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Tax Abolishes Histone H1 Repression of p300 Acetyltransferase Activity at the Human T-Cell Leukemia Virus Type 1 Promoter▽

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Upon infection of human T-cell leukemia virus type 1 (HTLV-1), the provirus is integrated into the host cell genome and subsequently packaged into chromatin that contains histone H1. Consequently, transcriptional activation of the virus requires overcoming the environment of chromatin and H1. To efficiently activate transcription, HTLV-1 requires the virally encoded protein Tax and cellular transcription factor CREB. Together Tax and CREB interact with three *cis*-acting promoter elements called viral cyclic-AMP response elements (vCREs). Binding of Tax and CREB to the vCREs promotes association of p300/CBP into the complex and leads to transcriptional activation. Therefore, to fully understand the mechanism of Tax transactivation, it is necessary to examine transcriptional activation from chromatin assembled with H1. Using a DNA template harboring the complete HTLV-1 promoter sequence and a highly defined recombinant assembly system, we demonstrate proper incorporation of histone H1 into chromatin. Addition of H1 to the chromatin template reduces HTLV-1 transcriptional activation through a novel mechanism. Specifically, H1 does not inhibit CREB or Tax binding to the vCREs or p300 recruitment to the promoter. Rather, H1 directly targets p300 acetyltransferase activity. Interestingly, in determining the mechanism of H1 repression, we have discovered a previously undefined function of Tax, overcoming the repressive effects of H1-chromatin. Tax specifically abrogates the H1 repression of p300 enzymatic activity in a manner independent of p300 recruitment and without displacement of H1 from the promoter.

Human T-cell leukemia virus type 1 (HTLV-1) was first isolated in 1979 (51) and has since been identified as the etiologic agent of adult T-cell leukemia and HTLV-1-associated myelopathy/tropical spastic paraparesis (17, 49, 55, 59). Upon infection, the provirus is randomly integrated into the host cell chromosome and subsequently packaged into chromatin (37). Of the estimated 12 million worldwide carriers, roughly 4% eventually develop adult T-cell leukemia or HTLV-1-associated myelopathy/tropical spastic paraparesis, often decades after the initial infection (58). The events that mediate transformation remain largely unknown. However, activation of transcription from the integrated provirus assembled into chromatin is a necessary step for the production of virally encoded proteins, including Tax. Tax is essential for efficient transcription of the viral genome (4, 5, 61–64) and contributes to malignant transformation through effects on other genes and cell components (21, 22).

Tax associates with the HTLV-1 promoter through protein-protein interactions between CREB (or other ATF/CREB family members) and protein-DNA contacts. The Tax/CREB complex interacts with three *cis*-acting DNA elements called viral cyclic AMP-response elements (vCREs) located within the U3 region of the HTLV-1 long terminal repeat (LTR) (1, 12, 20). Specifically, CREB binds as a dimer to the 8-nucleotide, off-consensus CRE core within each 21-bp vCRE (26). Tax interacts with CREB and the GC-rich sequences flanking the core CRE within the DNA minor groove, forming a ternary complex on the promoter (30, 42, 46). Several studies have shown that Tax and CREB binding to viral DNA promotes association of the pleiotropic coactivators p300 and CBP, forming a quaternary complex (18, 29, 33, 35).

CBP and p300 are cellular coactivators that likely regulate transcription in all metazoans (reviewed in reference 19). Their effect on transcription is, at least in part, through their intrinsic acetyltransferase activity that targets lysine residues within each of the four core histone proteins (48) as well as nonhistone targets (15). Chromatin regions enriched with acetylated nucleosomes correlate with areas of active chromatin (25). In vivo studies of the HTLV-1 LTR provide direct evidence for p300 and CBP promoter occupancy contributing to increased levels of histone H3 and H4 acetylation (40). Furthermore, inhibition of histone deacetylase complexes results in greater levels of histone acetylation and a concomitant increase in viral RNA transcripts (40, 41, 44). Therefore, it is no surprise that we and others have previously observed a requirement for p300 and acetyl coenzyme A (AcCoA) in potentiating the effects of Tax transactivation on chromatin templates in vitro (16, 43).

Nucleosomes form the basic repeating unit of chromatin and consist of a core histone octamer (two H2B/H2A dimers and one H3/H3 tetramer) wrapped by 146 bp of DNA (45). In addition to the core histones, a fifth or "linker" histone, H1, also contributes to the structure and function of chromatin. Approximately one histone H1 is associated per nucleosome in vivo (3); however, the precise location of H1 on the nucleosome remains to be determined. The linker histone acts, in part, to stabilize higher order chromatin structure but is not required for chromatin condensation (54). Functionally, H1 is classified as a transcriptional repressor, but no general mech-

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anism for H1 repression has emerged from the literature. Rather, chromatin containing histone H1 has been shown to inhibit various steps associated with transcriptional activation and even initiation (7, 23, 27, 28, 60).

Prior to Tax activation in cells, nucleosomes on the HTLV-1 promoter contain histone H1 (39). Therefore, when examining the mechanism of Tax activation biochemically, it is critical to do so using chromatin containing H1. In this study, we investigated the effects of H1-containing chromatin on HTLV-1 transcriptional activation through the use of a defined, recombinant assembly system, extending previous work examining viral activation in the context of chromatin (15, 16, 43). Upon optimization of H1 incorporation into chromatin, we observed a twofold reduction in HTLV-1 transcriptional activation. We demonstrated that H1 represses transcription through inhibition of p300 activity. Furthermore, we determined that H1 repression is not mediated through interference with p300 recruitment but directly through an effect on p300 acetyltransferase activity. In determining the mechanism behind H1 repression, we uncovered a previously undefined function of Tax. Specifically, Tax abrogated H1-mediated inhibition of p300 enzymatic activity in a manner independent of p300 recruitment and without displacing H1 from the promoter. We propose a model wherein transcriptional activation at the HTLV-1 promoter is tightly regulated through the opposing effects of Tax and H1 on p300 acetyltransferase activity.

MATERIALS AND METHODS

Protein purification. Native *Drosophila* core histones (6) and histone H1 (8) were purified from *Drosophila* embryos. Sf9 cells were infected with *Drosophila* nucleosome assembly protein 1 (dNAP-1_{His6}) baculovirus at a multiplicity of infection (MOI) of 5 and purified as described previously (14). Recombinant ATP-utilizing chromatin assembly and remodeling factor (ACF) was produced in Sf9 cells by coinfection of Acf1 and $ISWI_{FLAG}$ baculoviruses at MOIs of 10 and 5, respectively, and purified as described previously (14). Recombinant CREB (13) and Tax (65) were expressed and purified, respectively, from *Escherichia coli* according to published procedures (18, 24). Recombinant $p300_{His6}$ was purified from Sf9 cells infected at a MOI of 5 (32).

Chromatin assembly. Core histones were deposited onto the p-306/G-less template (2) that contains HTLV-1 LTR sequence encompassing the three viral CREs and core promoter regions through the use of the NAP/ACF recombinant chromatin assembly system previously described (14). Briefly, core histones were preincubated with dNAP-1 (1:6 [wt/wt]) on ice for 30 min in 25 mM HEPES (K^+, pH 7.6), 0.05 mM EDTA, 5% glycerol. ACF (25 ng per 150 ng DNA) was added to the core histone/dNAP-1 mix, followed by the addition of an ATP-regenerating system (3 mM ATP, 30 mM phosphocreatine, 1 μg/ml creatine phosphokinase). Supercoiled plasmid DNA was added to the assembly reactions and incubated for 4 to 18 h at 27°C under final conditions of 10 mM HEPES (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 1% polyethylene glycol, and 0.01% NP-40. Histone H1 was added 1.5 h postassembly to the chromatin template where indicated at a 1:25 H1:octamer molar ratio. Reactions were adjusted to assemble 150 ng (67 fmol) of DNA in a 7 μl volume (amount added to a single in vitro transcription reaction); however, actual assembly volumes ranged from 50 to 600 μl when larger amounts of DNA template were assembled.

