titration of the above
Histones	Titration of xenopus and human histones
Chromatin Assembly	Adding Nap1 during salt deposition method, adding Nap1 after salt deposition method, **ACF/dNap1 assembly method**
Ac-CoA	Three different lots
pCREB	Four different protein preparations, titration
Tax	Four different protein preparations of His-tagged Tax, one protein preparation of strep-tagged Tax, titration
p300	**Exogenous p300 addition**, three different preps of His-tagged p300, one preparation of strep-tagged p300
Nuclear extract	Two different nuclear extract preparations, addition of phosphatase inhibitors, incubation at 4°C vs 30°C, addition of ATP, titration

studying Tax-mediated, Ac-CoA-dependent in vitro transcription (48, 72, 104, 107, 138, 139). In addition, assembly by an ATP-dependent method as well as by a salt deposition method had supported Ac-CoA-dependent in vitro transcription (48, 72, 104, 107, 138, 139). Perhaps most importantly, in reference to the chromatin substrate, as the histone:DNA ratio is increased, basal transcription (in the absence of activator proteins) decreases, eventually becoming completely transcriptionally repressed. Compared to naked DNA, maximally activated transcription from a chromatin template is highly reliant on Ac-CoA (1, 104).

As mentioned, comparable efforts were used while troubleshooting Nap1-dependent nucleosome disassembly and Ac-CoA-dependent transcription, largely due in part because of the initial similar experimental set up, where in both experiments the chromatin substrate is preincubated with pCREB and Tax and then subsequently incubated with p300 and Nap1 (or nuclear extract in the case of in vitro transcription) and Ac-CoA. After many attempts and failed experiments using an immobilized fragment promoter template assembled into chromatin by the salt deposition method, we turned to the experimental conditions used in Georges et al and Konesky et al (104, 139).

Georges et al used a plasmid template (p4XTRE) assembled into chromatin by the ACF/Nap1 method. Therefore, we purified ACF from SF9 cells and assembled chromatin on the p4XTRE plasmid using the ACF/Nap1 method as described in (140), employing this chromatin substrate in in vitro transcription assays. Concurrently with this method, we began adding "exogenous" recombinant p300 to the assay to supplement the "endogenous" p300 supplied by the nuclear extract. Using these techniques, we

successfully observed Ac-CoA dependent transcription activation from the chromatin templates tested.

Interestingly, we found varying dependency on the addition of exogenous p300 for Ac-CoA-dependent transcription activation when comparing a plasmid chromatin template to a fragment chromatin template. Briefly, Ac-CoA dependent transcription activation is completely dependent upon exogenous p300 addition using a fragment chromatin template, whereas exogenous p300 addition enhances already Ac-CoA-dependent transcription activation from a plasmid chromatin template. These findings are discussed in more detail in Chapter 4.

Activation of transcription from in vitro assembled chromatin templates is extremely sensitive to the level at which the chromatin templates are assembled with nucleosomes. Therefore, perhaps the most important requisite for successful experimentation is an appropriate histone:DNA ratio at which basal transcription is repressed but able to become activated upon the experimental condition being tested. This is only achieved through meticulous titration of histone:DNA ratios, as variation is found in the quanitation of DNA and histone octamer and from researcher to researcher. An example of titrating the histone:DNA to find the "window" of Ac-CoA dependency is shown in Figure 3.1. Histone:DNA was titrated on two different chromatin templates, a plasmid chromatin template (Figure 3.1A), and a fragment chromatin template (Figure 3.1B). On both chromatin templates, as the histone:DNA ratio is increased (i.e. number of nucleosomes per template is increased), Ac-CoA independent transcription decreases as the Ac-CoA dependency for transcription activation increases (Figure 3.1A and B graphical representation). Significantly, using the above mentioned results,
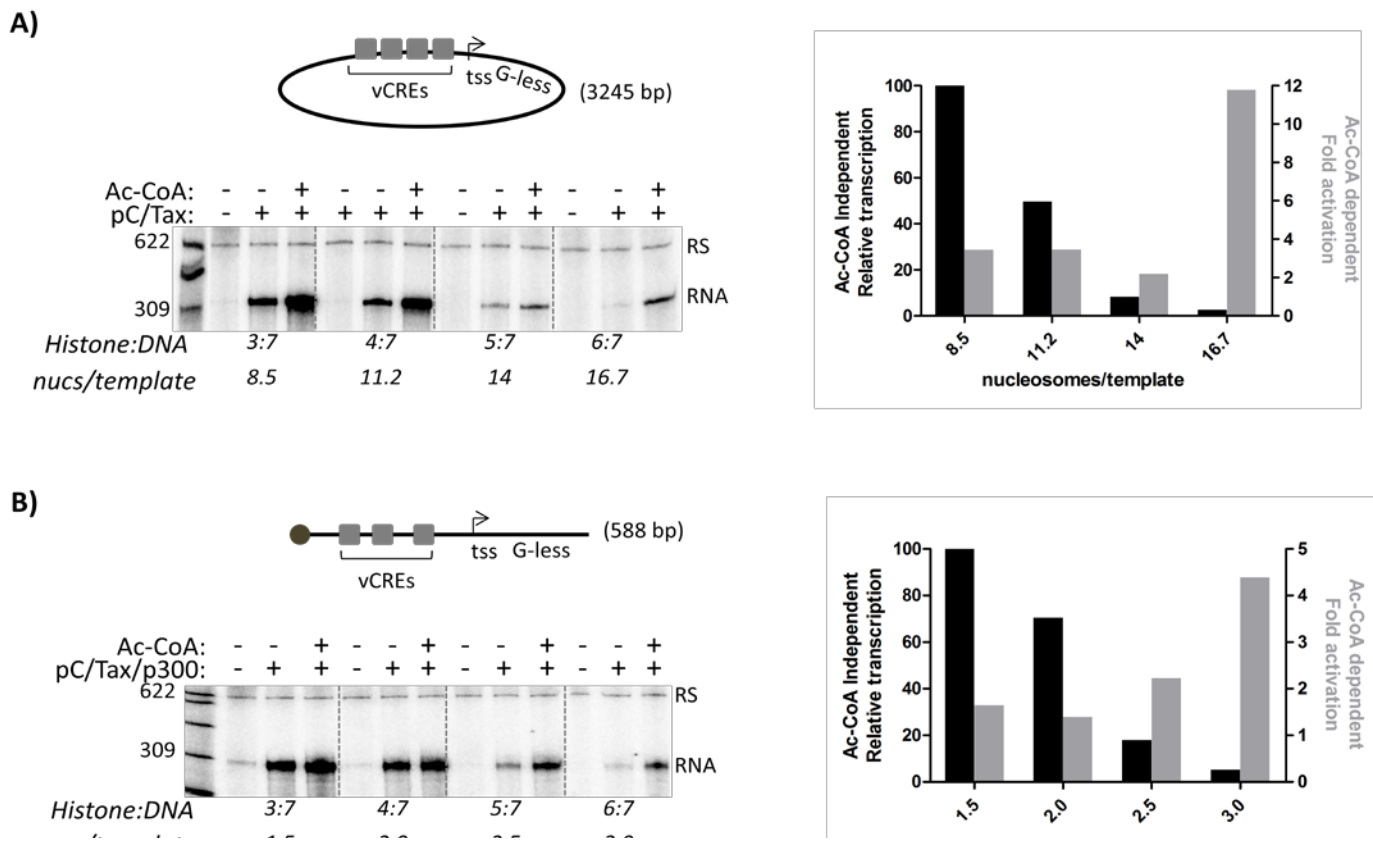
Figure 3.1. **Dissection of Ac-CoA independent activation and Ac-CoA dependent activation with increasing histone:DNA (µg:µg).** A) Plasmid chromatin template was assembled into chromatin using the ACF/Nap1 method at increasing histone:DNA as indicated. B)Fragment chromatin template was assembled into chromatin using the salt deposition method at increasing histone:DNA as indicated. Chromatin templates were incubated with the indicated recombinant proteins and Ac-CoA and tested in in vitro transcription using a CEM nuclear extract. Relative Ac-CoA independent transcription activation was determined by setting the lowest histone:DNA ratio in the absence of Ac-CoA but in the presence of activator or activator/coactivator to 100. Ac-CoA dependent fold activation was determined for each histond:DNA ratio by dividing the amount of transcript in the presence of activator and Ac-CoA by the amount of transcript in the presence of activators alone. All transcripts were normalized to the RS.

we have verified and recapitulated previous findings from the laboratory and from literature. These experiments are discussed below.

3.1c RECAPITULATION AND VERIFICATION OF PREVIOUS FINDINGS USING Ac-CoA DEPENDENT IN VITRO TRANSCRIPTION ACTIVATION CONDITIONS

Using the conditions found to support Ac-CoA dependent transcription activation (i.e addition of exogenous p300), we have confirmed previous reports of a transactivation mutant of Tax that fails to activate transcription from the HTLV-1 LTR, as well as verify a previously proposed transcriptionally defective chromatin template.

In 1990, mutational analysis of Tax by Smith and Green (141) produced 52 mutants of Tax that were subsequently used to study the transactivating properties of Tax. Of these Tax mutants, mutant number 47 (Tax M47,L319R, L320S) had severely impaired transactivation properties in vivo at the HTLV-1 LTR. As such, Tax M47 has been extensively studied and characterized by our laboratory, as well as others. *In vivo* and *in vitro* studies from our laboratory indicate that Tax M47 has decreased transcriptional activation from the HTLV-1 LTR [(106) and Sharma unpublished]. In agreement with this, we have recently shown that Tax M47 is defective for transcription activation from *in vitro* assembled chromatin templates in the presence and absence of exogenous p300 addition (Figure 3.2, lane 4 vs 8 and lane 6 vs 10).

In the studies presented in Chapter 2, we observed a strong correlation between a chromatin template being permissive to Nap1-dependent nucleosome disassembly also being permissive to Ac-CoA dependent transcription activation. From this
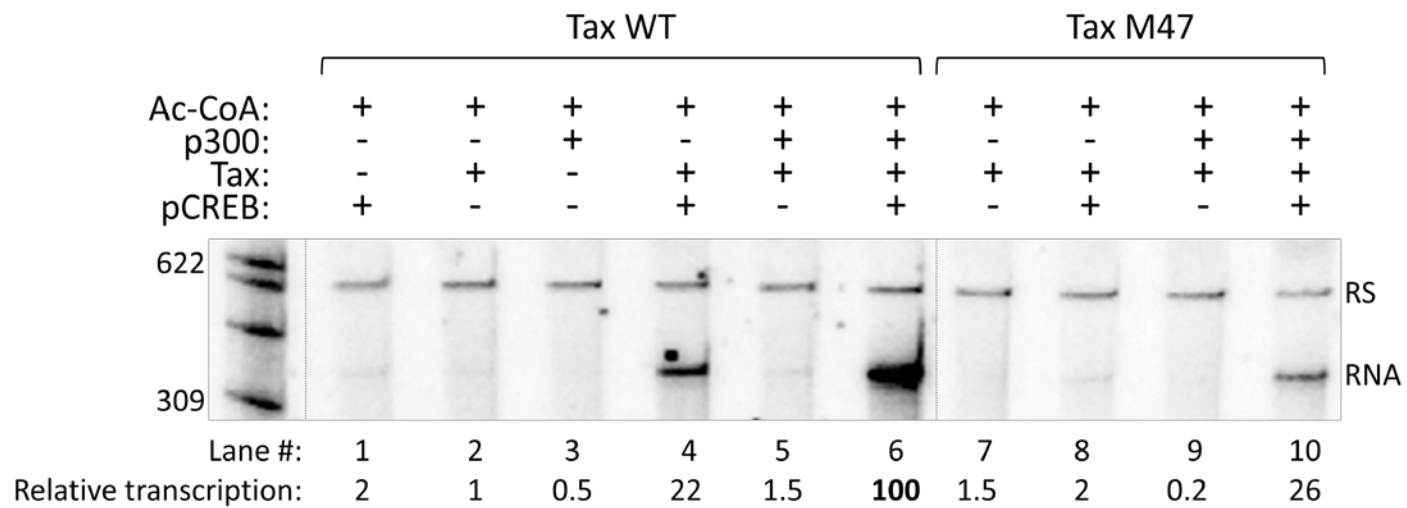
Figure 3.2. An in vitro transcription assay comparing the transactivating activity of wild-type Tax to a Tax mutant (Tax M47). Transcription reaction mixtures contained CEM nuclear extract and purified recombinant pCREB, Tax, Tax M47, p300 and Ac-CoA as indicated. The recovery standard, RNA transcript and size markers are indicated. Relative transcription was calculated by dividing the transcript with the recovery standard, with pC/Tax WT/p300/Ac-CoA transcription set to 100.

correlation, we predicted that a single mutation, histone H3 lysine 14 to arginine that

was defective for Nap1-dependent nucleosome disassembly would also be defective for

Ac-CoA dependent transcription activation. Using newly optimized Ac-CoA dependent

transcription activation conditions, we tested an immobilized chromatin template

carrying the H3K14R mutation in an in vitro transcription assay. Figure 3.3A shows that

a single mutation at H3K14R results in a decrease in transcription activation from the

HTLV-1 promoter template, compared to wild-type (lane 6 vs 12). A graphical

representation of the data from four independent experiments is shown in Figure 3.3B.

