

## Enhancement of Nuclear Factor- $\kappa$ B Acetylation by Coactivator p300 and HIV-1 Tat Proteins\*

Received for publication, August 15, 2001, and in revised form, October 30, 2001  
Published, JBC Papers in Press, December 5, 2001, DOI 10.1074/jbc.M107848200

Bansri Furia<sup>‡§</sup>, Longwen Deng<sup>‡§</sup>, Kaili Wu<sup>‡</sup>, Shanese Baylor<sup>‡</sup>, Kylene Kehn<sup>‡</sup>, Hong Li<sup>¶</sup>,  
Robert Donnelly<sup>¶</sup>, Tim Coleman<sup>\*\*</sup>, and Fatah Kashanchi<sup>‡ ††</sup>

From the Departments of <sup>¶</sup>Biochemistry and Molecular Biology and <sup>||</sup>Pathology and Pediatrics, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103, <sup>\*\*</sup>Human Genome Sciences, Inc., Rockville, Maryland 20850, and the <sup>‡</sup>George Washington University School of Medicine, Washington, D. C. 20037

Nuclear factor (NF)- $\kappa$ B transcription factors are involved in the control of a large number of normal cellular and organismal processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. Transcription of the human immunodeficiency virus type 1 (HIV-1) genome depends on the intracellular environment where the integrate viral DNA is regulated by a complex interplay among viral regulatory proteins, such as Tat, and host cellular transcription factors, such as NF- $\kappa$ B, interacting with the viral long terminal repeat region. CBP (CREB-binding protein) and p300, containing an intrinsic histone acetyltransferase (HAT) activity, have emerged as coactivators for various DNA-binding transcription factors. Here, we show that the p50 subunit as well as the p50/p65 of NF- $\kappa$ B, and not other factors such as SP1, TFIIB, polymerase II, TFIIA, or p65, can be acetylated by CBP/p300 HAT domain. Acetylation of p50 was completely dependent on the presence of both HAT domain and Tat proteins, implying that Tat influences the transcription machinery by aiding CBP/p300 to acquire new partners and increase its functional repertoire. Three lysines, Lys-431, Lys-440, and Lys-441 in p50 were all acetylated *in vitro*, and a sequence similarity among p50, p53, Tat, and activin receptor type I on these particular lysines was observed. All proteins have been shown to be acetylated by the CBP/p300 HAT domain. Acetylated p50 increases its DNA binding properties, as evident by streptavidin/biotin pull-down assays when using labeled NF- $\kappa$ B oligonucleotides. Increased DNA binding on HIV-1 long terminal repeat coincided with increases in the rate of transcription. Therefore, we propose that acetylation of the DNA binding domain of NF- $\kappa$ B aids in nuclear translocation and enhanced transcription and also suggest that the substrate specificity of CBP/p300 can be altered by small peptide molecules, such as HIV-encoded Tat.

Nuclear factor  $\kappa$ B (NF- $\kappa$ B)<sup>1</sup>- $\kappa$ B is an inducible transcription

\* This work was supported by National Institutes of Health Grants AI44357, AI43894, and 13969 (to F. K.) and by grants from the Alexander and Alexander Sinsheimer Foundation (to B. F. and L. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

†† To whom correspondence should be addressed: George Washington University School of Medicine, 2300 Eye St., N.W., Ross Hall, Rm. 552, Washington, D. C. 20037. Tel.: 202-994-1781 or 1782; Fax: 202-994-1780; E-mail: bcmfkk@gwumc.edu.

<sup>1</sup> The abbreviations used are: NF- $\kappa$ B, nuclear factor  $\kappa$ B; CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; CMV, cyto-

factor that regulates the expression of a wide variety of genes including cytokines, growth factors, cytokine receptors, and adhesion molecules (1), which are involved in the control of a large number of normal cellular and organismal processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. In addition, these factors are active in a number of disease states, including cancer, arthritis, inflammation, asthma, neurodegenerative diseases, and heart disease (2).

The prototype NF- $\kappa$ B transcription factor consists of two subunits, NF- $\kappa$ B1 (p50), the DNA-binding subunit, and RelA (p65), the transactivating subunit. NF- $\kappa$ B transcription factors bind to 10-bp DNA sites ( $\kappa$ B sites) as dimers. They bind specifically to a decameric sequence (5'-GGGACTTTC-3') in the  $\kappa$  light chain enhancer region (3). The activity of NF- $\kappa$ B is tightly regulated by interaction with inhibitory I $\kappa$ B proteins. The best studied Rel-I $\kappa$ B interaction is that of I $\kappa$ B  $\alpha$  with the NF- $\kappa$ B p50-RelA dimer, and this interaction blocks the ability of NF- $\kappa$ B to enter the nucleus and bind to DNA (4). Thus, in most cells, NF- $\kappa$ B is present as a latent, inactive, I $\kappa$ B-bound complex in the cytoplasm (4). When a cell receives any of a multitude of extracellular signals, I $\kappa$ B kinase leads to the phosphorylation of two conserved serines near the N terminus of I $\kappa$ B  $\alpha$ , which targets I $\kappa$ B  $\alpha$  for polyubiquitination and subsequent degradation by the 26 S proteasome (5). The unmasked NF- $\kappa$ B can then enter the nucleus to activate target gene expression. After translocation to the nucleus, NF- $\kappa$ B must gain access to the promoters and enhancers of the genes it regulates before it can stimulate their transcription (6).