MNase digestion. Micrococcal nuclease (MNase) digestion was performed on 2.1 μg of assembled DNA (98 μl assembled chromatin). First, 135 μl of MNase buffer (1.74 mM HEPES [pH 7.6], 121 mM KCl, 12% [vol/vol] glycerol, 2.4% [wt/vol] polyethylene glycol, 11.2 mM $CaCl_2$) were added to chromatin samples and incubated at 37°C for 5 min. Digestion was initiated by addition of 0.12 U MNase (Worthington) to templates lacking histone H1 and 0.48 U MNase to H1-containing templates at 37°C. After 1, 2, 4, and 8 min, 60 μl aliquots were removed and digestion was stopped with 12 μl Tris-EDTA (6 μl $T_{10}E_1$–6 μl 0.5 M EDTA). Samples were deproteinized with 9.6 μl 2.5 mg/ml proteinase K and 96 μl chromatin stop (20 mM EDTA, 200 mM NaCl, 1% [wt/vol] sodium dodecyl sulfate [SDS], 0.25 mg/ml glycogen) at 37°C for 30 min. The DNA was extracted with phenol-chloroform followed by ethanol precipitation. Nucleic acids were analyzed on a 1.2% Tris-borate-EDTA agarose gel and visualized with SYBR gold. Gene ruler 100 bp plus markers (Fermentas) were used as DNA size standards.

Sucrose gradients. For sucrose gradient analysis, 4.2 μg of DNA was assembled into chromatin with various H1:octamer molar ratios. Chromatin samples were resolved using 13 ml 15% to 40% sucrose gradients (10 mM Tris-HCl [pH 7.8], 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine). Gradients were centrifuged at 4°C in an SW41Ti (Beckman) rotor at 40,000 rpm for 4 h. Gradient fractions were collected (1.2 ml) from top to bottom. Proteins were trichloroacetic acid-deoxycholate precipitated using 800 μl of each fraction, resolved on 15% SDS-polyacrylamide gel electrophoresis (PAGE), and visualized with SYPRO ruby. DNA was deproteinized and precipitated from 200 μl of each gradient fraction, resolved on a 1% Tris-borate-EDTA agarose gel, and visualized with SYBR gold.

In vitro transcription assays. Following chromatin assembly, 150 ng (67 fmol) of assembled DNA in a 7 μl volume was added to transcription reaction mixtures containing TM buffer (1 mM dithiothreitol, 25 mM Tris-HCl [pH 7.9], 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol) and 50 μM AcCoA in a 30 to 40 μl final volume. Exogenous Tax and CREB (1.6 pmol each) were added after or during the chromatin assembly where indicated. p300 (0.3 pmol) was also added to transcription reactions where indicated. Preinitiation complexes were formed with the addition of 40 μg CEM (HTLV-1-negative T-cell line) nuclear extract (10), and samples were incubated for 60 min at 30°C. RNA synthesis was initiated with addition of 250 μM ATP, 250 μM CTP, 12 μM UTP, and 0.8 μM [α-^{32}P]UTP (3,000 Ci/mmol) and incubated for 30 min at 30°C. Following RNA synthesis, 100 U of RNase T1 were added and samples were incubated for 30 min at 37°C. Proteins were digested for 30 min at 50°C with 8.5 μl 10 mg/ml proteinase K and 10 μl transcription stop (250 mM NaCl, 1% SDS, 20 mM Tris-HCl [pH 7.5], 5 mM EDTA). A 622-bp DNA fragment, isolated from an HpaII digest of pBR322 and end labeled, was added for a recovery standard. RNA products and labeled DNA recovery standards were precipitated and resolved on 6.5% sequencing urea-PAGE. Radiolabeled HpaII-digested pBR322 served as molecular weight size markers. All radioactive experiments were imaged using STORM phosphorimaging and quantified with Image Quant 5.1 software. Experiments were performed a minimum of three times.

In vitro acetylation assays. For in vitro acetylation assays, 2 μg of core histones were assembled into chromatin (1.26 pmol DNA). Tax, CREB, and P-CREB (30 pmol each) were included in the chromatin assembly reaction where indicated. A slight excess (8.4 pmol) of p300 was used compared to that used in transcription studies. Final reaction volumes varied from 100 to 150 μl and contained 75 μM ^{14}C-labeled AcCoA (56 mCi/mmol specific activity) under final conditions that included TM buffer with 10 mM sodium butyrate and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated for 60 min at 30°C. Proteins were methanol-chloroform precipitated and resolved on 18% SDS-PAGE. Free (unassembled) core histones were acetylated for a marker. Experiments were performed at least three times.

DNase I primer extension footprinting. Experiments were performed as described previously (50) with the following modifications. Briefly, p-306/G-less plasmid DNA (56 fmol) was assembled into chromatin in a 25 μl volume. CREB was titrated (1.3, 2.7, 6.7, and 13.4 pmol) and added after chromatin assembly where indicated in a final volume of 50 μl in TM buffer. Binding reactions were incubated for 1 h at 30°C. Digestion was performed as described previously (50), except digestion was stopped by placing samples in a dry ice-EtOH bath for 10 min. Digested DNA (10 fmol) was incubated with 50 fmol of [γ-^{32}P]ATP-labeled primer, and primer extension was performed as described previously (50). To footprint the distal and middle vCREs, we used the primer 5′-TCGATAAGCT TCTAGACCTCCCAGTG-3′, which binds ~70 bp upstream of the distal vCRE within pUC13. To footprint the proximal vCRE we used the primer 5′-TCAG CCATATGCGTGCCATGAA-3′, which anneals to the promoter at position −47 relative to the transcription start site. Following primer extension, the DNA was precipitated and resolved on a 6.5% sequencing gel. Experiments were performed a minimum of three times. Percent accessibility is only shown for the distal vCRE, as the degrees of CREB binding for all of the vCREs are similar.

Immobilized chromatin templates. Biotinylated DNA fragments from −288 to +408 and −69 to +408 of the HTLV-1 LTR were prepared by standard PCR using biotinylated forward primers. PCR products were purified using preparative-cell electrophoresis (Bio-Rad) and bound to M 280 streptavidin Dynabeads (Dynal Biotech ASA) (10 μl beads/pmol DNA) as described by the manufacturer. Chromatin templates were assembled using 2 pmol biotinylated DNA. Tax (18 pmol), CREB or phospho-CREB (12 pmol), and H1 (1.25 molar excess per octamer) were added to the assembly reactions where indicated in a final volume of 50 μl in TM buffer. Chromatin was assembled for 4 h at 27°C. After assembly, factors were added as indicated (0.75 pmol p300) and the volume was adjusted