In summary, we have optimized in vitro transcription conditions for Ac-CoA

dependent activation of transcription and have used these optimized conditions to verify

previous observations related to Tax-mediated HTLV-1 transcription activation.
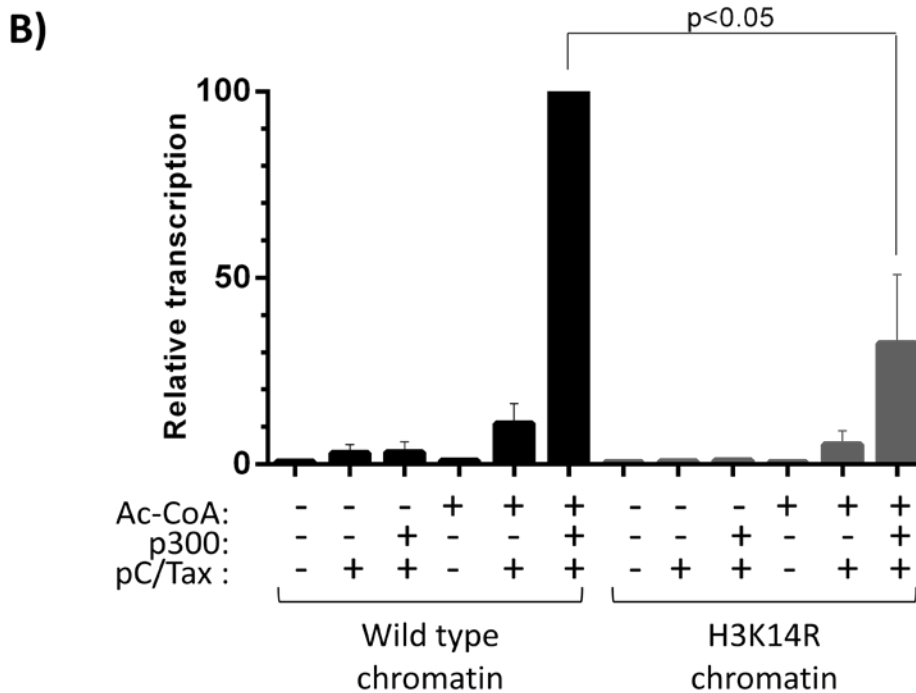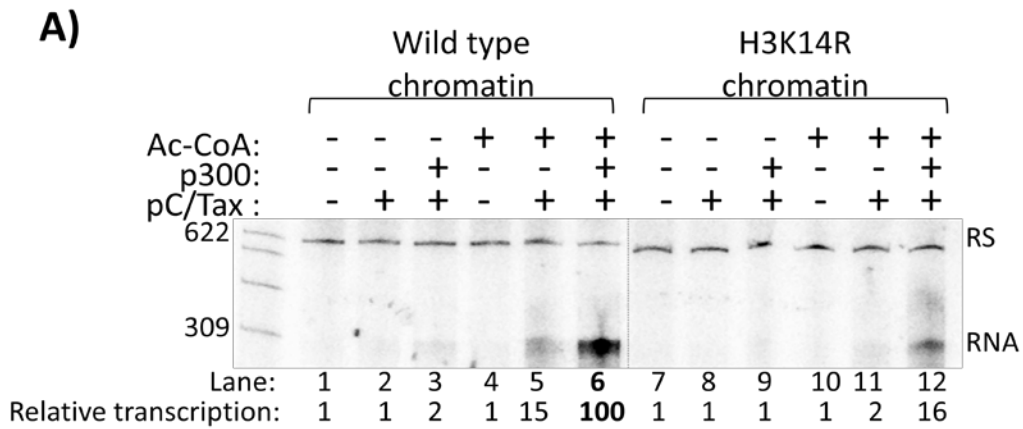
Figure 3.3. **Histone H3 lysine 14 to arginine mutation is defective for activator/coactivator/Ac-CoA dependent transcription activation from the HTLV-1 promoter *in vitro*.** A) An in vitro transcription assay comparing the activity of a wild-type chromatin template to a mutant H3K14R chromatin template. Transcription reaction mixtures contained HeLa nuclear extract and purified recombinant pCREB/Tax, pCREB/Tax/p300, pCREB/Tax/Ac-CoA, or pCREB/Tax/p300/Ac-CoA as indicated. The recovery standard, RNA transcript and size markers are indicated. Relative transcription was calculated by dividing the transcript with the recovery standard, with pC/Tax/p300/Ac-CoA activated wild-type chromatin transcription set to 100. **B)** Graphical representation of A. Data is plotted as a mean with standard deviation from four separate experiments. The p-value was determined using the repeated measures one way ANOVA test.

CHAPTER 4

A REQUIREMENT FOR THE p300 BROMODOMAIN IN HTLV-1 TRANSCRIPTION ACTIVATION[3]

4.1   SUMMARY

Transcription activation from the human T-cell leukemia virus type 1 (HTLV-1)
promoter assembled into chromatin is dependent upon the recruited histone
acetyltransferase (HAT), CBP/p300. Recruitment of CBP/p300 is mediated by the
cellular transcription factor, CREB and the virally encoded transactivating protein, Tax.
Strong transcription activation correlates with acetylation-dependent nucleosome
disassembly and mutation of a single histone lysine residue, histone H3 lysine 14
(H3K14) disrupts p300 acetylation-dependent nucleosome disassembly and subsequent
activation of transcription from the HTLV-1 promoter. These findings suggest that
nucleosome disassembly requires an acetyl-lysine "reader" protein, leading us to
hypothesize that the recruited p300 bromodomain may play a significant role in
facilitating transcriptional activation of the HTLV-1 model promoter. We used in vitro
assembled chromatin templates to assess the ability of a bromodomain deletion mutant
of p300 to activate HTLV-1 transcription. Compared to wild-type p300, a bromodomain

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[3] Chapter four describes a study of the p300 bromodomain during HTLV-1 transcription
activation. Transcription activation by a bromodomain deletion mutant of p300 was
examined in vitro on two different recombinant chromatin templates containing the
HTLV-1 promoter. A recombinant, isolated bromodomain of p300, as well as a
CBP/p300 specific small molecule inhibitor of the bromodomain, were utilized to
characterize the role of the p300 bromodomain in HTLV-1 transcription activation. I
performed the following experiments (Figure 4.1E, 4.2B and D, 4.3A-B, and 4.5A-B), Dr.
Qian Zhang performed the following experiments (Figure 4.1D and 4.2C), and an
undergraduate independent study student, Julio Flores Servin, performed the following
experiments (Figure 4.4A-B and 4.6C) under my guidance.

deletion mutant of p300 produced a dramatic reduction in HTLV-1 transcription activation. Further, the CBP/p300-specific bromodomain inhibitor, SGC-CBP30, produced a similar reduction of activation of transcription, suggesting that the p300 bromodomain is required for gene activation from this promoter. Importantly, p300 bromodomain deletion or addition of the isolated bromodomain in trans retains recruited HAT activity towards the nucleosomal template through its interaction with pCREB and Tax, demonstrating that the transcription defect is unrelated to promoter recruitment and p300 HAT activity.

4.2   INTRODUCTION

CBP/p300 are multidomain coactivator proteins involved in transcription at a large number of cellular genes. CBP/p300 coactivator function is generally thought to be exerted in two ways: **i)** through protein-protein interactions between numerous cellular transcription factors, coactivators and basal transcription machinery **ii)** and through recruited intrinsic acetyltransferase activity located in the core domain of p300 (see figure 4.2A). CBP/p300 interacts with over 400 different proteins and acetylates histones as well as non-histone substrates (58, 59, 61, 62). In addition to the above mentioned functional domains of CBP/p300, the coactivator proteins possess an acetylated lysine binding domain, called a bromodomain. The bromodomain allows for further substrate binding specificity and has been suggested to be important for recruitment of the coactivator protein to a chromatin template during transcription activation (63).

Bromodomains are found in numerous chromatin modifying enzymes and function as specific acetylated lysine binding domains. The bromodomain is a conserved structure consisting of a left-handed, four helix bundle connected with two interhelical loops (119). The loops form a hydrophobic pocket where acetyl-lysine binds. Despite bromodomain structure conservation across 61 known human bromodomain family members (142), the amino acid residues in the loops vary considerably from bromodomain to bromodomain and thus confer substrate specificity to the various bromodomain containing proteins (143). It is important to note, however, that the bromodomains of CBP and p300 have 96% sequence identity, and are therefore presumed indistinguishable in the experiments presented here.

HTLV-1 is a human retrovirus that recruits CBP/p300 for transcription activation of the integrated provirus from a repressed chromatin state. CBP/p300 is recruited to the HTLV-1 promoter through interaction with the serine 133 phosphorylated form of the cellular transcription factor CREB, and the viral transactivator protein, Tax bound at the viral enhancer elements (Figure 4.1A). At the HTLV-1 promoter, CBP/p300 is thought to primarily activate transcription through acetylation of histone tails. This is supported by the observation that inhibition of p300 HAT function using a p300 specific inhibitor, Lys-CoA, resulted in decreased transcription activation from in vitro assembled chromatin templates (104). In addition, p300 mediated HTLV-1 transcription activation requires recruited p300 HAT activity through the KIX domain of p300, as demonstrated by a reduction in transcription activation when recruitment through the KIX domain is blocked (72). More recent data has indicated that p300 HAT activity is also involved in the removal of HTLV-1 promoter nucleosomes *in vivo* and *in vitro* (106, 107), and that

acetylation of a single lysine residue on the H3 tail, lysine 14 (H3K14), is required for nucleosome disassembly and transcription activation (1) (unpublished data, see Chapter 3 Figure 3.3).

We initially became interested in the p300 bromodomain as a result of our most recent finding that nucleosome disassembly and Ac-CoA dependent transcriptional activation from the HTLV-1 promoter required the acetylation of only one residue, histone H3, lysine 14. This was unexpected, as we predicted that an accumulation of acetylation marks on the histone tails known to have a destabilizing effect on chromatin templates (115, 116, 144), rather than the acetylation of a single lysine residue, would have a greater effect on transcription activation. The requirement of a specific singly acetylated lysine residue suggests that the mechanism of acetylation for the promotion of gene activation in this particular context is through the recognition of H3K14ac by a bromodomain containing protein. We therefore hypothesized that the recognition of H3K14ac by the p300 bromodomain was involved in nucleosome disassembly and transcription activation. Using in vitro transcription, immobilized template assays, and HAT assays, we have found that the p300 bromodomain is required for Ac-CoA and p300-dependent transcription activation. Importantly, the requirement of the bromodomain for transcription activation is not related to the initial recruitment of the coactivator to the promoter chromatin template, but instead is related to a transcription activation event that is downstream of p300 recruitment to the promoter. These results are consistent with the p300 bromodomain functioning in H3K14ac recognition, leading to nucleosome disassembly and subsequent transcription activation.