Transcription of the human immunodeficiency virus type 1 (HIV-1) genome is largely dependent on constitutive DNA-binding proteins, inducible transcription factors (such as NF- $\kappa$ B), and the viral activator Tat (7–9). The best studied element in the HIV-1 regulatory region is the "enhancer region," which contains two NF- $\kappa$ B binding motifs (10, 11). Several different NF- $\kappa$ B subunit combinations have been shown to activate HIV-1 transcription strongly *in vivo* and *in vitro* (12–14), and various studies have reported that the NF- $\kappa$ B binding sites (15, 16) as well as the NF- $\kappa$ B (p50/p65 heterodimer) proteins (17, 18) are important for virus replication in peripheral blood lymphocytes and activated T cell lines *in vivo*. NF- $\kappa$ B activates HIV-1 transcription significantly through the reconfiguration of the chromatin structure in the proximal promoter region to

megalovirus; CREB, cAMP-responsive element-binding protein; GST, glutathione S-transferase; HAT, histone acetyltransferase; HIV-1, human immunodeficiency virus type 1; HPLC, high pressure liquid chromatography; I $\kappa$ B, inhibitory  $\kappa$ B protein; LTR, long terminal repeat; MALDI-TOF, matrix-assisted laser absorption ionization time-of-flight; pol, polymerase; TBP, TATA-binding protein; TF, transcription factor; TNE, 100 mM Tris, 50 mM NaCl, and 1 mM EDTA.

an arrangement that resembles that of integrated provirus *in vivo* (19).

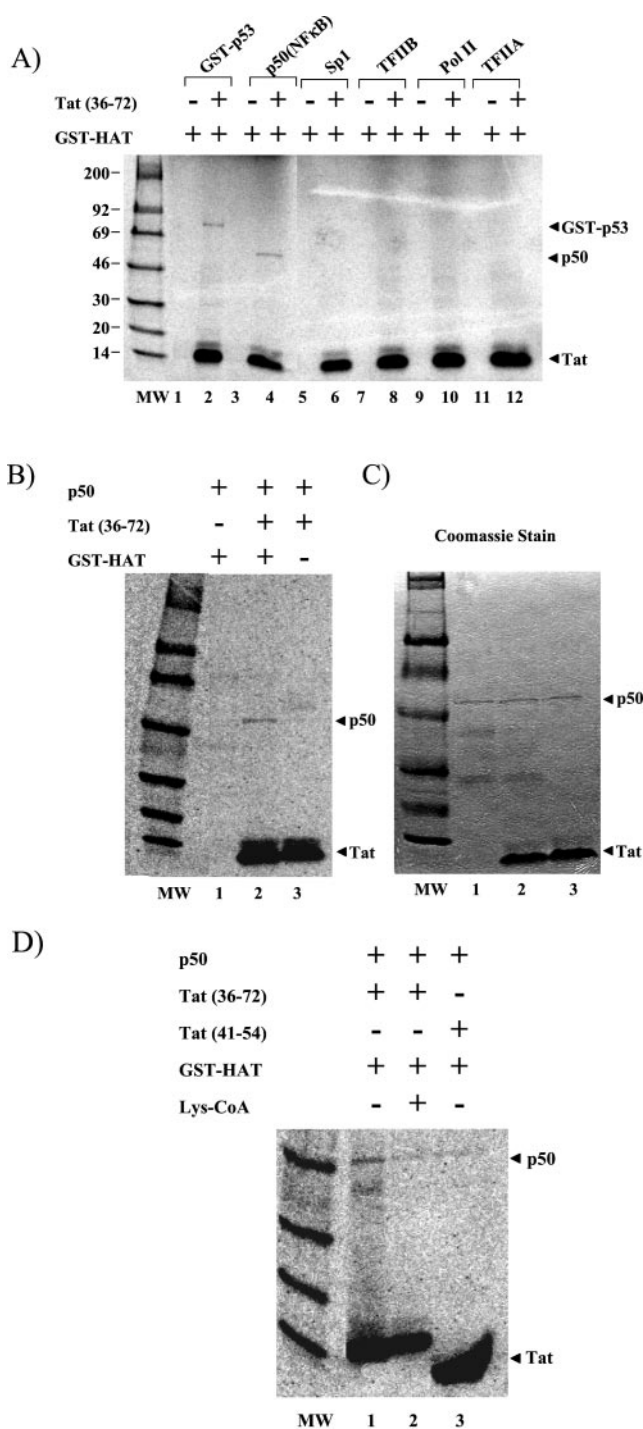
Among the factors associated with basal transcription complexes, CBP (cAMP-responsive element-binding protein-binding protein) and p300 have emerged as coactivators for various DNA-binding transcription factors. CBP/p300 are large proteins, containing 2,441 and 2,414 amino acids, respectively, and have the ability to interact simultaneously with various transcription factors such as nuclear hormone receptors, cAMP-responsive element-binding protein, c-Jun, v-Jun, c-Myb, v-Myb, Sap-1a, c-Fos, MyoD, YY1, NF- $\kappa$ B, p53 (20), and with other coactivators such as the p300/CBP associated factor (21–23). Both CBP/p300 proteins are transcriptional integrators (23) and contain an intrinsic histone acetyltransferase (HAT) activity.

CBP/p300 was also recently reported to interact with the HIV-1 Tat protein and serve as a coactivator of Tat-dependent HIV-1 gene expression (24, 25, 48–50). This superinduction in activated transcription on chromatin DNA has been attributed to the HAT activity of CBP/p300 on the integrated HIV-1 promoter. It is known that CBP/p300 acetylates Tat at a double lysine motif in a highly conserved region (24, 25, 50). In the absence of CBP/p300 and Tat crystal structures, it was shown previously that Tat can change the conformation of the CBP/p300 *in vitro* such that basal transcription factors such as TATA-binding protein (TBP) and TFIIB could bind with higher affinity to CBP/p300 and influence the activation process by aiding CBP/p300 to recruit new partners into the transcription machinery (25). In this study, we find that the p50 subunit of transcription factor NF- $\kappa$ B is acetylated by CBP/p300 in presence of HIV-1 viral protein Tat. Interestingly, acetylated p50 or p50/p65 binds with higher affinity to DNA containing NF- $\kappa$ B binding sites. This was observed in a gel shift assay and confirmed further by biotin pull-down and competition assays. Furthermore, acetylated p50 increases the transcription of HIV-1 compared with unacetylated p50. Therefore, our results suggest that acetylated p50 or p50/p65 binds with high affinity to DNA, which in turn increases the rate of transcription.