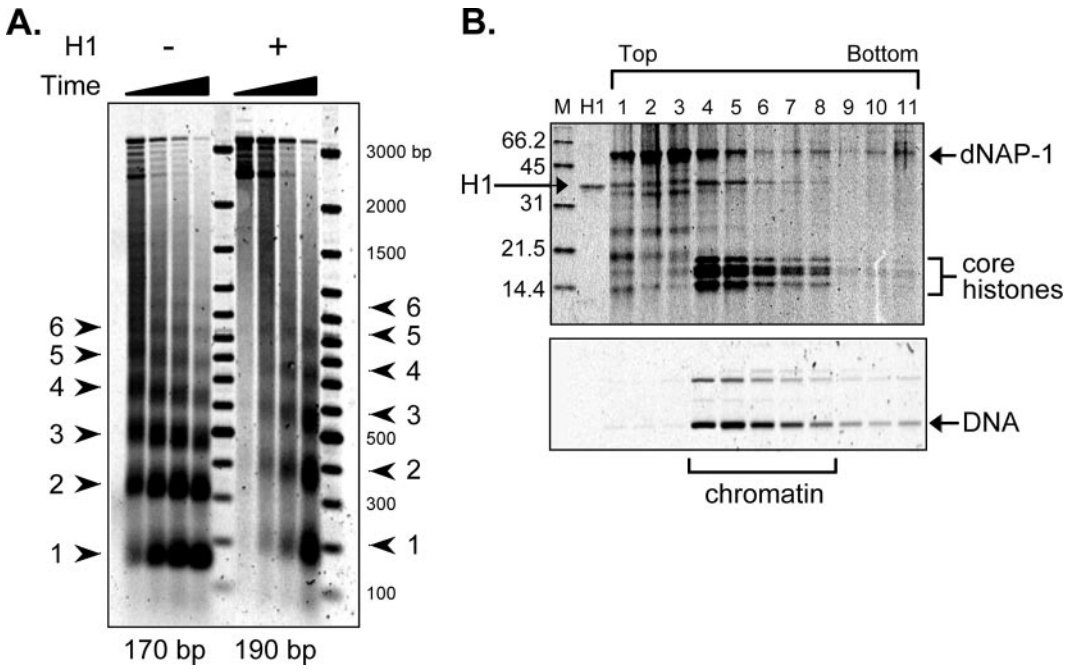

FIG. 1. Histone H1 is properly incorporated into chromatin through use of a recombinant assembly system. (A) Histone H1 incorporation (1.25 molar excess to octamer) increases nucleosome repeat length from ~170 to ~190 bp. Chromatin was assembled on the p-306/G-less template (0.94 pmol) through use of recombinant NAP-1 and ACF assembly factors in the absence or presence of histone H1 as indicated and digested with MNase for 1, 2, 4, and 8 min. Nucleosomal DNA was resolved on a native 1.2% agarose gel and visualized by SYBR gold staining. The numbers and positions of nucleosomes for each template are labeled numerically. Base pair size markers are indicated to the right. (B) Histone H1 is stably incorporated into chromatin. Chromatin containing H1 was purified over 15% to 40% sucrose gradients. Proteins from each fraction were resolved on 15% SDS-PAGE and visualized with SYPRO ruby (top). Molecular weight markers (M) are shown on the left. Positions of H1, core histones, and the major chromatin peak fractions are also indicated. DNA from each fraction was also resolved using agarose gel electrophoresis and SYBR gold (bottom).

to 100 μl in TM buffer. Binding reactions were performed for 45 min at 27°C. Immobilized templates were washed twice with 100 μl TM buffer containing 100 mM KCl, resuspended in SDS-PAGE loading dye, and processed by Western blotting. Experiments were performed a minimum of three times. Antibodies against CREB (SC-186), P-CREB (SC-7978-R), and p300 (SC-584) were purchased from Santa-Cruz Biotechnology. H3 (Ab 1791) and H1 (V7013) antibodies were purchased from Abcam and Biomeda, respectively. A monoclonal Tax antibody (Hybridoma 168B17-46-92) was obtained from the National Institutes of Health Aids Research and Reference Reagent Program.

RESULTS

Proper incorporation of histone H1 into in vitro-assembled chromatin templates. H1-containing chromatin was assembled with purified H1 and core histones by the use of a well-characterized, highly defined assembly system (14). Histones were deposited onto a plasmid harboring natural HTLV-1 promoter sequence (see Fig. 2A) through the use of NAP-1 and ACF. Typically, one molecule of H1 binds per core histone octamer in vivo (3); therefore, it is critical to assemble chromatin with the appropriate ratio of histone H1 to core histones. Chromatin samples were assembled at different H1-to-core histone octamer ratios. Since a nucleosome containing histone H1 protects 10 to 20 additional base pairs from micrococcal nuclease digestion (36, 57), we used increased nucleosome repeat length to verify proper and stoichiometric H1 incorporation. The nucleosomal repeat length increased from 169 bp in the absence of H1 to 186 bp upon H1 addition, confirming linker histone incorporation (Fig. 1A). We found that a slight molar excess of histone H1 (1.25:1) to histone octamer was needed to achieve a uniform increase in repeat length.

We tested directly for any unincorporated linker histone by separation from assembled chromatin templates on 15% to 40% sucrose gradients (Fig. 1B). At H1-to-octamer molar ratios up to and including 1:25, no free H1 protein was observed (H1 runs between the second and third largest major proteins at the top of the sucrose gradient). As the H1-to-octamer ratios were increased past 1:25, we observed significant levels of free, unassembled H1 (data not shown). Therefore, we chose templates assembled with an H1-to-octamer ratio of 1.25 for subsequent experiments. Together, these experiments confirm proper incorporation of histone H1 to the chromatin template by use of a recombinant assembly system.

H1-chromatin represses HTLV-1 transcriptional activation. Having optimized H1 incorporation, we next examined the effects of chromatin containing linker histone on HTLV-1 transcriptional activation. Plasmid templates containing the HTLV-1 viral promoter upstream of a G-less cassette (Fig. 2A) were assembled into chromatin in both the absence and the presence of histone H1. Exogenous Tax, CREB, and p300 were added to the transcription reactions after chromatin assembly and H1 incorporation, where indicated. All reactions contained AcCoA and nuclear extract prepared from an uninfected T-cell line (CEM), which contains the RNA polymerase II transcription machinery.