4.3   RESULTS

4.3a   ADDITION OF EXOGENOUS p300 ACTIVATES TRANSCRIPTION FROM VARIOUS CHROMATIN TEMPLATES

Previous studies that utilized in vitro reconstituted chromatin templates to study Tax-dependent HTLV-1 transcription activation have shown varying dependence on the addition of exogenous p300 for transcription activation, as compared to recruitment of "endogenous" p300 provided by the nuclear extract (1, 48, 72, 104, 105). We were interested in the transcriptional activation ability of a p300 bromodomain deletion mutant and therefore initially tested the transcriptional activity of pCREB/Tax-mediated transcription activation in the presence and absence of exogenous wild-type p300. We tested both a linear fragment template and a circular plasmid template containing the HTLV-1 promoter assembled into chromatin using the salt deposition method and ACF/Nap1 assembly method (140) , respectively (Figure 4.1A). In vitro transcription assays were performed as shown in Figure 4.1C. We found that the different chromatin templates display a varying level of dependence on the addition of exogenous p300 for Tax-mediated HTLV-1 transcription activation. Under our experimentally optimized conditions, Tax-mediated transcription activation from a linear fragment assembled into chromatin is completely dependent upon the addition of exogenous p300 (Fig 4.1D, lane 5). In contrast, Tax-mediated transcription activation from a plasmid template is Ac-CoA dependent and is enhanced upon the addition of exogenous p300 (Fig 4.1E, lane 5). In summary, our in vitro transcription system is sensitive to the addition of exogenous p300, allowing for a more tailored system to study the function of the p300 bromodomain in promoting HTLV-1 transcription activation.
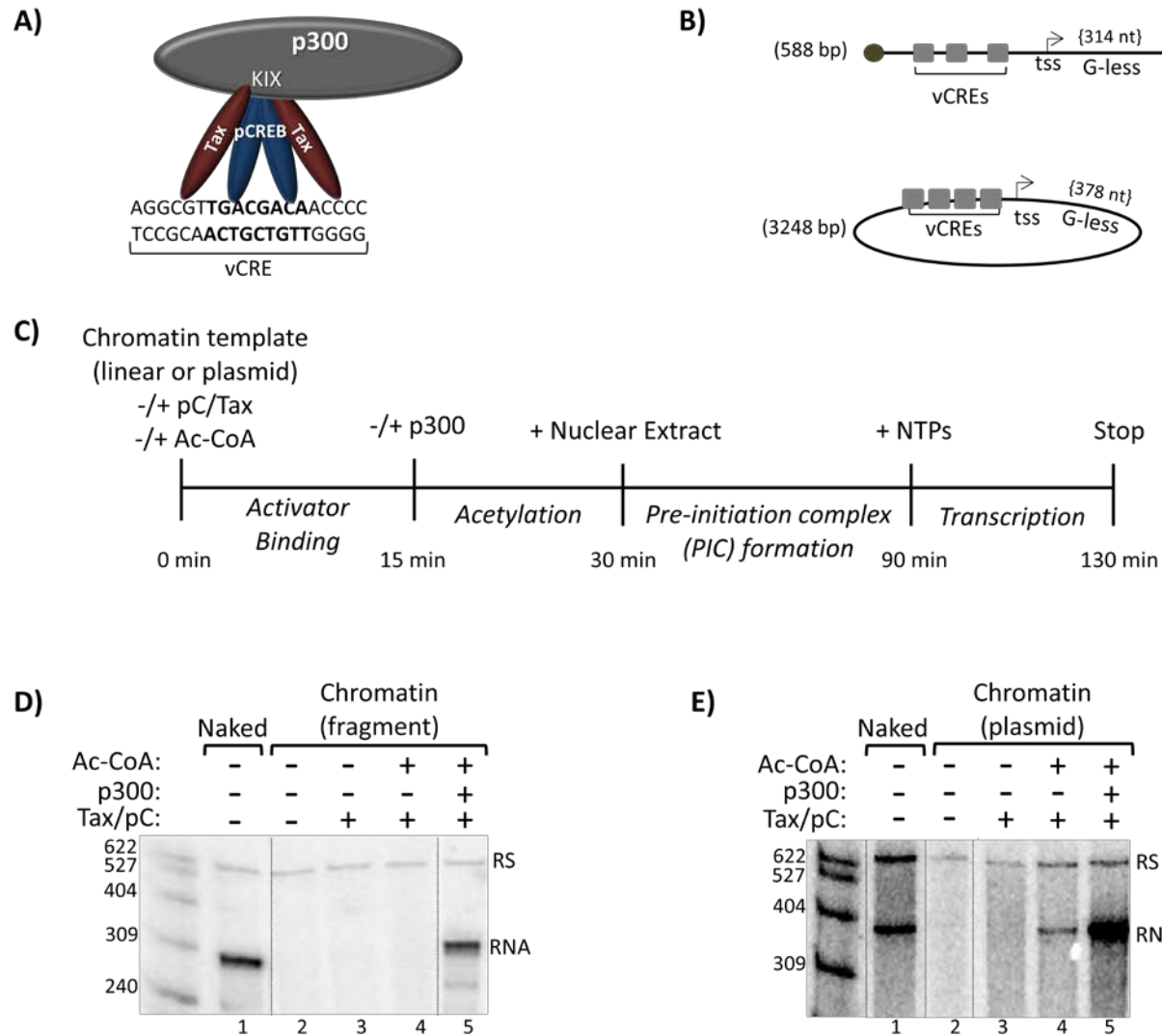
Figure 4.1. **p300 activates transcription from the HTLV-1 promoter.** A) A representative HTLV-1 enhancer element (vCRE), illustrating pCREB and Tax recruitment of p300 through the KIX domain. B) Schematic of the chromatin templates used. The linear fragment template is biotinylated and contains the natural HTLV-1 promoter and enhancer elements upstream of a 314 nt G-less cassette (top). The plasmid template is the 3.2 kbp p4xTRE plasmid, consisting of tandem 4 vCREs upstream of a 378 nt G-less cassette (bottom). (C) Schematic of the steps involved in the in vitro transcription assay. (D) In vitro transcription assay from the fragment template assembled into chromatin by the salt deposition method. (E) In vitro transcription assay from the plasmid template assembled into chromatin by the ACF/Nap1 method.

4.3b A p300 BROMODOMAIN DELETION MUTANT IS DEFECTIVE FOR AC-COA DEPENDENT HTLV-1 TRANSCRIPTION ACTIVATION

We were initially interested in determining if the p300 bromodomain was involved in promoting HTLV-1 transcription activation through recognition and binding of acetylated H3K14. We hypothesized that p300 bromodomain interaction with acetylated H3K14 would result in the destabilization and mobilization of promoter nucleosomes, ultimately activating transcription. Therefore, we predicted that p300 carrying a bromodomain deletion would be ineffective at activating HTLV-1 transcription. Using linear fragment and circular plasmid templates, we tested the ability of p300Δ1071-1241 (p300ΔBR) (Figure 4.2A-B) to activate transcription from chromatin templates in a activator and Ac-CoA dependent manner. Figure 4.2C shows again that on a linear fragment chromatin template, pCREB/Tax/p300 mediated transcription activation was Ac-CoA dependent (lane 5). As expected, as wild-type p300 was titrated, transcription activation increased (Fig 4.2C, lanes 5-8). However, when p300ΔBR was titrated over a 500-fold concentration range, it failed to activate transcription from the HTLV-1 promoter (Fig 4.2C, lanes 9-18). Figure 4.2D shows that on a circular plasmid chromatin template, pCREB/Tax-mediated transcription activation is Ac-CoA dependent (lane 2) and the addition of exogenous p300 enhanced Ac-CoA dependent transcription activation (lane 3). Unlike the fragment template, titration of wild-type p300 does not result in further transcription activation, probably due to the plasmid chromatin template already being saturated with both endogenous and exogenous p300 (Fig 4.2D, lanes 3-5). However, titration of exogenously added p300ΔBr resulted in a corresponding
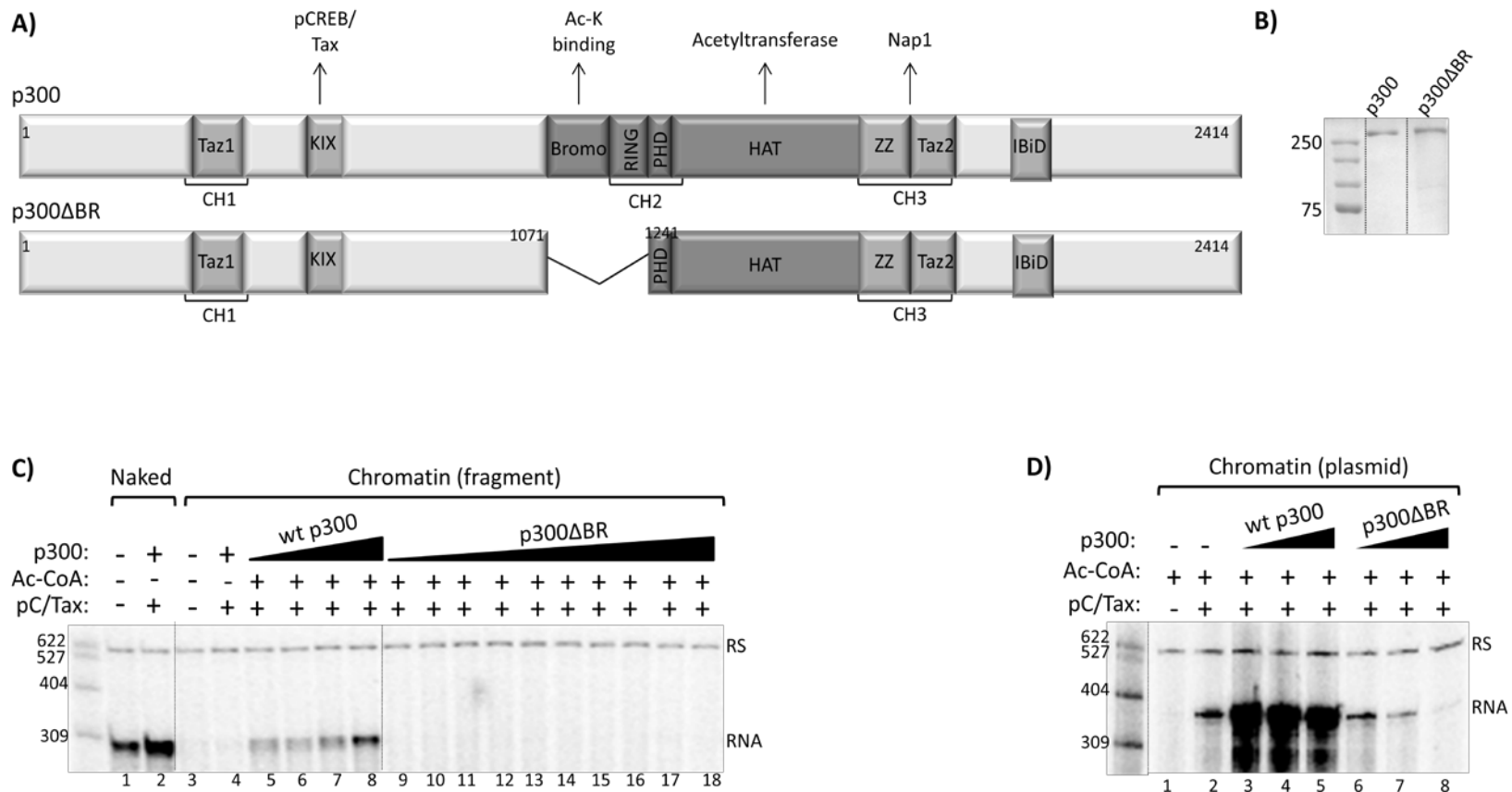
Figure 4.2. **A p300 bromodomain deletion mutant is defective for Ac-CoA dependent transcription activation** A) Schematic showing the p300 domains and the location of the bromodomain deletion. B) SDS-PAGE analysis of purified, recombinant wild-type p300 and p300ΔBromo. C) In vitro transcription assay utilizing the fragment chromatin template to test the transcription activity of the bromodomain deletion mutant of p300 (p300ΔBR). (D) In vitro transcription assay utilizing the plasmid chromatin template to test the transcription activity of the bromodomain deletion mutant of p300 (p300ΔBR).

decrease in transcription activation, likely illustrating a competition between endogenous wild-type p300 and exogenous p300ΔBr (Fig 4.2B, lanes 6-8).