#### EXPERIMENTAL PROCEDURES

**Cell Culture, Plasmids, Expression**—Log phase CEM (12D7) T lymphocytes cells were grown in culture at 37 °C up to  $1 \times 10^5$  cells/ml in RPMI 1640 medium containing 10% fetal bovine serum treated with a mixture of 1% streptomycin, penicillin antibiotics, and 1% L-glutamine (Invitrogen) (26–28). HIV-LTR-CAT reporter and eukaryotic Tat expression vectors (pcTat) have been described previously (27–29). GST-p300 (HAT) construction from amino acids 1,197–1,735 has been described previously (25). A 500-ml LB + ampicillin flask was inoculated with the overnight culture and was grown for 4 h at 37 °C. Isopropyl-1-thio- $\beta$ -D-galactopyranoside was added to a final concentration of 0.1 mM to induce fusion protein expression, and the culture was switched to 30 °C for an additional 4 h. Cells were collected by centrifugation in a GSA rotor at  $5,800 \times g$  for 10 min at 4 °C. For sonication, the bacterial pellet was resuspended in 25 ml of phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and was sonicated (Branson) for 35 pulses at the 3.5 microprobe setting. The resulting mixture was centrifuged in a GSA rotor at  $5,800 \times g$  for 10 min at 4 °C. A second centrifugation in a SS-34 rotor at  $23,500 \times g$  for 20 min at 4 °C clarified the extract of remaining debris. GST fusion proteins were bound to agarose beads overnight, washed the next day, and run on a 4–20% SDS-PAGE for both quality and quantity prior to use in HAT assays. CMV-p50 plasmid was obtained from Dr. L. E. Harrison (Dept. of Surgery, University of Medicine and Dentistry of New Jersey).

**Proteins and Peptides**—p50 and p65 proteins were expressed and purified from insect cells. The amplified viral stocks of p50 were used to infect Sf9 cells at a multiplicity of infection of 1–2. Infected cells were harvested at 60 h postinfection and lysed in a buffer containing 20 mM Hepes, pH 7.9, 139 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5% (v/v) Nonidet P-40. The lysate was diluted, and cell extracts were resolved by 10% SDS-PAGE and stained with Coomassie Blue. Protein concentrations were determined using Bio-Rad protein assay. For pro-



**FIG. 1. Acetylation of NF- $\kappa$ B p50 protein by CBP/p300 *in vitro*.** Panel A, 100 ng of GST-p53 (lanes 1 and 2), 100 ng of NF- $\kappa$ B p50 (lanes 3 and 4), 100 ng of Sp1 (lanes 5 and 6), 100 ng of TFIIB (lanes 7 and 8), 100 ng of pol II (lanes 9 and 10, both II<sub>0</sub> and II<sub>A</sub> forms), and 100 ng of TFIIA (lanes 11 and 12, all three recombinant subunits) were incubated with 100 ng of GST-p300 (HAT domain) without or with 300 ng of Tat (36–72) peptide and [<sup>14</sup>C]acetyl-CoA. Acetylated products were resolved on a Tris-glycine 4–20% polyacrylamide gel, dried, and exposed to a PhosphorImager cassette. Panels B and C show HAT reactions similar to that in panel A, but only for the p50 protein. Lane 1 serves as a control with GST-HAT and no Tat 36–72 peptide. Lane 2 indicates a positive reaction with both GST-HAT and Tat 36–72 peptide. Lane 3 is a control with no GST-HAT. Panel C represents the Coomassie Blue stain of the same gel as in panel B. Panel D illustrates the inhibition of p50 acetylation by the Lys-CoA (lane 2), used in similar assays as above, resolved on a Tris-glycine 4–20% polyacrylamide gel, dried, and exposed to a PhosphorImager cassette. Lane 3 contains Tat 41–54 peptide, which does not enhance acetylation by GST-HAT or full-length p300 (data not shown) *in vitro*.