H1-chromatin reduced relative transcription from the HTLV-1

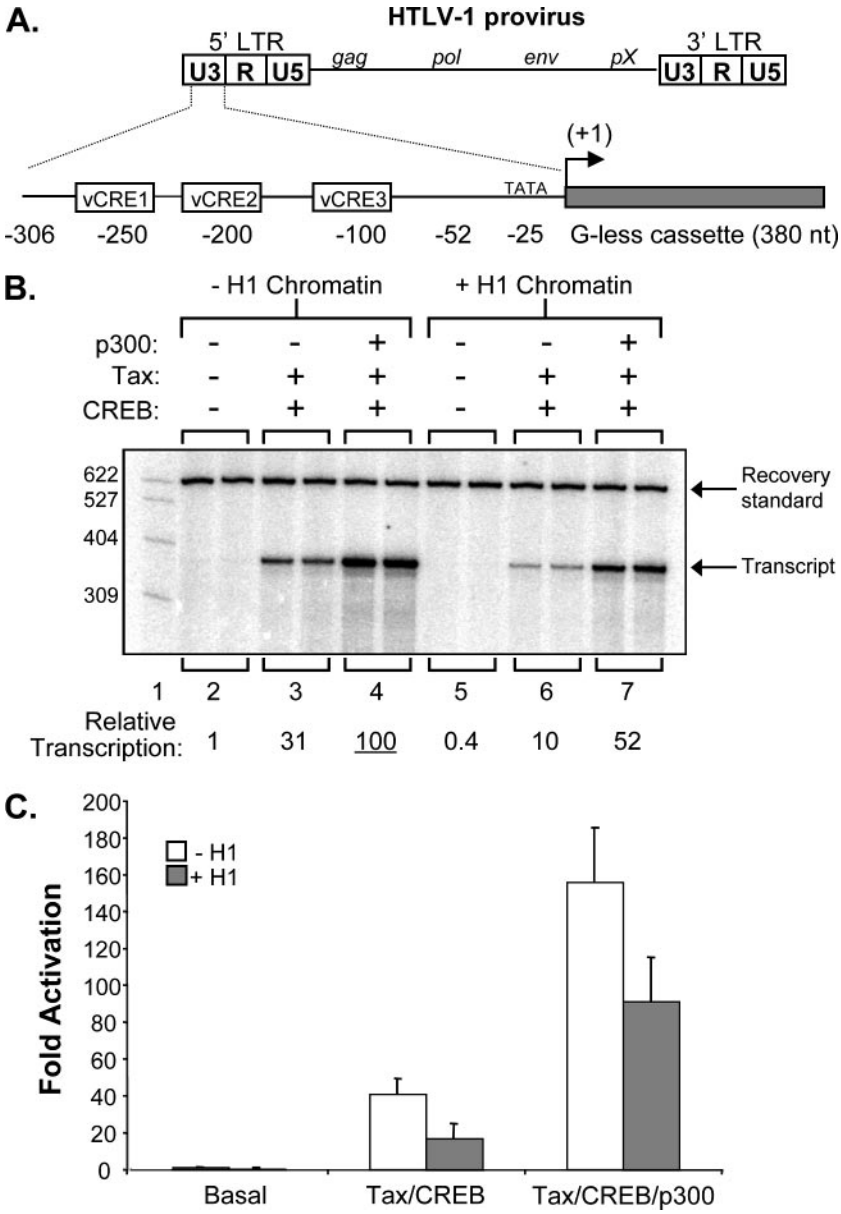

FIG. 2. Incorporation of histone H1 into chromatin represses HTLV-1 transcriptional activation. (A) Promoter schematic of the p-306/G-less construct relative to the HTLV-1 genome. The approximate positions of the TATA box and vCREs are indicated relative to the transcription start site at +1. (B) In vitro transcriptional analysis of chromatin containing histone H1. Chromatin (67 fmol p-306/G-less DNA) was assembled without or with H1 and transcription was assayed in the presence (+) or absence (−) of recombinant CREB (1.6 pmol), Tax (1.6 pmol), and p300 (0.3 pmol) as indicated. CREB, Tax, and p300 were added to transcription reaction mixtures after chromatin assembly (see Fig. 3A). All reaction volumes contained 50 μM AcCoA and ~40 μg CEM T-cell (uninfected) nuclear extract. Transcript levels were averaged between duplicate lanes and are expressed relative to the results seen with the maximally activated Tax/CREB/p300-dependent transcript, which is set to 100 (lane 4). The positions of RNA transcript and labeled DNA recovery standard are indicated to the right, while labeled DNA size markers (in nucleotides) are indicated to the left of the gel. (C) Graphical representation of quantitation data from five independent experiments measuring transcription from chromatin either lacking (open bars) or containing (gray bars) histone H1. The severalfold activation values were calculated relative to basal transcription from chromatin assembled in the absence of H1, which is set to 1.

promoter compared to that from chromatin lacking the linker histone (Fig. 2B, lanes 2 to 4 versus 5 to 7). Basal and Tax/CREB-mediated transcription levels were reduced about threefold (Fig. 2B, lane 2 versus 5 and 3 versus 6), while Tax/CREB/p300-mediated transcription was reduced twofold (Fig. 2B, lane 4 versus lane 7). The effects of H1 were chromatin specific, as H1 addition to free (unassembled) DNA at levels used in chromatin assembly had no significant impact on the levels of transcription (data not shown).

Tax counteracts H1-chromatin repression of transcriptional activation. As a primary step in elucidating the mechanism of H1 repression at the HTLV-1 promoter, we examined the ability of Tax and CREB to compete with H1. For these experiments, chromatin was assembled without or with H1. In

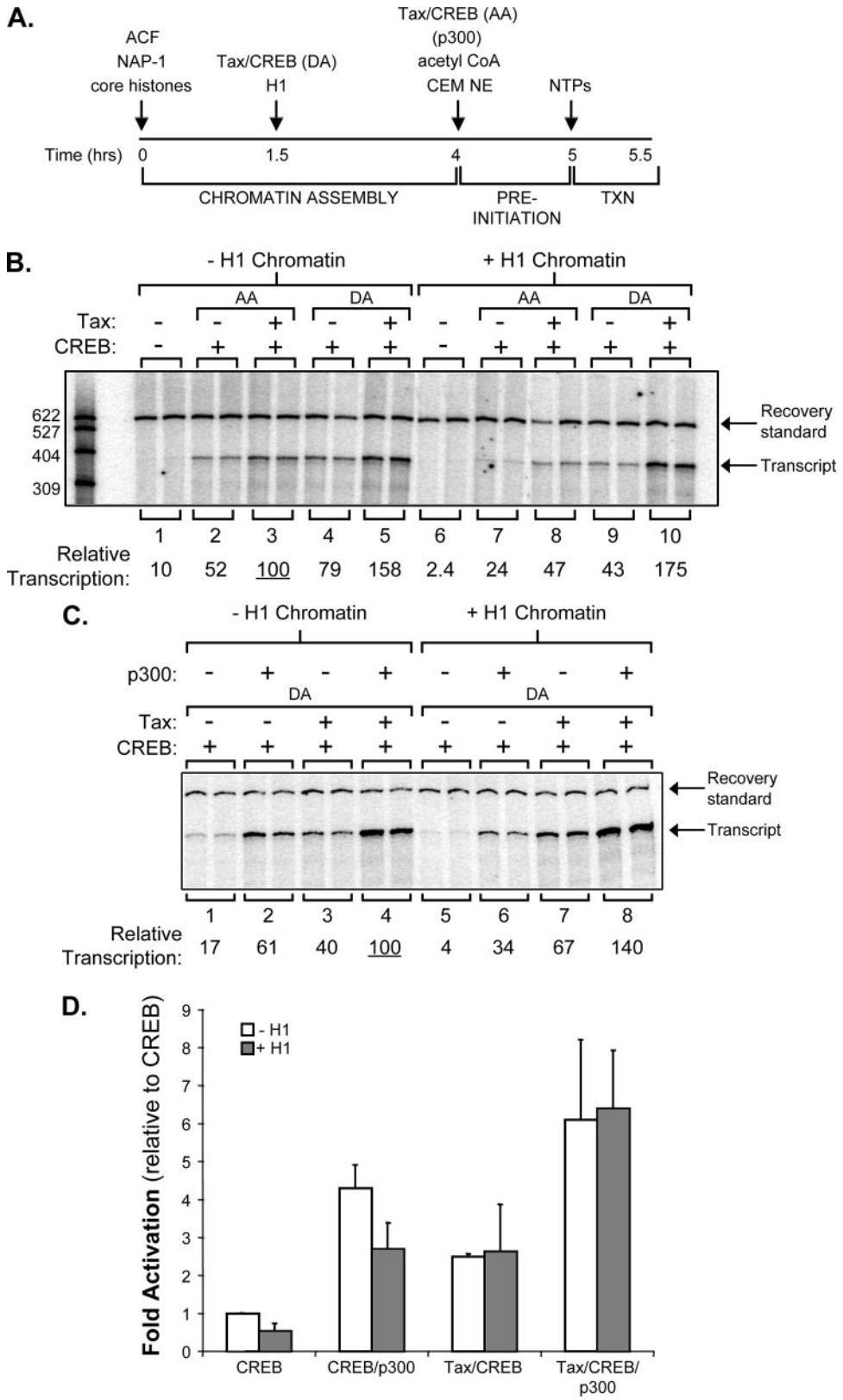

FIG. 3. Tax counteracts the H1-chromatin repression of transcriptional activation. (A) Timeline of chromatin assembly and factor addition for in vitro transcription reactions. (B) Tax and CREB, but not CREB alone, overcome H1 repression of transcriptional activation. Chromatin (67 fmol p-306/G-less DNA) was assembled without or with histone H1. Equivalent amounts of Tax and CREB (1.6 pmol each) were added either after chromatin assembly (AA) or during chromatin assembly (DA) as indicated for each chromatin template. Transcript levels were averaged between duplicate lanes and calculated relative to lane 3, which is set to 100. (C) Addition of p300 does not overcome H1 repression of CREB-mediated transcriptional activation.

addition, we were concerned that Tax function may require an as-yet-unidentified chromatin-remodeling component or that Tax be present during S phase of the cell cycle. The addition of Tax with CREB during assembly would bypass these potential early chromatin-remodeling steps. Linker histone addition 1.5 h into the assembly reaction resulted in the most consistent incorporation. Tax and CREB (or CREB alone) were added either after the 4-h chromatin assembly or during assembly at 1.5 h along with H1 (for a schematic of the experimental timeline, see Fig. 3A). Upon complete assembly (4 h), chromatin templates were incubated with AcCoA and nuclear extract, and transcription was initiated with the addition of NTPs (Fig. 3A). Where indicated, exogenous p300 was added to the transcription reactions after chromatin assembly.

As described above, H1-chromatin repressed Tax/CREB-mediated transcriptional activation twofold when added after assembly (Fig. 3B, lane 3 versus 8). When CREB alone was added after chromatin assembly, transcription also decreased twofold in the presence of H1-chromatin (Fig. 3B, lane 2 versus 7). Even when CREB was added during chromatin assembly, CREB-alone activation was repressed twofold by chromatin containing H1 (Fig. 3B, lane 4 versus 9). However, addition of Tax together with CREB during chromatin assembly completely counteracted the H1-chromatin repression of CREB transcriptional activation (Fig. 3B, lane 5 versus 10). We performed micrococcal nuclease digestion analysis to verify that Tax and CREB addition during assembly did not compromise the integrity of our chromatin templates. We obtained ladders of the same quality as those seen when chromatin was assembled in the absence of transcription factors (data not shown).