4.3c   THE BROMODOMAIN OF p300 IS REQUIRED FOR PROMOTER RECRUITMENT AND SUBSEQUENT HAT ACTIVITY IN THE ABSENCE OF ACTIVATORS

Our results are in agreement with a previous study showing that deletion of the bromodomain was inhibitory to transcription activation from the IRF-1 promoter (63). In that study, the authors stated that the transcription defect was likely due to a disruption in p300 interaction with chromatin. A subsequent study showed that p300 interaction with chromatin required the bromodomain (145). However, in our system, p300 recruitment to the chromatin template is facilitated by the pCREB/Tax interaction with the KIX domain of p300. To ensure that deletion of the bromodomain did not result in impaired recruitment of p300 within our system, we tested the ability of p300ΔBR to be recruited to our chromatin template using an immobilized template assay in the presence of radiolabeled Ac-CoA. Figure 4.3A shows that in the absence of activators, wild-type p300 has HAT activity directed towards the nucleosomal template (lower panel) and is recruited to the template, although to a lesser degree than when activators are present (lanes 1 and 3). As expected, the bromodomain deletion mutant, is not recruited to the chromatin template in the absence of activators and does not have HAT activity towards the template (Fig 4.3A, lower panel, lane 2). This difference is not seen, however, when wild-type p300 and p300ΔBr are recruited to the chromatin template in the presence of pCREB and Tax through their well-established interaction with the p300
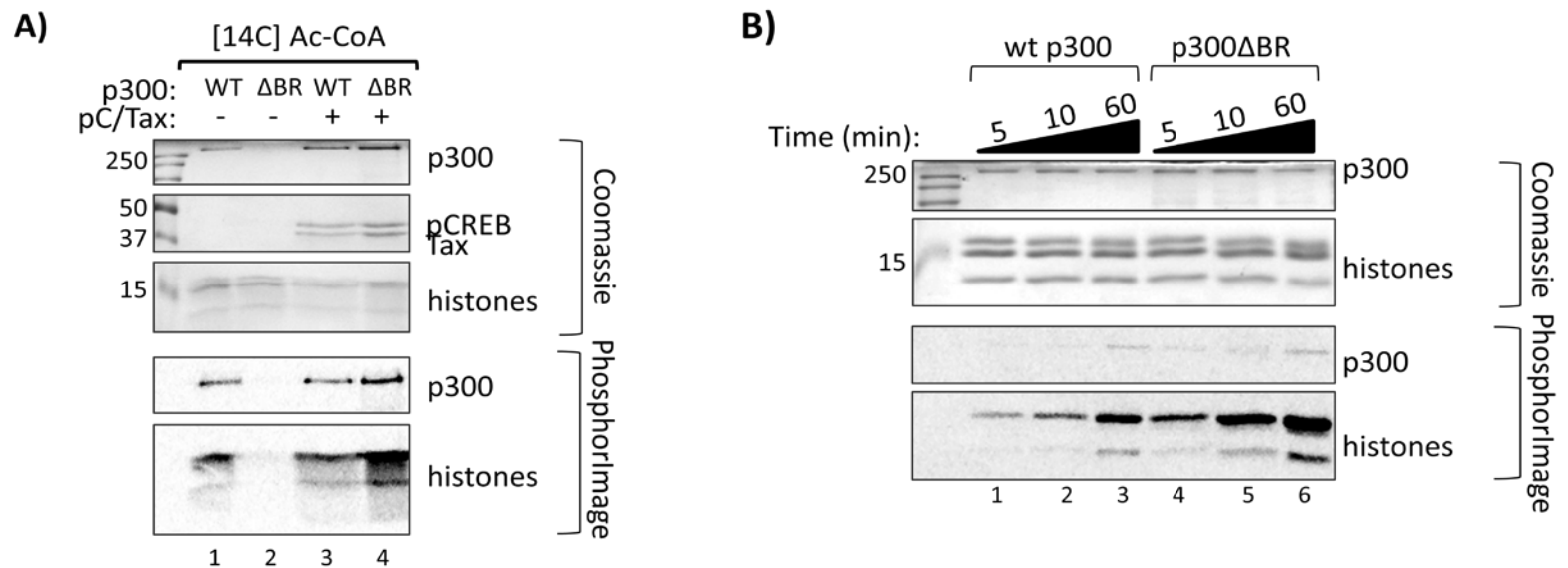
Figure 4.3. **A p300 bromodomain deletion mutant acetylates histones at a faster rate than wild-type p300.** A) The recruitment of wild-type p300 and p300ΔBR to the HTLV-1 chromatin promoter was analyzed in the presence and absence of the transcriptional activators, pCREB and Tax, using 750 ng of chromatin in an immobilized template assay in the presence of [14C] Ac-CoA. B) wild-type p300 and p300ΔBR HAT activity was measured over time using histones free in solution and [14C] Ac-CoA.

KIX domain (Fig 4.3A, upper panel, lanes 3 and 4). Consistent with their similar recruitment to the promoter, both wild-type p300 and p300ΔBR acetylate nucleosomal histones in the presence of pCREB and Tax. (Fig 4.3A, lower panel lanes 3 and 4). Significantly, this data indicates that the transcription defect we observe from deletion of the bromodomain is not a promoter recruitment/HAT defect, but is related to a transcription initiation event downstream of promoter recruitment.

We noticed that in the presence of pCREB and Tax, p300ΔBR had increased HAT activity compared with wild-type p300 acetylation of nucleosomal templates. This is likely accounted for by increased recruitment of p300ΔBR to the chromatin template. To further assess the enhanced HAT activity of p300ΔBR, we compared the HAT activity of wild-type p300 and p300ΔBR towards free histones, which is independent of recruitment. Figure 4.3B shows a time course of HAT activity using [14C] Ac-CoA. Both wild-type p300 and p300ΔBR acetylated free histones, although p300ΔBR acetylated at a faster rate. This observation is consistent with a recent paper showing the 3D structure of the core domain of p300 (aa 1048-1664). The authors found that, in addition to the previously identified bromodomain, PHD domain and HAT domain, the p300 core consisted of an additional, previously unidentified RING domain. Deletion of the RING domain resulted in an increase in p300 autoacetylation and p53 acetylation (65). The bromodomain deletion construct used in this, and other previous studies (Δ1071-1241), encompasses this RING domain.

In light of the structural paper, we wanted to target the bromodomain function more specifically and avoid confounding results with this newly identified RING domain in which deletion may affect HAT activity and thus transcription activity.

4.3d BROMODOMAIN INHIBITION RESULTS IN DECREASED TRANSCRIPTION ACTIVATION WITHOUT DISRUPTION OF THE QUATERNARY COMPLEX

In an effort to directly analyze the function of the p300 bromodomain, we purified the isolated bromodomain (aa 1039-1196), hypothesizing that its inclusion in our assays would compete for full length p300 bromodomain binding to acetylated lysines (Fig 4.4A). We first tested the binding specificity of the isolated bromodomain to our chromatin template. Using immobilized chromatin templates assembled with either mock or p300 pre-acetylated histones, we show that the isolated p300 bromodomain binds only to acetylated chromatin (Fig 4.4B). We then titrated the isolated bromodomain in our in vitro transcription system. Figure 4.5A shows that as the isolated bromodomain concentration increases, transcription activation decreases, likely through a disruption in interaction between the bromodomain that is contained in the full length protein and its substrate. Importantly, addition of the isolated bromodomain does not result in a disruption of the pCREB/Tax/p300 complex, nor does it affect recruited HAT activity towards the nucleosomal template (Figure 4.5B).