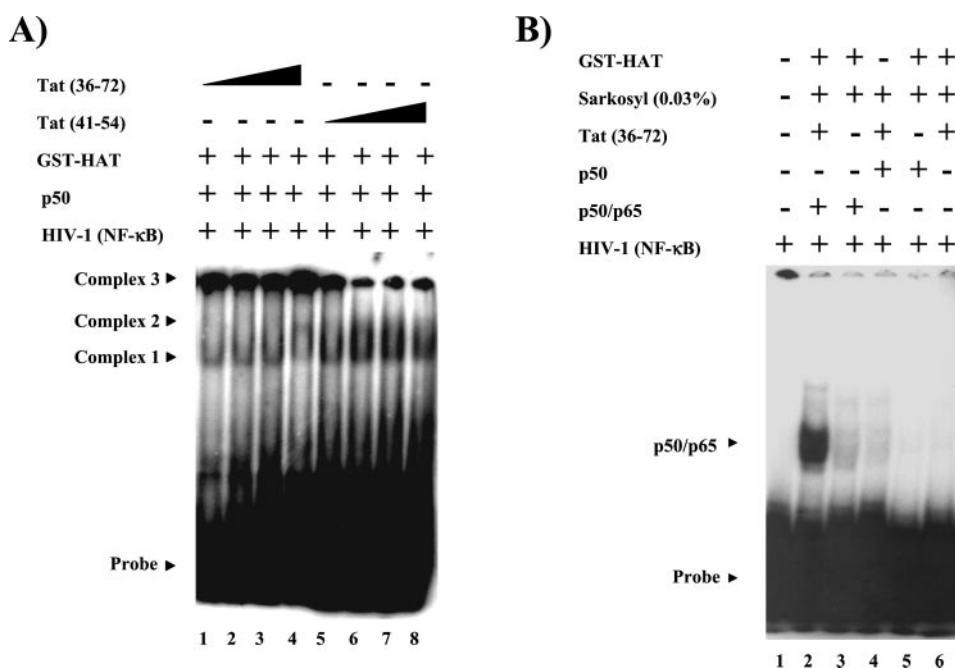


FIG. 2. Electrophoretic mobility shift assay to detect DNA binding activity of acetylated p50. Panels A and B, the HAT assay reaction mixtures of 100 ng of p50 with or without Tat peptides and GST-HAT were incubated with  $^{32}$ P-labeled probe at room temperature for 30 min and separated on a 6% DNA retardation polyacrylamide gel (Invitrogen). Panel A shows positive and negative controls. Lanes contain probe (2 ng), p50, GST-HAT, and Tat 36–72 peptide in lanes 1–4 (0.2, 0.5, 1, and 2  $\mu$ g, respectively), and Tat 41–54 peptide in lanes 5–8 (0.2, 0.5, 1, and 2  $\mu$ g, respectively). Three complexes were apparent where complexes 1 and 3 were well separated. Panel B represents a similar experiment in the presence of sarkosyl. Lane 1 contains probe alone, lane 2, 200 ng of probe plus p50/p65 complex incubated with 500 ng of Tat (36–72) and GST-HAT. The reaction was incubated at room temperature for 30 min prior to the addition of 0.03% sarkosyl. Lane 3 is similar to lane 2 without any added Tat. Lanes 4 and 5 contain p50 alone with and without Tat, and lane 6 serves as control without the addition of any p50 or p50/p65 complex.

tein purification, recombinant viruses were used to infect 200-ml Spinner flasks containing  $\sim 1.5 \times 10^6$  Sf9 cells/ml. After centrifugation the cell extracts were bound to an agarose column. After binding, the column was washed with buffer D (20 mM Hepes, pH 7.9, 0.5 mM EDTA, 1 mM dithiothreitol, 0.2% (v/v) Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and 15% (v/v) glycerol) containing 150 mM NaCl, and bound protein eluted with buffer D containing 400 mM NaCl, and dialyzed against buffer D (30). Tat peptides used in this study, Tat 36–72 and Tat 41–54, have been described previously (25).

**HAT Assay**—The HAT assay was performed in 30- $\mu$ l reactions at 37  $^{\circ}$ C for 60 min in a buffer containing 2  $\mu$ l of purified GST-p300 HAT (1 mg/ml), 200–400 ng of substrate proteins or peptides in 20 mM Hepes-NaOH, pH 7.4, 1 mM dithiothreitol, 10 mM sodium butyric acid, and 1  $\mu$ l of [ $^{14}$ C]acetyl-CoA (65 mCi/mmol, ICN). Proteins and peptides were resolved on a 4–20% or 15% SDS-PAGE. Gels were dried and exposed to a PhosphorImager cassette for further analysis.

**Electrophoretic Mobility Shift Assay**—The DNA probe with NF- $\kappa$ B binding site was end labeled with [ $\gamma$ - $^{32}$ P]ATP using T4 kinase assay. Gel mobility shift assays (16  $\mu$ l, final reaction volume) were carried out in binding buffer (30 mM Tris-HCl, pH 8.0, 12% glycerol, 70 mM KCl, 1.3 mM dithiothreitol, 0.01% Nonidet P-40, 5.5 mM MgCl<sub>2</sub>) and contain 1 ng of labeled NF- $\kappa$ B DNA probe. Reactions were incubated for 30 min at room temperature, and DNA bound complexes were separated on a pre-run 6% DNA retardation gel (Invitrogen) containing 0.5  $\times$  TBE buffer, at 7 watts for 2.5 h at 4  $^{\circ}$ C. The gel was then dried and exposed to PhosphorImager. Oligonucleotides with NF- $\kappa$ B binding sites were synthesized with a 5'-biotin label. The sequence of both strands is as follows.

5'-TACAAGGGACTTTCCGCTGGGGACTTTCCAG-3'  
3'-ATGTTCCCTGAAAGCGACCCCTGAAAGGTC-5'

Corresponding strand oligonucleotides were then annealed at 65  $^{\circ}$ C for 1 h and then at 37  $^{\circ}$ C for 2 h to form double strand DNA.

**Streptavidin Bead Pull-down Assay**—Synthesized oligonucleotides, which are biotin-labeled at the N terminus, were used in the pull-down assays. The biotin-labeled oligonucleotides were incubated with the HAT assay extracts in gel shift buffer (5 $\times$ ) at 4  $^{\circ}$ C for 2 h. Streptavidin beads (Roche Molecular Biochemicals) were added to the mixture and incubated for 2 h at 4  $^{\circ}$ C. Beads were washed twice with TNE<sub>300</sub> + 0.1% Nonidet P-40 buffer. The bound proteins were separated on a 4–20% SDS-PAGE (Invitrogen) and then Western blotted with appropriate antibodies.

**Immunoblotting**—Proteins transfers were carried out overnight at 80 mA, at room temperature, onto polyvinylidene difluoride membranes (Millipore Corp.). During the last 30 min of the transfer, the amperage was increased to 240 mA. Membranes were blocked with 5% milk solution (nonfat dry milk and TNE<sub>50</sub> + 0.1% Nonidet P-40) at 4  $^{\circ}$ C for 2 h, with gentle rocking. Membranes were washed with TNE<sub>50</sub> + 0.1% Nonidet P-40 and were incubated with primary antibody overnight at 4  $^{\circ}$ C. p50 and p65 antibodies (Santa Cruz Biotechnology) were used in 1:1,000 dilution. The next day, membranes were washed once, and the protein G labeled with  $^{125}$ I (50  $\mu$ l/10 ml of solution; Amersham Biosciences, Inc.) was placed on membranes for 2 h with gentle rocking. Membranes were finally washed three times with TNE<sub>50</sub> + 0.1% Nonidet P-40, air dried, and were placed on a PhosphorImager cassette overnight and scanned the next day.