We next tested the effect of exogenous p300 addition on transcriptional repression by H1. The addition of exogenous p300 after assembly (see schematic in Fig. 3A) on both chromatin templates increased the relative level of transcription in the presence of both CREB and Tax/CREB (Fig. 3C and D). However, H1 incorporation still produced about a twofold reduction in CREB/p300-mediated transcription (Fig. 3C, lane 2 versus 6, and Fig. 3D), indicating that p300 cannot counteract H1 repression and that H1-chromatin may function through inhibition of CREB binding. While p300 addition to chromatin templates assembled with Tax/CREB augmented transcriptional activation in the presence of H1 (Fig. 3C, lane 7 versus 8, and Fig. 3D), exogenous p300 was not required for Tax and CREB to completely counteract H1-chromatin repression (Fig. 3C, lane 3 versus 7, and Fig. 3D).

CREB binding to the vCREs is not reduced in the context of H1-chromatin. To directly test the hypothesis that H1-chromatin represses transcription through an inhibition of CREB binding to the vCREs, we performed DNase I primer extension footprinting analysis. CREB binding was determined using the same templates and salt concentrations as in transcription studies. Chromatin was assembled without or with histone H1 followed by incubation with increasing concentrations of CREB. The templates were digested with DNase I, and DNA cleavage was analyzed by primer extension.

Surprisingly, incorporation of H1 into chromatin produced no measurable decrease in CREB binding at the vCREs. At the lowest ratio of CREB per vCRE binding site (the same fourfold molar excess as that used in transcription reactions), CREB addition after chromatin assembly resulted in similar DNase I protection on either chromatin template (Fig. 4 lane 3 versus 10). As CREB concentrations were increased to a 40-fold molar excess relative to binding sites, DNase I protection continued to increase by essentially equal levels on both chromatin templates (Fig. 4, lane 6 versus 13). We have also footprinted the promoter proximal vCRE and found no change in the ability of CREB to bind upon H1 incorporation (data not shown). Based on these data, we concluded that the mechanism by which H1-chromatin represses transcriptional activation is not through an effect on CREB binding.

As would be expected, when CREB was added during chromatin assembly, H1 incorporation had no effect on CREB binding and addition of Tax during assembly did not increase CREB binding to H1-chromatin (data not shown). Therefore, Tax functions at a step downstream of CREB binding in counteracting H1-chromatin repression.

Chromatin containing histone H1 inhibits p300 activity. Both in vitro and in vivo studies have demonstrated the importance of p300 in HTLV-1 transcriptional activation (Fig. 2B and 3C; see also references 15, 16, 40, and 43). In vitro, the Tax/CREB complex has been shown to directly recruit p300 and stimulate the intrinsic acetyltransferase activity of this coactivator (15, 44). Therefore, we tested whether H1-chromatin affected Tax/CREB stimulation of p300 activity by use of histone acetyltransferase (HAT) assays. The same plasmid template (Fig. 2A) used in transcription and footprinting studies was assembled into chromatin containing or lacking linker histone. Upon assembly, chromatin was incubated with Tax, CREB, p300, and ^{14}C-labeled AcCoA. The core histone proteins were resolved using SDS-PAGE, and ^{14}C incorporation was analyzed as described in Materials and Methods.

Addition of Tax/CREB after chromatin assembly increased relative acetylation above basal levels from both chromatin templates (Fig. 5A, lane 2 versus 3, and Fig. 5B). However, incorporation of H1 decreased basal histone acetylation (Fig. 5A, lane 2 versus 4, and Fig. 5B). H1 also decreased Tax/CREB-stimulated acetylation about threefold when these activators were added after chromatin assembly (Fig. 5A, lane 3 versus 5, and Fig. 5B). Repression of p300 histone acetylation paralleled the reduction in transcription observed for chromatin containing H1 when Tax and CREB were added after assembly (Fig. 2B, lane 4 versus 7, and Fig. 2C). Free H1 (not incorporated into nucleosomes during assembly) had no effect on p300 HAT activity (data not shown). To verify that the

Chromatin was assembled as described above. Tax and CREB (1.6 pmol each) were added during chromatin assembly (DA), while p300 (0.3 pmol) was added after chromatin assembly (AA) to the transcription reaction volumes as indicated. Transcript levels were averaged between duplicate lanes and calculated relative to lane 4, which is set to 100. (D) Graphical representation of three independent transcription experiments from chromatin lacking (open bars) or containing (gray bars) histone H1 assembled with factors added during chromatin assembly (DA). The severalfold activation values were calculated relative to CREB-alone transcription from chromatin assembled without H1, which is set to 1.

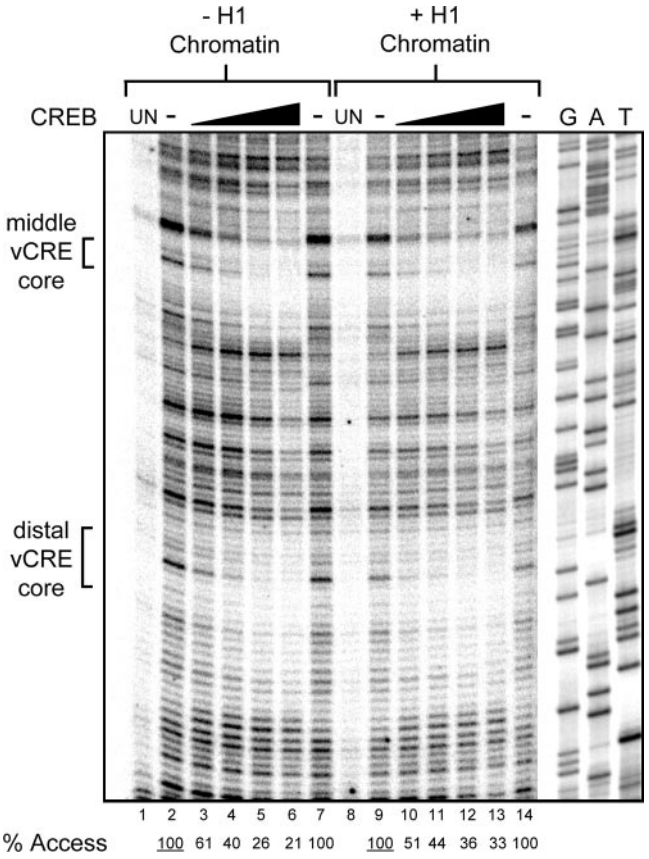

FIG. 4. H1-chromatin repression does not result from inhibition of CREB binding. The results of DNase I primer extension footprinting analysis of CREB binding on chromatin templates (56 fmol p-306/G-less DNA) assembled in the absence and presence of histone H1 are shown. CREB was added after chromatin assembly to each template and titrated from a 4- to 40-fold molar excess as indicated by triangles (1.3, 2.7, 6.7 and 13.4 pmol of CREB). Undigested DNA (UN) and no-protein (−) lanes are labeled for both chromatin templates. ^{32}P-labeled DNA products from the primer extension reaction were resolved on an 8% sequencing gel. Protection was observed at both the middle and distal vCRE core regions as CREB was titrated onto the DNA. Protein binding was measured as a percentage of DNA accessibility, with the digested, no-protein (−) lanes set as 100 percent accessible. The percentages of accessibility were calculated for the distal vCRE, although similar levels of protection were calculated for the middle and proximal vCREs.