To further characterize p300 bromdomain function, we obtained a small molecule inhibitor of the CBP/p300 bromodomain, called SGC-CBP30. The inhibitor binds to the CBP/p300 bromodomain with low nanomolar affinity and is reported to be 40-fold selective for the CBP/p300 bromodomain over BRD4 (another cellular bromodomain containing protein). There currently are no published reports using this bromodomain inhibitor, however the structure of the CBP bromodomain bound with SGC-CBP30 has been submitted to the PDB (Figure 4.6A). We tested the inhibitor in our in vitro transcription system and found that upon its inclusion, p300 and Ac-CoA dependent
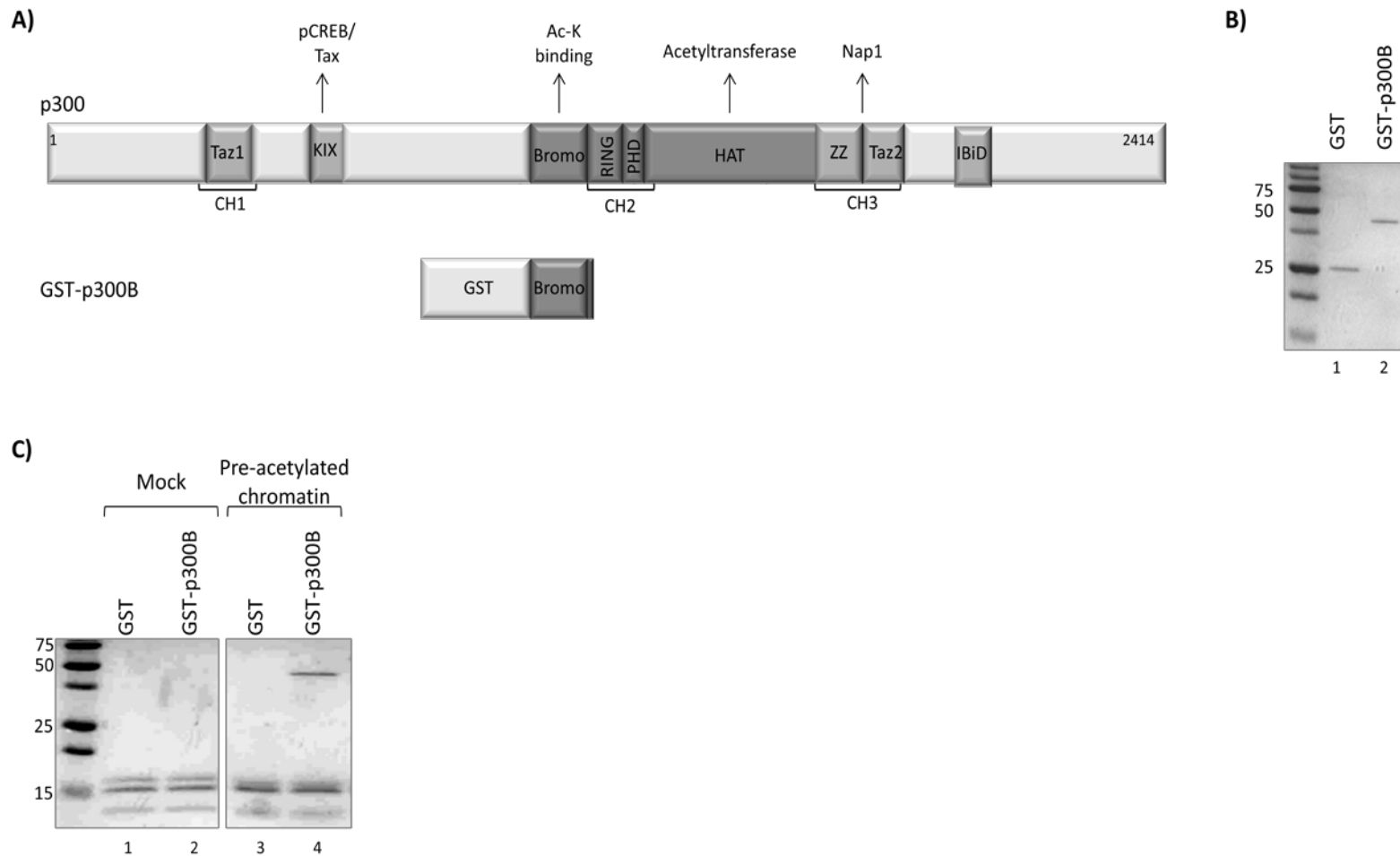
Figure 4.4. **The isolated p300 bromodomain binds specifically to acetylated chromatin.** A) Schematic and B) SDS-PAGE analysis of full length, wild-type p300 and GST-tagged p300 bromodomain (aa 1039-1196). C) An immobilized template assay was used to determine the specificity of binding of the isolated bromodomain of p300. Briefly, histones were acetylated with p300 prior to chromatin assembly by salt deposition. Chromatin was assembled, incubated with GST or GST-p300B, and then washed three times prior to the bound fraction being analyzed by SDS-PAGE.
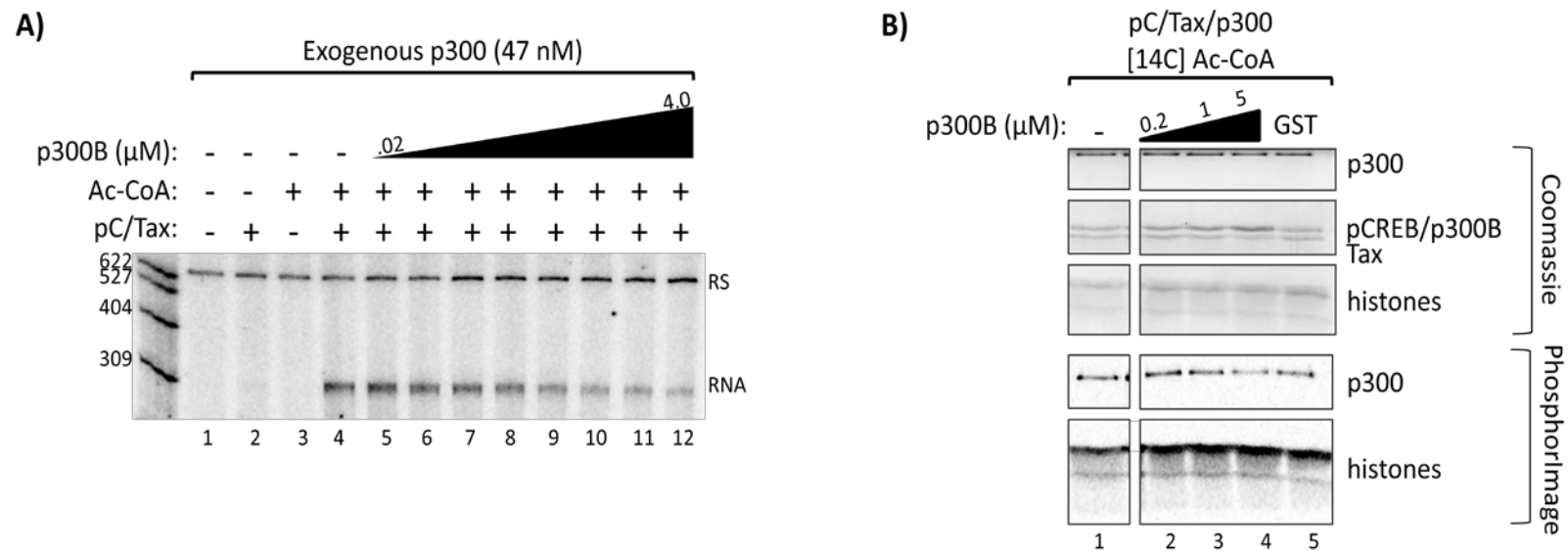
Figure 4.5. **The isolated p300 bromodomain inhibits transcription activation without disrupting the quaternary complex.** A) The isolated bromodomain of p300 was added in trans to an in vitro transcription assay. B) An immobilized template assay was used to determine if the isolated p300 bromodomain disrupted binding of pC/Tax/p300 to the HTLV-1 promoter (quaternary complex). Chromatin was assembled on the immobilized template, incubated with pC/Tax/p300 and increasing amounts of GST-p300B or GST in the presence of [14C] Ac-CoA, and then washed three times prior to the bound fraction being analyzed by SDS-PAGE.
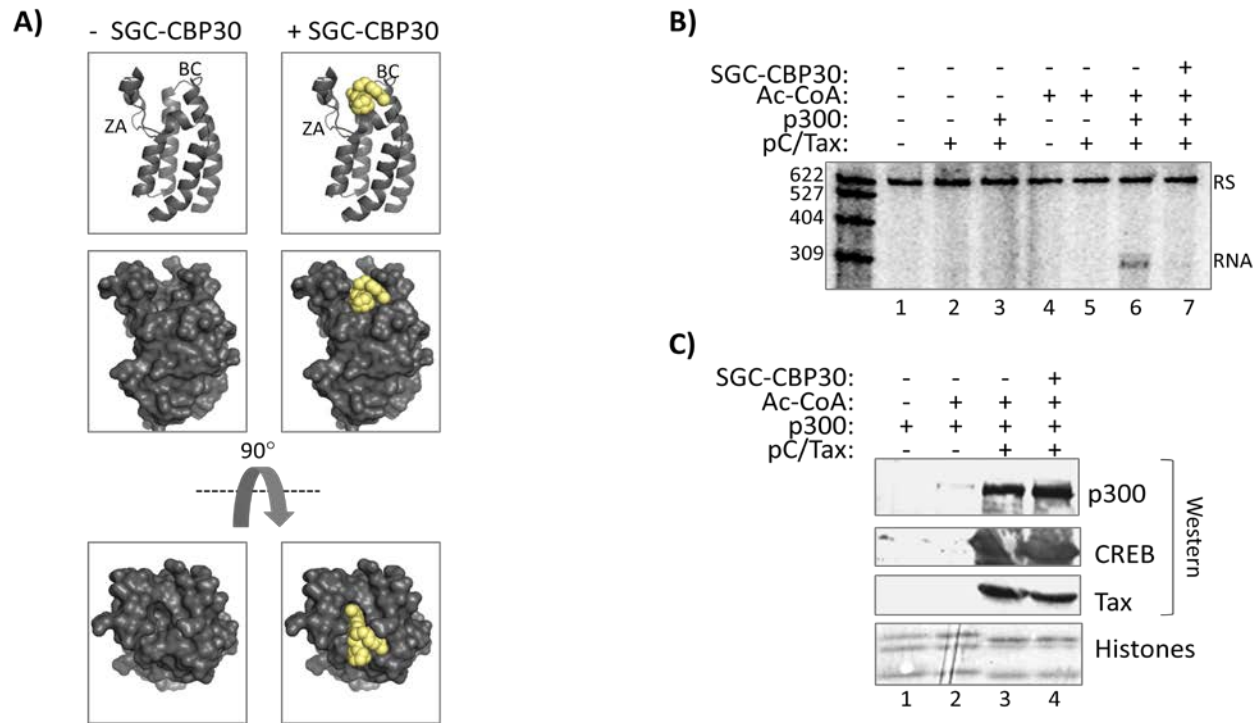
Figure 4.6. **A CBP/p300 specific bromodomain inhibitor reduces transcription activation without dirsupting the quaternary complex.** A) Crystal structure of the CBP bromodomain bound to the CBP/p300 specific bromodomain inhibitor, SGC-CBP30 (deposited into the Protein Data Bank by Filippakopoulos et al, accession code 4NR7). PyMOL was used to obtain the structure. The ZA and BC loops making up the hydrophobic acetyl-lysine binding pocket are labeled. The inhibitor is shown in yellow. Ribbon structure is shown in the top panel, and surface representation is shown in the middle and bottom panels. B) In vitro transcription assay showing p300 and Ac-CoA dependent transcription activation inhibited by SGC-CBP30. C) An immobilized template assay was used to determine if the CBP/p300 specific bromodomain inhibitor, SGC-CBP30, disrupted binding of pC/Tax/p300 to the HTLV-1 promoter (quaternary complex). Chromatin (1 µg) was assembled on the immobilized template and incubated with p300 either in the absence or presence of pCREB , Tax, Ac-CoA and SGC-CBP30. The chromatin templates were washed three times prior to the bound fraction being analyzed by SDS-PAGE and western blot analysis.

75

transcription activation was abolished (Figure 4.6B, lane 7). Again, we wanted to make sure that we were not disrupting the pCREB/Tax/p300 complex at the promoter, so we utilized our immobilized chromatin template to look at activator and coactivator binding in the presence of the bromodomain inhibitor, SGC-CBP30. Figure 4.6C shows that in the absence of activators and Ac-CoA, p300 is not recruited to the chromatin template (lane 1). In the presence of Ac-CoA only, p300 is slightly bound to the chromatin template, likely through its bromodomain (Figure 4.6C, lane 2) and consistent with our experiment in Figure 4.3A. In the presence of Ac-CoA and activators, p300 is robustly recruited to the chromatin template through its KIX domain (Figure 4.6C, lane 3). Importantly, this recruitment is unaffected in the presence of the bromodomain inhibitor, SGC-CBP30 (Figure 4.6C, lane 4). The work with the inhibitor, combined with the p300 bromodomain deletion mutant and bromodomain competition experiments show that the bromodomain is specifically involved in HTLV-1 transcription activation and cumulatively support that the bromodomain functions in transcription activation beyond just promoter recruitment as was previously proposed (63).

4.4 DISCUSSION

The work described herein involved extensive optimization of our in vitro transcription system (discussed in Chapter 3) that ultimately culminated in adding recombinant, full length p300 to the in vitro transcription reaction. We found varying dependence on exogenous p300 addition for HTLV-1 transcription activation using two different chromatin templates (Figure 4.1). This variability, nonetheless, is in agreement with past studies utilizing in vitro assembled chromatin templates to study Tax-mediated

HTLV-1 transcription activation. In previous studies using a circular plasmid template assembled into chromatin by the ACF/Nap1 method, the activators pCREB and Tax were able to utilize endogenous p300 from the nuclear extract for transcription activation and showed an enhanced level of activation upon the addition of exogenous p300 (72, 104). Alternatively, in previous studies using a fragment template assembled into chromatin using drosophila extracts, transcription activation was found to be completely dependent upon the addition of exogenous p300 (105), while other studies utilizing a fragment chromatin template showed no dependence on the addition of exogenous p300, regardless of the chromatin assembly method used (1, 107). The work presented here implies that differences in the exogenous p300 dependency for transcription activation could be due to the chromatin assembly method used, the chromatin template used, and/or the level of endogenous p300 contained within the nuclear extract.