**Lymphocyte Transfection**—Lymphocyte (CEM, 12D7) cells were grown to mid log phase and were processed for electroporation according to a procedure published previously (26). The cells were centrifuged and then washed with phosphate-buffered saline without Mg<sup>2+</sup> or Ca<sup>2+</sup> twice and resuspended in RPMI 1640 at  $4 \times 10^5$  cell/0.25 ml. The CEM cells (0.25 ml) were transfected with the plasmid DNAs of p50, Tat, and HIV-LTR-CAT (wild type and  $\Delta$ -65). The mixture of cells and plasmid DNAs was then transferred to a cuvette and electroporated with fast charge rate, at 230 V, and capacitance of 800 microfarads. Cells were then plated in 10 ml of complete RPMI 1640 medium for 18 h prior to harvest and CAT assay.

For CAT assays, standard reaction was performed by adding the cofactor coenzyme A to a microcentrifuge tube containing cell extract and radiolabeled chloramphenicol, in a final volume of 50  $\mu$ l and incubated at 37  $^{\circ}$ C for 1 h. The reaction mixture was then extracted with ethyl acetate. It was then separated by TLC on silica gel plates (Bakerflex silica gel TLC plates) using the chloroform:methanol (19:1) solvent system. The resolved reaction products were then detected by exposing the plate to a PhosphorImager cassette.

**Peptide Synthesis**—All peptides were prepared on a Tris (alkoxy)benzylamide polyethylene glycol-polystyrene resin by continuous flow solid phase synthesis on a PerSeptive Biosystems Pioneer synthesizer (Framingham, MA) using HBTU-activated Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) amino acids. Peptide purification was achieved by conventional reversed phase HPLC on Vydac C18 (Hesperia, CA) in an overall yield of 25–30% based on starting resins. The purity of the peptides was confirmed further by analytical reversed phase HPLC,

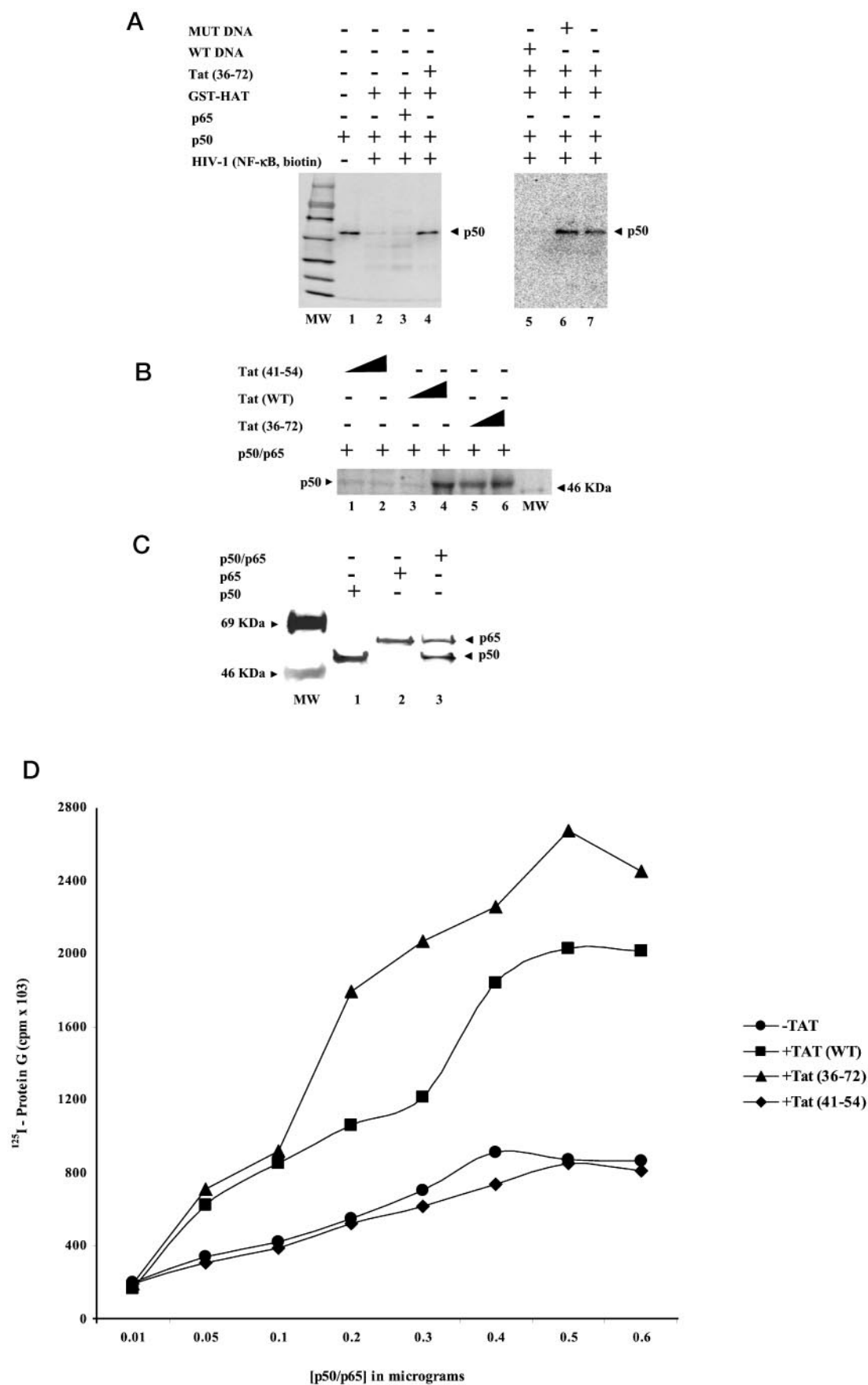


FIG. 3. Acetylation of p50 increases its DNA binding ability. *Panel A*, biotin-labeled NF- $\kappa$ B oligonucleotides from HIV-1 LTR were incubated with HAT assay extracts ( $5 \times$  reaction) in gel shift buffer at  $4^\circ\text{C}$  for 2 h, and oligonucleotides were then pulled down using streptavidin beads (Roche Molecular Biochemicals) and washed twice with TNE<sub>300</sub> + 0.1% Nonidet P-40 buffer. The bound peptides were then separated on the Tris-glycine 4–20% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and Western blotted with anti-p50 antibody (Santa

capillary zone electrophoresis, and matrix-assisted laser absorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Amounts of each peptide were determined by Bio-Rad protein assay as well as running small aliquots on 4–20% or 15% SDS-PAGE followed by silver staining (Silver Stain Plus, Bio-Rad).