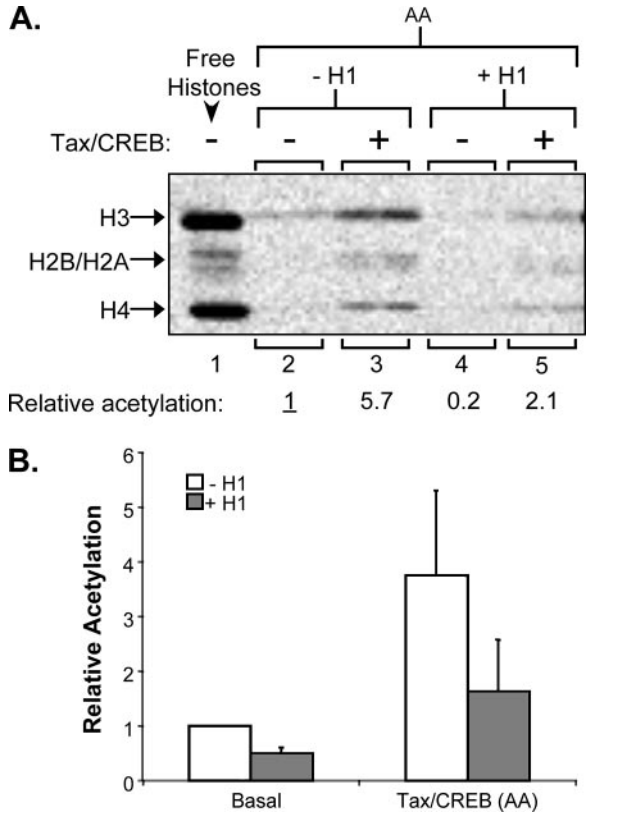

FIG. 5. H1-chromatin repression operates through an effect on p300. (A) In vitro histone acetyltransferase activity of p300 was measured by ^{14}C-labeled AcCoA incorporation into histones present on chromatin templates (1.26 pmol p-306/G-less DNA) assembled in the absence or presence of linker histone. Acetylation reactions were performed with recombinant p300 (8.4 pmol) in the presence (+) or absence (−) of Tax and CREB (30 pmol each) added after chromatin assembly (AA). Histones were resolved via 18% SDS-PAGE. Free histones were acetylated by p300 for size markers, and positions of each core histone are indicated on the left. Acetylation levels were calculated relative to chromatin lacking H1 in the absence of Tax and CREB (set to 1) and are displayed as the average values between duplicate lanes. (B) Tax/CREB-mediated acetylation by p300 is vCRE dependent. The p-52/G-less construct (Fig. 2A, 1.26 pmol DNA) was assembled into chromatin without or with histone H1, and acetylation reactions were performed as above. (C) Graphical representation of acetylation levels on chromatin assembled without (open bars) or with (gray bars) linker histone. Tax and CREB were added after chromatin assembly (AA). Data were calculated from three independent experiments, and acetylation levels are relative to basal from templates lacking H1, which is set to 1.

increase in acetylation required vCRE-mediated recruitment of Tax and CREB, we assembled a plasmid carrying the HTLV-1 promoter deleted to −52 (see schematic promoter diagram in Fig. 2A). This construct, which lacks the vCREs, was assembled into chromatin in the absence or presence of linker histone. The results obtained demonstrated that p300-mediated histone acetylation requires Tax/CREB binding to the vCREs (data not shown).

These data indicate that H1-chromatin reduces Tax/CREB-mediated transcriptional activation through an effect on p300 recruited to the HTLV-1 promoter. The data support a model in which H1 inhibits p300 recruitment and/or enzymatic activity.

Tax completely overcomes the H1-chromatin repression of p300 activity. Having determined that incorporation of linker histone into our chromatin templates affects p300 function, we turned our focus to the mechanism of Tax in overcoming H1-chromatin transcriptional repression. This function of Tax is most pronounced when it is added together with CREB during chromatin assembly. Therefore, we examined the ability of Tax and CREB to stimulate p300 HAT activity when added during chromatin assembly either in the absence or presence of histone H1 (see schematic of experimental time line in Fig. 3A).

Tax/CREB addition during chromatin assembly abrogated H1-chromatin repression of p300 histone tail acetylation (Fig. 6A, lanes 3 and 5, and Fig. 6C). The degree to which these

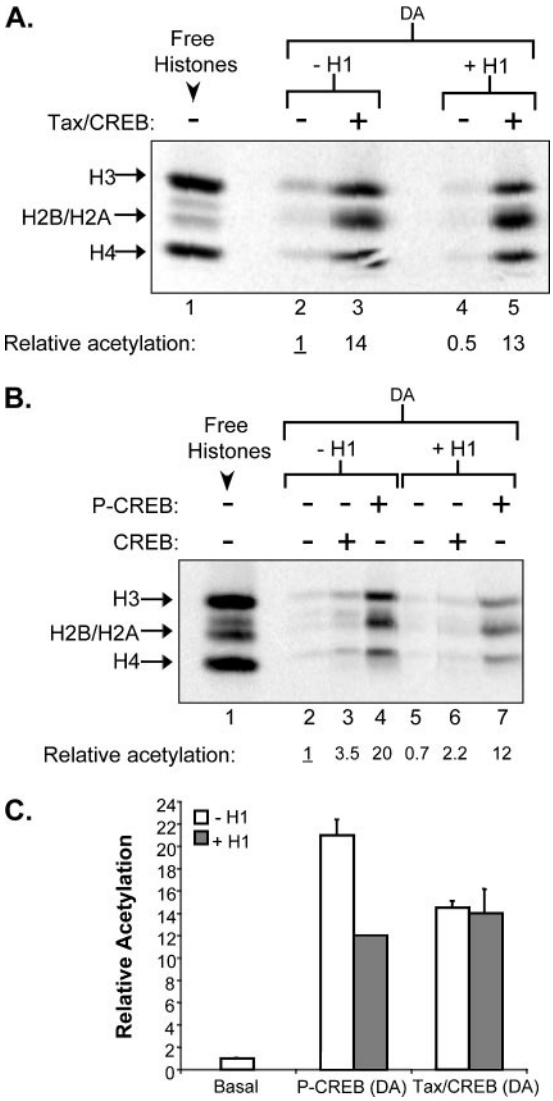

FIG. 6. Tax completely overcomes H1 repression of p300 activity. (A) Addition of Tax and CREB during chromatin assembly (DA) (Fig. 3A) reverses the effect of histone H1 on p300 activity. In vitro histone acetyltransferase activity of p300 was measured by ^{14}C-labeled AcCoA incorporation onto chromatin templates (1.26 pmol p-306/G-less DNA). Acetylation reactions were performed with recombinant p300 (8.4 pmol) in the presence (+) or absence (−) of Tax and CREB (30 pmol each) added during chromatin assembly (DA) on chromatin templates containing (+) or lacking histone H1 (−) where indicated. Free histones were acetylated by p300 for size markers, and positions of each histone are labeled on the left. Acetylation levels were calculated relative to chromatin lacking H1 in the absence of Tax and CREB (lane 2, set to 1). (B) Chromatin containing H1 represses P-CREB-mediated p300 HAT activity. Acetylation reactions were performed as described above except that either P-CREB or CREB (30 pmol each) was added without Tax to each reaction mixture during chromatin assembly (DA) on chromatin templates assembled without or with histone H1. The positions of acetylated free histones are labeled on the left. Acetylation levels were calculated relative to chromatin lacking H1 in the absence of CREB and P-CREB (lane 2, set to 1). (C) Graphical representation of acetylation levels from factor addition during chromatin assembly (DA) on templates lacking (open bars) or containing (gray bars) histone H1. Data were calculated from two independent experiments for P-CREB and from three independent experiments for Tax/CREB. Acetylation levels are relative to basal from chromatin lacking H1, which is set to 1.

results mirror the derepression observed in transcriptional activation with Tax/CREB addition during chromatin assembly is striking (Fig. 3C, lane 4 versus 8, and Fig. 3D), suggesting that Tax functions through an effect on p300 activity in counteracting H1 repression of transcription. Unlike transcription reactions, HAT assays were performed using only purified proteins (no nuclear extract). Therefore, we can be certain that only Tax is required (together with CREB) to overcome the repressive effects of H1-chromatin.