It has been well demonstrated that HTLV-1 transcription activation involves the robust recruitment of the coactivator protein, p300, to the viral promoter. The various domains of p300 that have been previously shown to contribute to HTLV-1 transcription activation include the KIX domain, the HAT domain, and the CR2 domain (iBiD) (66, 72, 104). Cumulatively, our data shows that HTLV-1 transcription activation is activator (pCREB and Tax), coactivator (p300) and Ac-CoA dependent, demonstrating the importance of the previously characterized functional domains of p300 required for transcription activation. From the studies described herein, we have identified the bromodomain of p300 to be an additional domain required for HTLV-1 transcription activation. Importantly, we have extensively shown that the requirement for the

bromodomain in transcription activation is not for the initial recruitment of the coactivator to the chromatin template. Instead, our data indicates that the p300 bromodomain functions in transcription activation at an event post promoter recruitment.

Our data further suggests that the p300 bromodomain functions in the context of the full length protein through cooperation with other domains of p300, such as the KIX and HAT domains. Figure 4.7 directly shows that p300 promoter recruitment via the p300 bromodomain is not sufficient for transcription activation, rather transcription activation requires p300 recruitment and interaction with pCREB and Tax at the vCREs, presumably mediated by the KIX domain of p300 (lane 5 vs lane 11). A possible explanation for the requirement of activator mediated recruitment of p300 is that the pCREB/Tax interaction with p300 is a more stable interaction than the p300 bromodomain/acetylated chromatin interaction. Although the p300 bromodomain interaction with chromatin has been reported as a stable interaction (64), we find that p300 recruitment to the HTLV-1 chromatin template is more robust in the presence of activators, leading to increased targeted local concentration of p300 and increased histone acetylation, as evidenced by faster migrating histones on a SDS-PAGE when activators are present (Figure 4.6C). Another possible theory is that the pCREB/Tax interaction with p300 through the KIX domain orients the coactivator in such a way to interact with other factors needed for transcription activation (i.e. basal transcription machinery).

As part of its coactivator function, p300 is known to interact with over 400 different proteins. Therefore, there are numerous possible p300 bromodomain interactions and it is highly probable that there is more than one p300 bromodomain
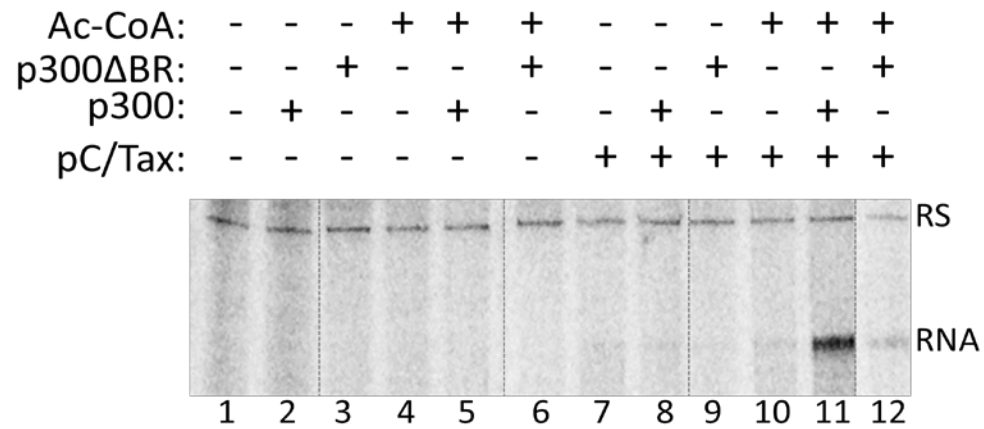
Figure 4.7. **p300 mediated HTLV-1 transcription activation requires activators and Ac-CoA**. In vitro transcription was performed using a fragment chromatin template. Wild-type p300 and p300ΔBR mediated transcription activation was assessed in the presence and absence of the activators (pCREB and Tax) and in the presence and absence of Ac-CoA.

interaction required for HTLV-1 transcription activation. In our studies, we blocked the transcription activation required interaction/s of the bromodomain of p300 using two approaches: adding the isolated bromodomain in trans to compete for and block full length p300 binding with its binding partner/s, and inhibition of the bromodomain in the full length p300 protein by adding a CBP/p300 specific bromodomain inhibitor. We have shown that the isolated bromodomain of p300 specifically binds our chromatin templates when acetylated, in contrast to Manning et al (64) that only showed bromodomain binding to acetylated histones free in solution and not to acetylated nucleosomes. Additionally, from the experiment in figure 4.5B, we show that the isolated bromodomain specifically binds to our immobilized chromatin template without disrupting the quaternary complex. However, from this experiment, we cannot determine if the interaction with chromatin is direct or indirect, as both chromatin and p300 are acetylated. Similarly, in our in vitro transcription experiment, the isolated p300 bromodomain could be interacting with acetylated chromatin, acetylated p300, or an acetylated protein present in the nuclear extract. Therefore, it is possible that the transcription defect we observe when the p300 bromodomain is deleted or blocked is due to a disruption in p300 interaction with chromatin, a disruption of a p300/p300 intermolecular interaction, or disruption in p300 interaction with some other required cellular factor. Additionally, whether this unidentified interaction with the p300 bromodomain is functionally relevant *in vivo* and whether the p300 bromodomain is involved in histone removal from the HTLV-1 promoter remains to be determined.

4.5   MATERIALS AND METHODS

4.5a   PURIFICATION OF RECOMBINANT PROTEINS

pCREB was purified as previously described (133). GST and GST-p300B (aa 1039-1196) were purified as described in (146). His-tagged p300ΔBR was expressed in SF9 insect cells and purified as previously described (63). ISWI and Acf1 were coexpressed in SF9 insect cells and purified as previously described (140). *Xenopus* histones and octamer were prepared as in (136). *Drosophila* Nap1 was expressed in E.coli and purified according to (140). C-terminally strep-tagged Tax was made using site directed, ligase independent mutagenesis to delete the existing C-terminal His$_6$-tag and insert a C-terminal Strep 2 tag (WSHPQFEK). Tax protein was purified from *E.coli* using batch strep-tactin chelate chromatography (Strep-Tactin Superflow, Qiagen) according to the manfacturer's instructions, and dialyzed against 50 mM Tris pH 8, 100 mM KCl, 4 mM MgCl2, 5 µM ZnSO4, 20% glycerol, 2 mM DTT. Wild-type p300 was made by inserting the p300 coding sequence (accession number Q09472) between BamHI and EcoRI in the baculovirus pVL1393 plasmid. The clone was custom made by Genewiz, is codon optimized for expression in SF9 insect cell culture and contains an N-terminal His tag and two C-terminal tags, Strep 2 (WSHPQFEK) and FLAG (DYKDDDDK) for purification. Insect cell culture and optimization of baculovirus expression were performed by Kinnakeet Biotech. The p300 protein was purified from SF9 cell pellets using batch strep-tactin chelate chromatography (Strep-Tactin Superflow, Qiagen) according to the manfacturer's instructions, and dialyzed against

50 mM Tris pH 7.9, 100 mM KCl, 6.25 mM MgCl2, 10 µM ZnSO4, 20% glycerol, 1mM

DTT. Nuclear extract from a HTLV-1 negative human T-cell line (CEM) was prepared as

in (147).

4.5b   DNA IMMOBILIZATION

A fragment of DNA containing the HTLV-1 promoter and enhancer elements

linked to a 313 bp G-less cassette was produced by PCR amplification, using an

upstream biotinylated primer (5'-GTCTGAAAAGGTCAGGGCC-3'), non-biotinylated

downstream primer (5'-GGATATATGAGATGAGTAGG-3') and the -306 HTLV-1

plasmid as template. The 588 bp PCR product was purified using an Amicon-Ultra 15

centrifugal filter device, 50K MW cutoff (Millipore). Biotinylated fragment was bound to

streptavidin coated magnetic Dynabeads (M280, Invitrogen) per manufacturer

instructions, at 3 µg DNA/100 uL of slurry of Dynabeads. Percent DNA binding to the

Dynabeads was determined by spectrophotometric analysis at A260 of the supernatant

after binding, compared to the A260 of the prebound reaction mixture. Immobilized DNA

was stored in 1X TE buffer (10 mM Tris, 1 mM EDTA) at 4°C.

4.5c   CHROMATIN ASSEMBLY

The salt deposition method, previously described in (107), was used to assemble

chromatin on the immobilized 588 bp HTLV-1 fragment template. When using the

p4XTRE/G-less template (plasmid template), the ACF/Nap1 assembly system was used

as described in (104, 140, 148). Chromatin assembly was performed at empirically

determined histone:DNA (mass:mass) that gave complete transcription repression in the absence of activators/coactivator/Ac-CoA, but was activated in the presence of activators/coactivator/Ac-CoA.

4.5d *IN VITRO* TRANSCRIPTION

In vitro transcription assays were performed as previously described (104, 107) under the following conditions. Briefly, 35 fmol of the plasmid chromatin template or 130-200 fmol of fragment chromatin template was incubated in the presence and absence of 33 nM pCREB, 66 nM Tax, and 10 nM p300 and 100 µM Ac-CoA. All reactions contained 20-30 µg CEM nuclear extract. In experiment 4.2D, wild-type p300 and p300ΔBR were titrated from 10-30 nM. SGC-CBP30 was used at a final concentration of 10 µM as indicated in experiment 4.5A. Final reaction volumes were 30 µL in 25 mM Tris, 50 mM KCl, 6.25 mM MgCl2, 10% glycerol and 0.5 mM EDTA. A schematic of the order of addition of components and incubation periods is shown in Fig 4.1.

4.5e IMMOBILIZED TEMPLATE ASSAY

Chromatin assembled on the immobilized HTLV-1 promoter was incubated with the recombinant proteins indicated, and 100 µM Ac-CoA as indicated at 30°C, 1200 RPM for 1 hour in a buffer containing 50 mM Tris, 100 mM KCl, 6.25 mM MgCl2, 1 mM EDTA, 20% glycerol, 0.1% NP-40, 5 ng/µL polydA:dT and 2 mM DTT. Reaction mixtures were magnetized, the supernatant was removed and the bound fraction

washed three times in the buffer conditions just described (- polydA:dT). The bound

fractions were resuspended in SDS sample dyes and loaded onto 15% SDS-PAGE

gels. Bound proteins were analyzed by coomassie staining and/or Western blot as

indicated.

4.5f   TIME COURSE HAT ASSAY

p300 (167 nM) was incubated with histone octamer (300 nM) at 30°C for 10

minutes in a buffer containing 50 mM Tris, 100 mM KCl, 6.25 mM MgCl$_2$, 1 mM EDTA,

20% glycerol and 2 mM DTT. A mixture of [14C] Ac-CoA and non-radiolabeled Ac-CoA

was added to a final concentration of 100 μM. The reaction mixture was incubated for

the times indicated and subsequently squelched by the addition of SDS-PAGE dyes.

Samples were loaded onto a 15% SDS-PAGE, stained by coomassie and analyzed by

PhosphorImaging.

CHAPTER 5

FUTURE DIRECTIONS

The studies presented here identified acetylation at histone H3, lysine 14 as a key requirement for Nap1 dependent nucleosome disassembly and transcription activation. A single lysine to arginine mutation at lysine 14 on the H3 N-terminal tail prevented Nap1-dependent nucleosome disassembly from the HTLV-1 promoter template. We later showed that this single mutation also decreased activator/coactivator/Ac-CoA dependent transcription activation by more than 50%. In agreement with the observations just mentioned, lysine to arginine mutation at lysines 9, 18 and 23 on the H3 N-terminal tail leaving lysine 14 wild-type, in a background of K→R mutations at the other N-terminal histone tails, did not affect Nap1-dependent nucleosome disassembly or transcription activation, suggesting that acetylation at H3K14 is sufficient for nucleosome disassembly and transcription activation. However, additional lysines were still available for acetylation by p300 in the H3K14 wt only mutant. Therefore, while we have shown that H3K14ac is required, we cannot be conclusive in the statement that sole acetylation at H3K14 is sufficient for nucleosome disassembly and transcription activation. We would like this to be addressed in future studies, described below.