**Mass Spectrometry**—The unacetylated or acetylated peptides or proteins were digested with either trypsin or proline-specific endopeptidase (Seikagaku Corp). The digested or undigested peptides were desalted using C<sub>18</sub> ZipTips (Millipore) according to manufacturer's instructions. A 1- $\mu$ l aliquot of sample was taken for peptide mass mapping on a PerSeptive Biosystem DEPRO MALDI-TOF mass spectrometer using  $\alpha$ -cyano-4-hydroxycinnamic acid as the matrix. Analysis was performed in the linear delayed extraction mode, with external calibration. The analysis of acetylation mass was performed through the ProFound Web site located at Rockefeller University (<http://prowl.rockefeller.edu>) as well as other public data bases (as of October 2001) including <http://www.ncbi.nlm.nih.gov/pubmed/>; <http://au.expsy.org/>; <http://www.ncbi.nlm.nih.gov/BLAST/>; <http://pfam.wustl.edu/>; <http://pbil.univ-lyon1.fr/pbil.html>; [http://www.ch.embnet.org/software/COILS\\_form.html](http://www.ch.embnet.org/software/COILS_form.html); and <http://www.nbrf.georgetown.edu/pirwww>.

## RESULTS

**The p50 Subunit of NF- $\kappa$ B Is Acetylated by the HAT Domain of CBP/p300**—The HIV-1 promoter has two NF- $\kappa$ B binding sites in its enhancer region (19). NF- $\kappa$ B plays a major role in the regulation of HIV-1 gene expression (11). It is also known that the effects of Tat on the enhancer are primarily mediated by Tat-induced nuclear translocation of NF- $\kappa$ B (9). However, to date the mechanism by which this occurs remains unclear.

We and others have shown previously that CBP/p300 acetylates Tat at a double lysine motif (amino acids 50 and 51) in a highly conserved region, and moreover the minimal HAT domain of CBP/p300 was capable of performing this acetylation (24, 25, 50). Therefore, we investigated the effect of acetylation on other transcription factors and enhancer-binding proteins that regulate HIV-1 transcription in the presence of a minimal HAT domain and Tat. The plasmid GST-HAT was expressed in *Escherichia coli* and purified using glutathione-agarose beads, washed, and the HAT protein was eluted with 10 mM glutathione and dialyzed against transcription buffer. We initially performed HAT assays on several substrates such as p53 (positive control for the HAT assay), p50 (NF- $\kappa$ B subunit), p65, SP1, TFIIB, pol II, and TFIIA. We incubated 0.1  $\mu$ g of each substrate protein with [<sup>14</sup>C]acetyl-CoA, Tat 36–72 peptide, and GST-HAT for 1 h at 30 °C. We have consistently used Tat 36–72 in most of our assays because it is easier to obtain this peptide *in vitro*, and it has coactivator acetylation activity similar to that of the full-length wild type Tat protein (50). The products were then resolved on a Tris-glycine 4–20% polyacrylamide gel and analyzed by autoradiography. As shown in Fig. 1A, the p53, p50, and Tat proteins were specifically labeled by [<sup>14</sup>C]acetyl-CoA, whereas no signals were detected in reactions containing SP1, TFIIB, pol II, TFIIA (lanes 5–12), or p65 (NF- $\kappa$ B subunit, data not shown). The acetylation of p50 and p53 was observed in lanes 2 and 4, which contained Tat peptide compared with lanes 1 and 3, where there was no addition of Tat peptide. Labeling of p50 was completely dependent on the presence of both GST-HAT and Tat (Fig. 1B, lane 2) and not just GST-HAT or Tat alone (lanes 1 and 3, respectively). Fig. 1C shows the

Coomassie Blue stain of the same gel as in panel B, which confirms that there were similar amounts of p50 protein present in all lanes. Collectively, these results indicate specific acetylation of p50 by GST-HAT and that the presence of Tat along with p300 HAT domain was important for the observed acetylation.

Finally, we asked whether the observed HAT activity could selectively be inhibited by Lys-CoA, a specific inhibitor of p300 HAT activity (31). When Lys-CoA was added to the reaction mixture, a more than 6-fold inhibition of p50 was observed (Fig. 1D, 4,623 cpm for p50 in lane 1 versus 678 cpm for p50 in lane 2). Moreover, Tat 36–72 peptide was essential for acetylation of p50, and the smaller 41–54 Tat peptide did not enhance this acetylation (compare lanes 1 and 3).

**Acetylation of p50 Activates Its Ability to Bind DNA**—Next, we asked whether acetylation of p50 could alter its affinity for NF- $\kappa$ B binding to DNA. To address this question we synthesized DNA probe with two NF- $\kappa$ B binding sites from the HIV-1 LTR, end-labeled, PAGE-purified, and used in a gel shift assay. The HAT assay was performed first with p50 followed by its use in gel shift assay with labeled probe. DNA-bound complexes were separated on a pre-run 6% DNA retardation gel (Invitrogen). Results of such an experiment are shown in Fig. 2. The probe used was specific for NF- $\kappa$ B binding, and GST-HAT on its own could not bind to the probe (data not shown). Fig. 2A indicates that as the amount of added Tat 36–72 peptide was increased, the binding ability of acetylated p50 also increased (Fig. 2A, lanes 1–4). However, there seem to be three complexes apparent after the addition of acetylated p50 to the reaction. Complex 1 was the original unacetylated p50 bound to DNA, complex 2 was a slightly slower migrating complex, and complex 3 was simply unable to enter the 6% (or a 4% gel, data not shown) acrylamide gel. Complex 3 was much less apparent in the control lanes, where only Tat peptide 41–54 was added prior to p50 acetylation (lanes 5–8). We next asked whether the p50-DNA or p50/p65-DNA complex could withstand non-ionic detergent treatments such as sarkosyl. Sarkosyl is frequently used to determine how stable DNA-protein complexes are in *in vitro* assays. Results of such an experiment are shown in Fig. 2B, where, upon addition of 0.03% sarkosyl, only the acetylated form of p50/p65 could withstand the detergent and be stable in a gel shift assay (lane 2). Although complex 3 disappeared under these conditions, acetylated or unacetylated p50 alone could not withstand the detergent treatment. Thus, these experiments point to the fact that acetylated p50 in the context of p50/p65 binds with higher affinity to DNA.