Phosphorylation of CREB at Ser133 is a necessary step in the recruitment of p300 and subsequent transcriptional activation of cellular promoters (34, 47). Tax is thought to bypass the need for CREB phosphorylation in p300 recruitment (33). By extension, CREB phosphorylation may have the same effect as Tax in counteracting H1-chromatin repression of p300. To test the ability of phosphorylated-CREB (P-CREB) alone to overcome the effects of H1-chromatin, P-CREB was added during chromatin assembly without and with H1 and p300 HAT activity was assayed as before. We observed a twofold reduction in P-CREB-mediated p300 HAT activity caused by the presence of H1 (Fig. 6B, lane 4 versus 7, and Fig. 6C), demonstrating that P-CREB is not sufficient for counteracting the repressive effects of H1-chromatin on p300 even when added during assembly.

p300 acetyltransferase activity, not recruitment, is inhibited by chromatin containing H1. To further define the mechanism of H1 repression, we used immobilized chromatin templates containing HTLV-1 DNA. The promoter fragment carries the three viral CREs, while the control fragment lacks viral CREs (Fig. 2A). First, each fragment was bound through a 5′ biotin moiety to magnetic streptavidin beads. These bead-bound fragments were then assembled into chromatin without or with H1 using the recombinant assembly system. Tax and P-CREB were added either after or during chromatin assembly, and p300 was added after chromatin assembly where indicated. The templates were isolated using magnetic separation and washed, and the remaining bound proteins were resolved by SDS-PAGE and quantified by Western blot analysis. The differences in Tax, P-CREB, and p300 binding to the promoter fragment versus the control fragment demonstrated that their binding is vCRE specific (Fig. 7A).

We compared p300 promoter occupancy in the presence of P-CREB alone or P-CREB plus Tax added during or after assembly on chromatin templates without and with histone H1. In the absence of Tax and P-CREB, there was no significant binding of p300 to the promoter (Fig. 7B, lanes 1 and 2). A significant increase in p300 recruitment was observed in the presence of P-CREB (Fig. 7B, lanes 3 to 6). Addition of Tax together with P-CREB further enhanced p300 promoter occupancy above that seen with P-CREB alone (Fig. 7B; compare lanes 3 to 6 to lanes 7 to 10). Despite the differences in the amounts of p300 recruited to the promoter by P-CREB and Tax/P-CREB, p300 promoter occupancy was unaffected by chromatin assembly with H1 (Fig. 7B and C). These results clearly demonstrate that assembly of chromatin with H1 does not inhibit p300 recruitment to the promoter. Therefore, our data strongly support a model wherein H1 carries out its repressive effects via inhibition of p300 acetyltransferase activity. In addition, Tax/CREB, but not CREB alone, reverses this inhibition, resulting in a high degree of HTLV-1 transcriptional activation.

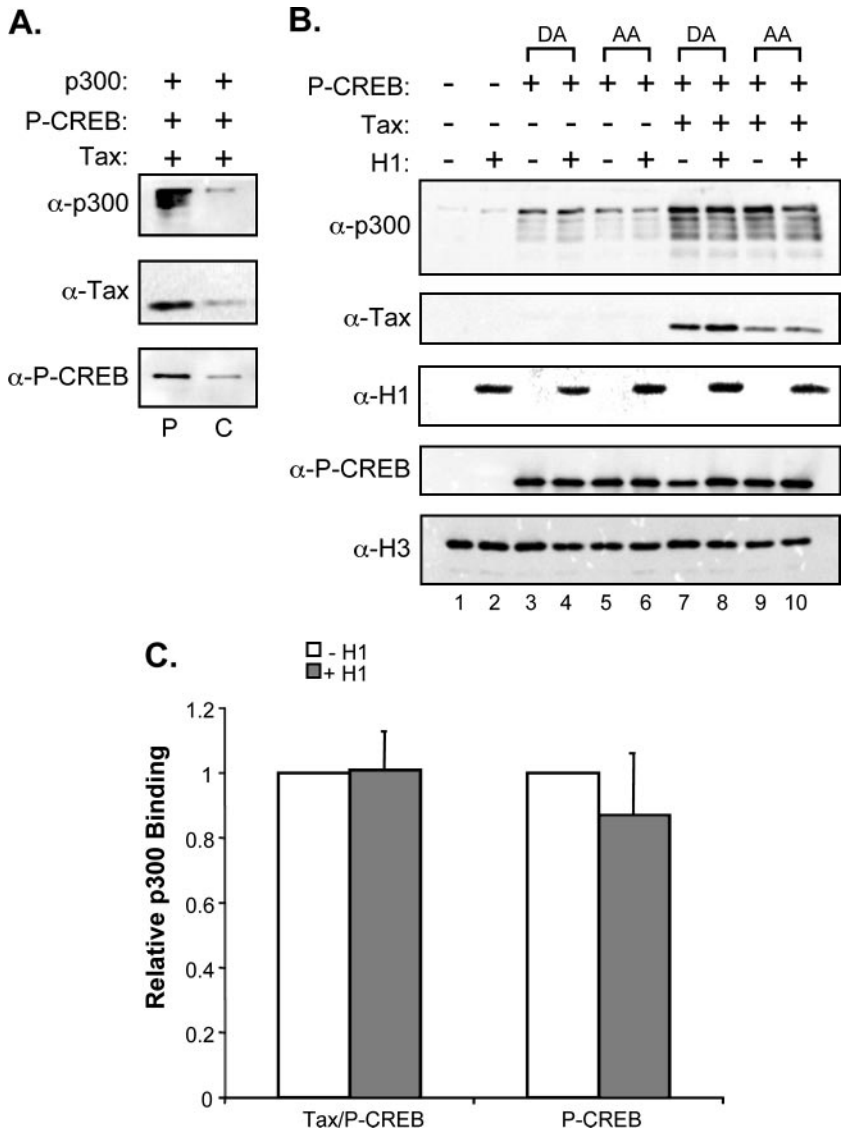

FIG. 7. Recruitment of p300 is not affected by chromatin containing H1. (A) Factor binding is vCRE dependent. Chromatin was assembled in the absence of linker histone on promoter (P; three vCREs) or control (C; no vCREs) DNA (2 pmol biotinylated fragment DNA). Recombinant Tax (18 pmol), P-CREB (12 pmol), and p300 (0.75 pmol) were added to the assembled chromatin templates. Upon isolation and washing of each template, the bound proteins were analyzed by Western blotting. Antibodies are indicated to the left of each blot. (B) Factor binding to the promoter fragment assembled into chromatin either in the absence (−) or presence (+) of histone H1. Tax and P-CREB were added during (DA) or after (AA) chromatin assembly, as indicated. All reaction mixtures contained p300, which was added after chromatin assembly. Bound proteins were detected using Western blot analysis with the indicated antibodies. (C) Graphical representation of p300 binding from either Tax/P-CREB or P-CREB-mediated recruitment in the context of chromatin lacking (open bars) or containing (gray bars) linker histone. Data were averaged between three independent experiments, and p300 binding is relative to chromatin assembled without H1 for each condition (set to 1).

Tax and CREB do not displace histone H1 from the promoter. We tested the hypothesis that Tax functions in counteracting H1 repression by displacing H1 from the promoter during chromatin assembly. Although micrococcal nuclease digestion experiments verified that global H1 incorporation was preserved, this assay could not detect a highly localized loss of H1 molecules at the promoter (data not shown). In contrast, approximately 3 chromatosomes form on each 700-bp bead-bound promoter fragment. Therefore, if Tax caused localized H1 displacement, the reduction would be detected by Western blotting. Surprisingly, the amount of linker histone assembled into chromatin does not change when Tax is added together with P-CREB either during or after chromatin assembly (Fig. 7B, lane 8 versus 10). Thus, Tax does not function in counteracting H1 repression through displacement of histone H1.