Additionally, we have identified another domain of the coactivator protein, p300 that is required for transcription from the HTLV-1 promoter. The KIX, HAT and CR2 (iBiD) domains had been previously identified and characterized to function in HTLV-1 transcription activation (66, 72, 104). The data presented herein cumulate in the

discovery that the p300 bromodomain is an additional required domain for HTLV-1

transcription activation. We conclusively show that the requirement for the p300

bromodomain is not out of necessity for recruitment of p300 to the HTLV-1 promoter, as

p300 is robustly recruited to the chromatin template by the activators, pCREB and Tax.

Rather, the p300 bromodomain is functioning in HTLV-1 transcription activation post the

p300-recruited HAT activity. We have yet to determine the exact nature of its function

and whether this requirement is relevant *in vivo*. These findings, nonetheless, are

potentially very exciting as bromodomain proteins are increasingly targeted for

therapeutic treatment of human disease (149-151). Of most relevance to our studies, a

BET-family specific bromodomain inhibitor (JQ1) was recently shown to have anti-

tumour and anti-proliferative effects in HTLV-1 infected cells by blocking Tax-mediated

NF-κB activation (152).

Described below are initial approaches and preliminary data for studying the

above mentioned, adding to the knowledge of not only Tax-mediated HTLV-1

transcription activation, but potentially the general mechanisms of p300-mediated

eukaryotic gene activation.

5.1 IS H3K14 ACETYLATION SUFFICIENT FOR NUCLEOSOME DISASSEMBLY AND TRANSCRIPTION ACTIVATION?

Using mixed octamer chromatin templates containing lysine to arginine mutations

at sites of acetylation within the histone N-terminal tails, we have found acetylation at

H3K14 to be required for Nap1-dependent nucleosome disassembly and

activator/coactivator/Ac-CoA dependent HTLV-1 transcription activation. Our data also

suggests that acetylation at H3K14 might be sufficient for Nap1-dependent nucleosome disassembly, as a mutant chromatin template containing K→R mutations throughout the nucleosome except for histone H3, lysine 14 was permissive to nucleosome disassembly. Conversely, the H3K14 only mutant (H3K9,18,23R, H2A/H2B/H4K-R) still retained wild-type lysines at positions 4, 27, 36 and 37. Importantly, lysine residues 27, 36 and 37 have been shown to be acetylated by p300 at the HTLV-1 promoter, *in vitro* (144). Therefore, we cannot definitively claim that acetylation at H3K14 is sufficient for nucleosome disassembly and transcription activation.

As such, we have cloned, expressed and purified another H3 tail mutant, called H3K→R^7 (K4, 9, 18, 23, 27, 36, 37R) to be used in future studies aimed to determine if H3K14 acetylation is truly sufficient for Nap1-dependent nucleosome disassembly and transcription activation. Another way of approaching this question would be to use a preacetylated chromatin template at H3 lysine 14 and perform experiments in the absence of Ac-CoA. Preliminary studies have been performed using such a chromatin template (Figure 5.1). Neumann et al describes a method used to genetically install an acetyl group at site-specific lysines in recombinant histones (153). H3K14ac was made using this method, assembled into histone octamer, assembled into chromatin on our immobilized HTLV-1 promoter and then tested in Nap1-dependent nucleosome disassembly. We found that in the absence of Ac-CoA, a chromatin template containing H3K14ac was not permissive to nucleosome disassembly, suggesting that preacetylation at H3K14 is not sufficient (Figure 5.1, lane 5). However, preacetylation at H3K14 in the presence of Ac-CoA also did not support nucleosome disassembly, as compared to a wild-type chromatin template (Figure 5.1, lanes 2 and 6). This result is
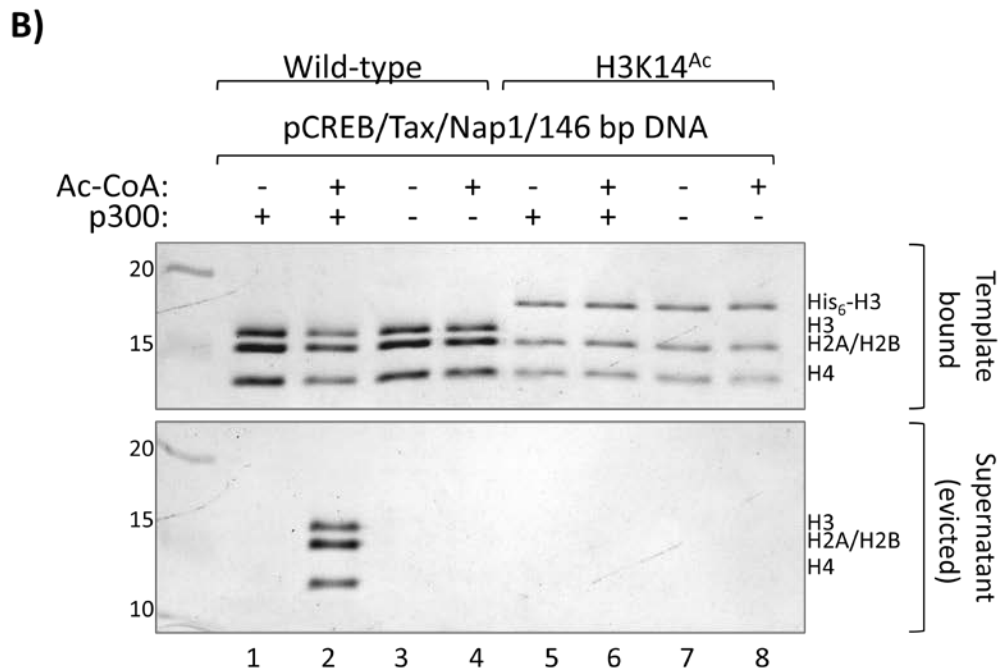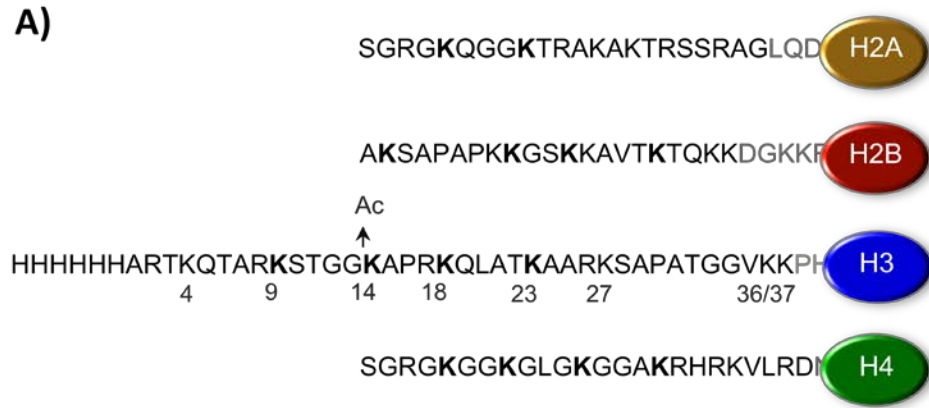
Figure 5.1. A) **Schematic of the N-terminal histone tails in a H3K14ac octamer.** The lysines located within the H3 tail are indicated, as is the His_6 tag on the N-terminus. B) **Nap1-dependent nucleosome disassembly assay using a chromatin template pre-acetylated at histone H3, lysine 14 only.** Wild-type or H3K14ac octamer was used to assemble chromatin on an immobilized HTLV-1 promoter by the salt deposition method. The immobilized chromatin template was then incubated with pCREB and Tax, p300, Ac-CoA, Nap1 and 146 bp DNA (Widom "601" sequence) as indicated. Immobilized templates were washed 3 times prior to analysis of the bound and supernatant (evicted) fractions by SDS-PAGE and coomassie staining.

suggestive of two things: Either preacetylation is not permissive to nucleosome disassembly and the act of acetylation by p300 is required, or the N-terminal histidine tag on H3K14ac inhibits nucleosome disassembly. It would not be surprising that a histidine tag on the amino-terminus of the histone H3 tail would be inhibitory, as the addition of a histidine tag, in principle, turns H3K14 into H3K20. Therefore, future experiments utilizing genetically installed H3K14ac should consider cleaving the histidine tag. Additionally, it remains to be tested if preacetylation at H3K14 supports transcription activation in the absence of Ac-CoA. One might argue that there are other functionally relevant targets of acetylation during transcription activation (104). However, recent preliminary data suggests that Ac-CoA is needed only prior to nuclear extract addition and preinitiation complex (PIC) formation (data not shown).

In summary, chromatin templates assembled from the H3KR^7 and H3K14ac octamers will be most useful in future studies addressing the sufficiency of acetylation at H3K14 for nucleosome disassembly and subsequent transcriptional activation. Importantly, these studies would provide us a better understanding of the mechanism of Nap1-mediated nucleosome disassembly at the HTLV-1 promoter.

5.2 IS THE REQUIREMENT FOR THE p300 BROMODOMAIN IN HTLV-1 TRANSCRIPTION ACTIVATION FUNCTIONALLY RELEVANT *IN VIVO*?

We have identified a requirement for the p300 bromodomain during HTLV-1 transcription *in vitro* and wish to determine if the same is true *in vivo*. We propose using the CHOK-1 cell line that contains the integrated HTLV-1 promoter driving a luciferase gene and performing luciferase assays to measure transcription activation *in vivo*. The

development of this cell line is described in (154) and has been used by our lab in the past to study Tax-mediated transcription activation *in vivo* (106). Importantly, transient transfection with a Tax expression plasmid activates transcription from the integrated HTLV-1 promoter. To study the effect of blocking the p300 bromodomain on HTLV-1 transcription activation *in vivo*, we envision two approaches. The first approach involves the inhibition of endogenous CBP/p300, and the second involves first the overexpression of p300 (or a p300 bromodomain mutant) and subsequent blocking of both endogenous and overexpressed CBP/p300 with either the SGC-CBP30 bromodomain inhibitor or overexpression of the isolated p300 bromodomain.