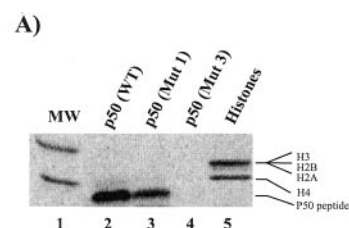
To confirm further that acetylation of p50 increases its affinity for DNA, we performed biotin pull-down assays. We synthesized oligonucleotides containing NF- $\kappa$ B binding sites, which also contained biotin moiety at the 5'-end. The biotin-labeled oligonucleotides were then incubated with acetylated p50-HAT or p50/p65-HAT reaction (same reactions used for gel shift analysis, 5 $\times$  reaction), and incubated at 4 °C for 2 h. Streptavidin beads (Roche Molecular Biochemicals) were then added to pull-down biotin-labeled oligonucleotides. The bound DNA-protein complexes were then washed in TNE<sub>300</sub> plus 0.1%

Cruz Biotechnology). Lane 1 serves as a positive control for migration of p50 on SDS-PAGE. Acetylated p50 was pulled down (lane 4) when the reaction contained Tat peptide. The right panel represents the competition assay where unlabeled oligonucleotides (40-fold excess of either wild type or mutated oligonucleotides, mutations on the 5'-GGACTT-3' to 5'-AAGACC-3') were added along with biotin probe, for 1 h before the pull-down assay. Panel B, similar to panel A, except a p50/p65 complex with varying concentrations of Tat was used before pull-down. Lanes 1 and 2, 250 and 750 ng of Tat 41–54; lanes 3 and 4 contained 250 and 750 ng of Tat wild type (WT); and lanes 5 and 6 contained 250 and 750 ng of Tat 36–72. 200 ng of p50/p65 was added to the reaction mixture before pull-down. Panel C, 500 ng of each free p50, free p65, and p50/p65 complex used in panels A and B were ran on a 4–20% SDS-PAGE and stained with Coomassie Blue. Panel D, stoichiometry of the acetylation reaction and its relation to p50 DNA binding. Samples were incubated with increasing concentrations of p50/p65 complex with fixed amount of various forms of 500 ng of Tat, followed by pull-down, washed, and Western blotted for the presence of p50. Antigen-antibody complexes were detected with <sup>125</sup>I-protein G. Blots were subsequently exposed to PhosphorImager cassette for 24 h, and bands were counted using the ImageQuante software.

Nonidet P-40, separated on the Tris-glycine 4–20% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and Western blotted with anti-p50 rabbit polyclonal antibody. Results of such an experiment are shown in Fig. 3A. Lane 1 shows positive control protein alone, indicating the migration of p50 protein on SDS-PAGE following Western blot. Biotin-labeled oligonucleotides were able to bind efficiently to p50 in the presence of GST-HAT and Tat (lane 4). However, in the presence of added free p65 or unacetylated p50, the complex could not withstand the wash conditions used here (300 mM salt and 0.1% Nonidet P-40) and therefore could not be retained on DNA (lanes 2 and 3). The complex was specific to p50 because a 40-fold excess of cold specific (lane 5), and not the mutant competitor (lane 6), could effectively out compete the complex. Next, we asked whether a p50/p65 complex could also withstand the wash conditions used here. Therefore, we designed an experiment where various forms of Tat (wild type, 36–72, and 41–54) at various concentrations (0.25 and 0.75  $\mu$ g) were used in acetylation reactions followed by pull-down, and Western blotted for p50. Results in Fig. 3B indicate that both Tat 36–72 and the wild type Tat, but not the 41–54 mutant, were capable of acetylating the p50 in the p50/p65 complex, thereby increasing its DNA binding activity. A Coomassie Blue stain of free p50, free p65, and p50/p65 complex used above is shown in Fig. 3C. To determine the stoichiometry of the acetylation reaction and its relation to DNA binding, we incubated increasing concentrations of p50/p65 complex with fixed amount of various forms of Tat, followed by pull-down and Western blot for p50 using specific antibody, and detected the antigen-antibody complex with  $^{125}$ I-protein G. Blots were subsequently exposed to PhosphorImager cassette for 24 h, and bands were counted using the ImageQuante software. Fig. 3D indicates that in the presence of wild type or 36–72 Tat, p50 DNA binding increases more so compared with the absence of Tat or mutant 41–54 peptide. Collectively, these results suggest that the acetylated form of p50 either alone or complexed with p65 binds with higher affinity to DNA.