DISCUSSION

Despite the general characterization of histone H1 as a transcriptional repressor (36), there is no clearly defined mechanism for H1 repression. Rather, the data presented to date

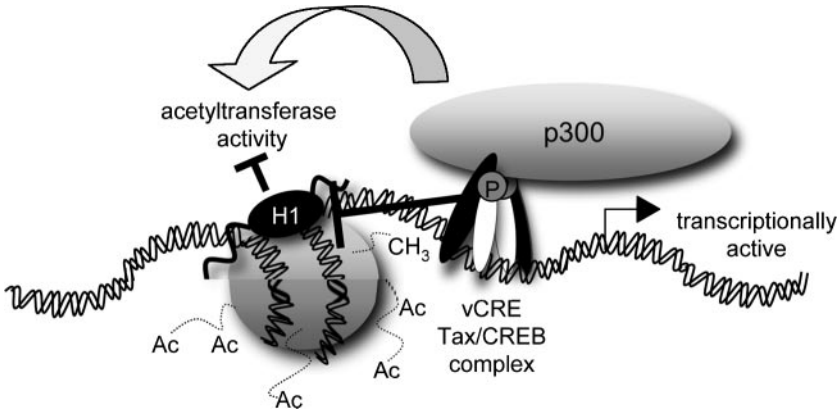

FIG. 8. Model for HTLV-1 transcriptional regulation in a chromatin context. H1 incorporation into nucleosomes inhibits p300 acetyltransferase activity, and Tax functions to abolish H1-chromatin repression, providing a means for tighter regulation of HTLV-1 transcription.

suggest that the effects of chromatin containing histone H1 are, to some degree, promoter specific (7, 9, 27, 31, 38, 53). Additionally, recent microarray data collected from mice embryonic stem cells null for three H1 isoforms demonstrated that mammalian H1 acts both negatively and positively in gene regulation (11).

In this study, we examined the effect of histone H1 on Tax-mediated activation of HTLV-1 transcription. Incorporation of H1 into the chromatin template repressed HTLV-1 transcriptional activation. The reduction in transcription was not attributable to decreased transcription factor binding, as we observed no change in either CREB or Tax promoter occupancy upon H1 incorporation. Rather, chromatin containing H1 inhibited p300 acetyltransferase activity directly without affecting p300 recruitment to the promoter. Additionally, we have discovered a previously undefined function of Tax, which is to overcome H1-mediated inhibition of p300 enzymatic activity.

H1 represses CREB transcriptional activation but not CREB binding. Although the histones, including H1, are evicted from the HTLV-1 promoter region upon Tax transactivation, Tax must first interact with chromatin that contains linker histone (39). Therefore, we examined steps prior to transcription initiation and RNA polymerase II recruitment in determining the role of H1 at the HTLV-1 promoter. Since H1-chromatin reduced transcriptional activation, we performed competition studies between CREB, Tax, and H1 and indirectly assayed factor binding by use of transcription assays. When Tax and CREB were added at the same time as histone H1 during chromatin assembly, the activators completely counteracted the repressive effects of H1-chromatin on transcriptional activation. However, CREB, in the absence of Tax, was not capable of overcoming H1 repression (Fig. 3B). Additionally, exogenous p300 failed to reverse the repressive effects of histone H1 on CREB-mediated activation (Fig. 3C). It is interesting that Tax must be present during chromatin assembly to abrogate H1 repression. This requirement may reflect the need for an additional remodeling activity or the need for Tax to be present during chromatin assembly in the cell cycle S phase.

Previous studies have shown that H1 interferes with transcription factor binding to the DNA (27, 56). We directly tested this possibility and found, through DNase I footprinting, that the repressive effects of H1 do not result from inhibition of CREB/vCRE interactions (Fig. 4). Additionally, we found no change in CREB or Tax binding to the vCREs on immobilized chromatin templates assembled in the absence or presence of H1 (Fig. 7B). These data are in agreement with Cheung et al., who demonstrated that estrogen receptor α binding is not affected by chromatin containing histone H1 (7). Further, Koop et al. found that H1 did not inhibit progesterone receptor binding to the mouse mammary tumor virus promoter. Instead, H1 increased the efficiency of progesterone receptor binding (31).

H1 represses transcription through inhibition of p300 activity. Additional mechanisms for H1 transcriptional repression include effects on factors with HAT activity (23). Given the presence of p300/CBP on the HTLV-1 promoter in vivo and the role of these coactivators in transcriptional activation, we examined the effects of H1-containing chromatin on p300 enzymatic activity and recruitment (16, 40, 43). While we have shown that the histone tails are not the primary targets of p300 in HTLV-1 activation, the nonhistone target(s) remains to be identified (15). Therefore, we used HAT assays as a general measure of p300 enzymatic activity. We found that H1-chromatin inhibited both Tax/CREB-mediated (targeted) and basal (nontargeted) p300 HAT activity (Fig. 5A). Thus, the twofold reduction in transcriptional activation observed could be attributed to the effects of H1-chromatin on p300 acetyltransferase activity (Fig. 2B). In agreement with our HAT data, others have previously demonstrated linker histone repression of nontargeted, recombinant PCAF (p300/CBP-associated factor) HAT activity (23). In contrast, Cheung et al. found no affect of H1 on estrogen receptor α-mediated stimulation of p300 HAT activity (7).

Intriguingly, we discovered that Tax and CREB addition during chromatin assembly abolished the repressive effects of H1-chromatin on p300 HAT activity (Fig. 6A), consistent with the derepression of transcription observed using H1-chromatin in this context (Fig. 3B and C). These data indicate that the function of Tax in counteracting histone H1 is attributable to an effect on p300 and suggest that Tax antagonizes H1 repression of HTLV-1 transcription via modulation of p300 function.

H1-chromatin directly represses p300 acetyltransferase activity. Herrera et al. attributed H1 repression of PCAF activity to steric hindrance of H3 N-terminal tail accessibility by the

H1 tails, but they did not examine whether PCAF interactions with the chromatin template were altered by H1 incorporation (23). To determine whether H1-chromatin directly inhibited p300 acetyltransferase activity or whether the observed repression was indirect and operated through an effect on p300 recruitment, we used immobilized chromatin templates. H1 incorporation into chromatin did not decrease P-CREB-mediated or Tax/P-CREB-mediated p300 recruitment, confirming that histone H1 inhibits p300 acetyltransferase activity at the HTLV-1 promoter.

Tax counteracts H1-chromatin repression of p300 enzymatic activity. Having defined the step in HTLV-1 transcriptional activation that H1 represses, we further probed the mechanism of Tax function in this process. Using immobilized chromatin templates, we demonstrated that Tax does not simply function through displacement of histone H1 at the promoter (Fig. 7B). Intriguingly, we observed a threefold increase in Tax promoter occupancy with addition during chromatin assembly relative to the level of Tax occupancy when added after chromatin assembly (Fig. 7B). However, we did not observe a concomitant increase in p300 binding. In fact, the level of p300 recruitment remained constant, regardless of when Tax and P-CREB were added to the assembly reaction mixtures. Furthermore, the increase in p300 recruitment observed with Tax/P-CREB over that seen with P-CREB alone, when added after chromatin assembly, did not correlate with counteracting H1 repression of p300 activity or transcriptional activation. The simplest interpretation of our findings is that Tax directly targets the inhibition of p300 enzymatic activity to abrogate H1-chromatin repression of HTLV-1 transcription.

H1 and Tax regulation of the HTLV-1 promoter. We propose a model that defines a role for histone H1 in regulation of the HTLV-1 promoter during early events in transcriptional activation (Fig. 8). Incorporation of linker histone into chromatin does not affect CREB, Tax, or p300 association with the promoter. Rather, H1 inhibits p300 directly through reduction of acetyltransferase activity, correlating well with the same degree of transcriptional repression of HTLV-1 transcription. In addition, when Tax is present with CREB during chromatin assembly, the effects of H1 on p300 acetyltransferase activity are alleviated (Fig. 8). While the exact mechanism has yet to be elucidated, we propose several models for Tax function in overcoming H1 repression of p300 HAT activity. Tax may alter p300 substrate accessibility. Addition of Tax during assembly could also affect higher-order chromatin structure, which, in turn, could lead to effects on protein-protein and protein-DNA interactions. Additionally or alternatively, Tax could disrupt H1 interactions that allosterically inhibit p300 activity, as AcCoA has been shown to allosterically regulate p300 inhibitory activity (52). Either mechanism is consistent with previous findings that the globular domain of H1/H5 is not sufficient for inhibition of HAT activity. Rather, the tails of H1 are responsible for mediating decreased acetylation (23). Coupled with our finding that histone H1 is not displaced, these data suggest that p300 repression is mediated by the H1 tails rather than the globular domain. Future experiments will be directed at differentiating between these possible mechanisms.

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