We have used the first approach involving inhibition of endogenous CBP/p300 and have performed preliminary *in vivo* experiments. CHOK-1 cells were seeded and transfected with a Tax expression plasmid for 12 hours, followed by a 2 hour treatment with the CBP/p300 specific bromodomain inhibitor, SGC-CBP30, prior to cell lysis and analysis of cell lysate luciferase activity. We find that treatment with the inhibitor results in a modest dose-dependent inhibition of HTLV-1 transcription, *in vivo* (Figure 5.2A). Additionally, we have also performed preliminary experiments involving the second approach of overexpressing p300. We obtained a p300 mammalian expression vector (pCDNA3.1-p300, Addgene) and cotransfected it with a Tax expression vector, prior to performing luciferase assays on cell lysates. Figure 5.2B shows that cotransfection of Tax and p300 results in higher HTLV-1 transcription activation than Tax transfection alone. As mentioned, future experiments could then utilize the overexpression of a p300 bromodomain mutant, comparing it to the overexpression of wild-type p300. The preliminary experiments presented here certainly require further methods optimization
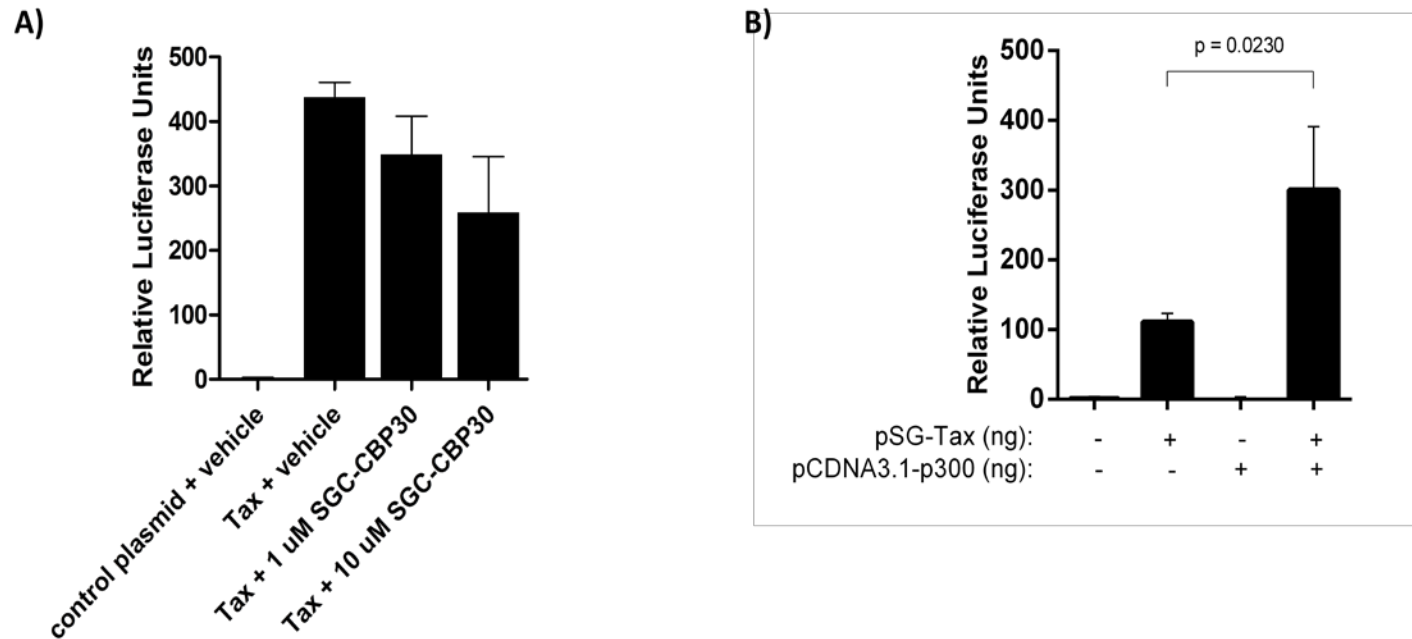
Figure 5.2. A) **The CBP/p300 specific bromodomain inhibitor, SGC-CBP30, inhibits Tax activation of the HTLV-1 promoter** *in vivo*. CHO-K1 cells were transfected with pSG-Tax and treated with the bromodomain inhibitor, SGC-CBP30 for 2 hours just prior to cell lysis 24 hours following transfection. Data was normalized to Renilla activity. Averages from triplicate wells are represented. B) **p300 enhances Tax activation of the HTLV-1 promoter** *in vivo*. CHO-K1 cells were transfected with pSG-Tax, pCDNA3.1-p300, pSG-Tax/pCDNA3.1-p300 or pUC19 as a control. Luciferase assays were performed 48 hours following transfection. Data was normalized to Renilla activity. Averages from triplicate wells are represented .

as well as replicate measures to determine statistical significance.   Lastly, in regards to the use of a dominant negative p300 bromodomain mutant, we propose the mutation N1132A, based on a recently published report and supported by our own data (65). Delvecchio el at made a N1132A mutation in the isolated bromodomain/RING/PHD (BRP) of p300 and performed a binding assay with a modified histone peptide array. The authors found that the N1132A mutation inhibited almost all binding to acetylated peptides. The asparagine at position 1132 directly interacts with the acetyl-lysine in the hydrophobic pocket of the CBP/p300 bromodomain (Figure 5.3B). Under my guidance, an undergraduate research student (Julio Flores Servin) made this same mutation in our isolated p300BRP module and has shown through immobilized chromatin template assays and GST-pulldown assays that this mutation disrupts the specific acetyl-lysine binding of the isolated p300BRP module to chromatin and p300 (Figure 5.3C and D). As such, we are confident in utilizing this mutation as a dominant negative p300 bromodomain mutant for use in *in vivo* studies concerning the role of the p300 bromodomain in HTLV-1 transcription activation.

5.3   IS THE p300 BROMODOMAIN INVOLVED IN PROMOTER NUCLEOSOME DISASSEMBLY?

As already mentioned and hypothesized, we are interested in whether the transcription activation defect caused by deletion or blocking of the p300 bromodomain is a defect in promoter nucleosome disassembly prior to PIC formation and transcription activation. *In vitro* and *in vivo* approaches designed towards answering this question are described below.
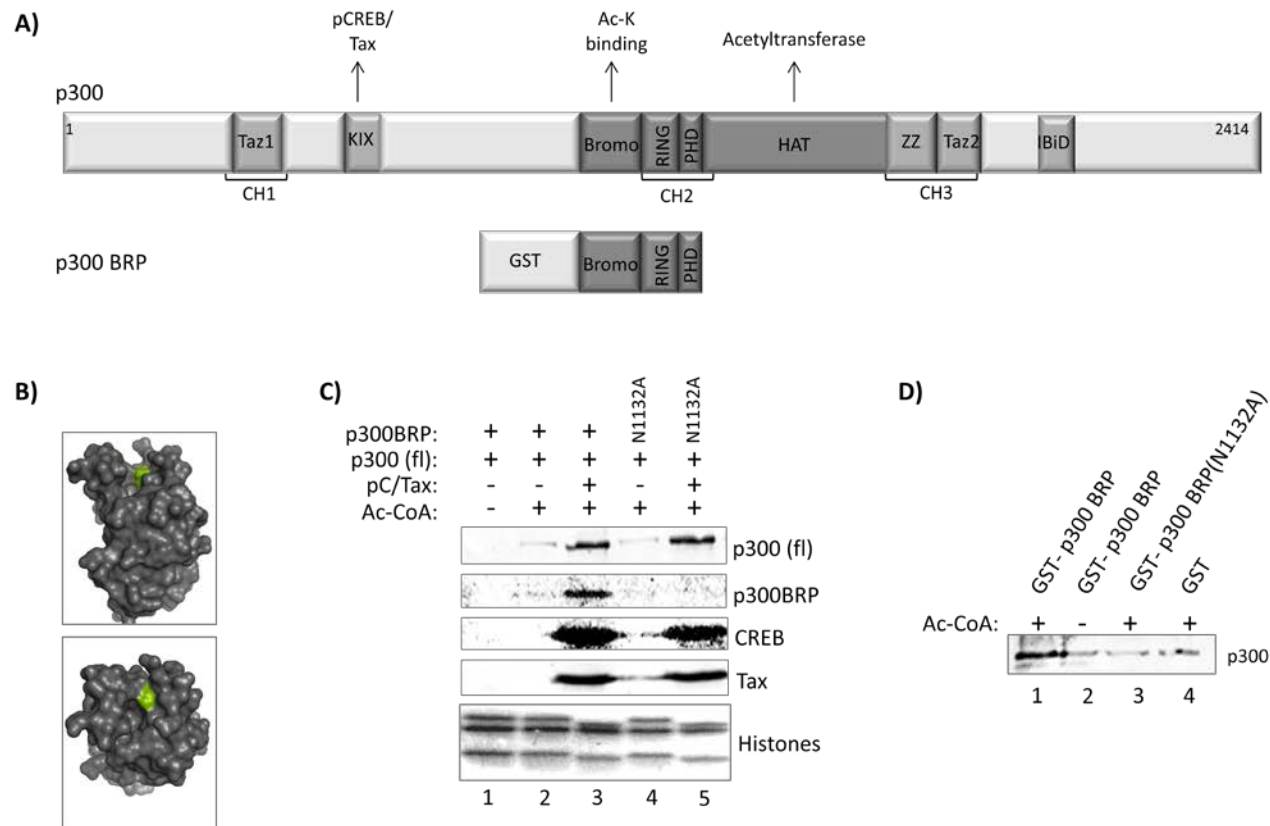
Figure 5.3. A) **Schematic of the domains of p300 and GST-p300BRP.** B) **Crystal structure of the CBP bromodomain, illustrating the location of N1132, colored in green** (accession code 4NR7). C) **N1132A mutation in the p300 BRP module disrupts acetylation-dependent bromodomain binding to the immobilized HTLV-1 promoter.** The HTLV-1 promoter was immobilized, assembled into chromatin by the salt deposition method and then subsequently incubated with pCREB, Tax, p300 and p300BRP, p300BRP N1132A and Ac-CoA as indicated. Templates were washed three times prior to analysis of the bound fraction by SDS-PAGE and Western blot. D) **N1132A mutation in the p300 BRP module disrupts acetylation-dependent bromodomain binding to full length p300.** Purified GST-p300 BRP and GST-p300 BRP(N1132A) were incubated with glutathione-agarose beads, prior to incubation with full length p300 in the presence and absence of Ac-CoA, as indicated. The reactions were washed three times and the bound fraction analyzed by SDS-PAGE and Western blot.

Studying the requirement for the p300 bromodomain in promoter nucleosome disassembly during HTLV-1 transcription activation *in vivo* largely depends upon the results described in Chapter 5.2. As such, we would employ whatever methods found successful that resulted in a decrease in transcription activation *in vivo* when blocking the p300 bromodomain, prior to performing chromatin immunoprecipitation (ChIP) assays to look at histone occupancy within the HTLV-1 promoter region. We would also want to ask if histone occupancy correlates with Pol II occupancy, as we have previously suggested that promoter nucleosome depletion precedes PIC formation (1).

To study this *in vitro*, we could perform the Nap1-dependent nucleosome disassembly assay on an immobilized chromatin template, comparing the activities of wild-type p300 and the p300 bromodomain deletion mutant (p300ΔBR) in removal of histones from the HTLV-1 promoter. We could also perform the Nap1-dependent nucleosome disassembly assay in the presence of the CBP/p300 specific bromodomain inhibitor, SGC-CBP30 to see if its inclusion inhibits nucleosome disassembly. Other *in vitro* methods that could be used to look at possible differences in the chromatin architecture prior to transcription activation include DNase 1 and micrococcal nuclease (MNase) digestions.

5.4   WHAT IS THE FUNCTIONALLY RELEVANT p300 BROMODOMAIN INTERACTION DURING HTLV-1 TRANSCRIPTION ACTIVATION?

From the studies presented herein, we know that the bromodomain of p300 is involved in and required for HTLV-1 transcription activation, as disruption of its binding to its binding partner results in decreased transcriptional activation. Naturally then, we

next want to know what the functional interaction of the p300 bromodomain is. This is

perhaps the most important question and the most difficult to answer. Over 400 different

proteins have been reported to interact with p300 (58). From our studies alone, we

know that p300 could be interacting with the acetylated chromatin template,

autoacetylated p300, and/or another acetylated protein provided by the nuclear extract

during activation of transcription. Mass spectrometry analysis of an immobilized

chromatin template under transcription activating conditions, in the absence and

presence of the bromodomain inhibitor SGC-CBP30 might identify a functionally

relevant interaction. However, this method would not enable us to identify the interaction

if the target protein is already recruited to the chromatin template through another

interaction and just not interacting with the p300 bromodomain. To help target the study,

it might be useful to first incubate immobilized p300 (using either its His, FLAG or Strep

tags) with a nuclear extract in the presence of Ac-CoA and the SGC-CBP30

bromodomain inhibitor and then identify by mass spectrometry, nuclear proteins that

bind to p300 in a bromodomain dependent manner. Regardless of the outcome of the

above study, we would gain a lot of insight into the identification of acetylated nuclear

proteins that are recruited to the HTLV-1 promoter during transcription activation,

ultimately increasing our knowledge of HTLV-1 transcription regulation.

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