**Site of p50 Acetylation by p300 HAT Domain**—To determine the site of p50 acetylation, we initially compared *E. coli* recombinant p50 (unmodified) and acetylated p50 by running both samples on gels to purify, trypsin in gel digested, eluted, and cleaned peptides with C<sub>18</sub> Zip Tips, and performed MALDI-TOF mass spectrometry on peptides. Our initial set of experiments pointed toward lysine residues downstream of the nuclear localization sequence as the target of modification (data not shown). Upon close examination of modified peptide masses from digested acetylated p50, we noticed a sequence similarity to other p300 HAT-modified substrates and possible acetylation sites from amino acids 426 to 446 on p50 (for amino acid sequence, see Fig. 6A). Therefore, we synthesized this particular peptide and all of its possible lysine mutants to determine the actual acetylation site(s) on p50. Results of such an experiment are shown in Fig. 4A, where three lysines, Lys-431, Lys-440, and Lys-441, were all acetylated *in vitro*. A triple lysine mutant (Mut 3) showed no acetylation (lane 4), and Lys-431 (Mut 1) mutant showed a more than 40% drop in acetylation (lane 3). A complete set of all peptides synthesized and utilized in the presence of HAT and Tat (either Tat 36–72 or wild type protein) is shown in panel B. Interestingly, mutation of Lys-431 alone (Mut 1) drops the  $^{14}$ C labeling by ~40%, and a double mutant of Lys-440 and Lys-441 together (Mut 7) also reduced labeling by ~40%. From these *in vitro* labeling studies, it can be concluded that the Lys-431 is acetylated preferentially, and the other two lysine sites (Lys-440 and Lys-441) are less preferentially acetylated in p50.

**Functional Significance of Acetylated p50 Subunit of NF- $\kappa$ B**



B)

Peptide	p50 + HAT + Tat (36-72)	p50 + HAT + Tat (WT)
WT	7,701	8,319
Mut 1	4,488	3,657
Mut 2	1,412	1,635
Mut 3	264	109
Mut 4	1,287	1,991
Mut 5	3,408	3,596
Mut 6	4,692	3,968
Mut 7	4,941	4,156

FIG. 4. **Site of acetylation on p50 protein.** Panel A shows acetylation of wild type (WT, lane 2) p50 peptide (for amino acid sequence, see Fig. 6A, top row) a single lysine mutant at position 431 (Mut 1, lane 3), and a triple lysine mutant at positions 431, 440, and 441 (Mut 3, lane 4). Lane 5 serves as positive control for the acetylation reaction. Panel B represents all of the possible lysine mutants in p50 and their acetylation reaction either with wild type Tat protein or Tat peptide along with the HAT domain.

**in Transcription**—To determine the effect of p50 acetylation on HIV-1 transcription, we performed transient transfection assays using CEM (12D7) T cells. We used wild HIV-LTR-CAT, a p50 expression vector and log phase growing CEM cells. Results of such an experiment is shown in Fig. 5, where upon electroporation of cells with reporter HIV-LTR-CAT, we observed a significant drop of transcriptional activity in presence of CMV-p50 (lane 2). However in the presence of CMV-p50 and pcTat the transcriptional activity increased (lane 3) nearly the same as in presence of pcTat alone (lane 4). When transfecting  $\Delta$ -65 (NF- $\kappa$ B<sup>-</sup>/SP1<sup>+</sup>) LTR-CAT we observed no significant transcriptional differences in presence of CMV-p50 and pcTat or pcTat alone (data not shown). These results imply that p50 alone in the absence of Tat might have an inhibitory function possibly resulting from binding to I $\kappa$ B or simply not being acetylated. Interestingly, when cells were electroporated with either Tat 36–72 or 36–54 peptides (which contain both core and basic domains), we observed an increase in the level of p50 activation (Fig. 5, lanes 6 and 7). This level of activation was not evident with a Tat peptide spanning the C-terminal domain (lane 8). Control experiments using Tat peptides alone (in the absence of p50 expression vector) showed no activated transcription on HIV-1 LTR (lanes 9–12). Collectively, these data suggest that Tat region 36–54 is sufficient for activation of p50, possibly by means of activating the p300 HAT domain and increasing the DNA binding properties of the NF- $\kappa$ B protein.

#### DISCUSSION

Members of the p300 and CBP family appear to be present only in higher eukaryotic cells. Many viruses have evolved mechanisms to control both viral and host transcriptional ma-

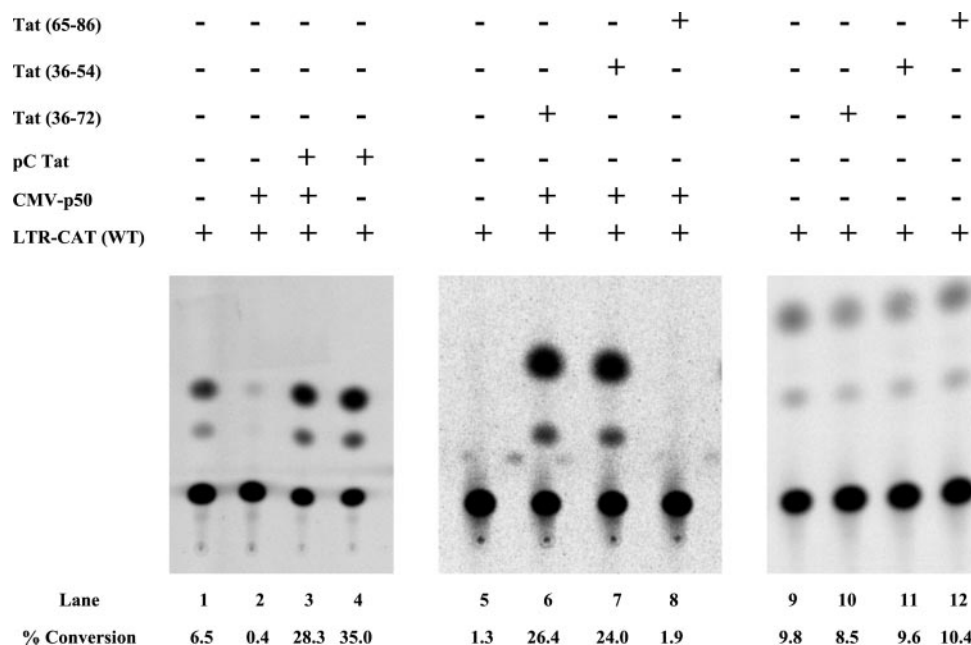


FIG. 5. **Increased LTR transactivation activity with acetylated p50.** CAT assays were performed from lysates of transfected CEM (12D7) cells with 5  $\mu$ g of LTR-CAT, cotransfected without or with either 1  $\mu$ g of pC Tat, 5  $\mu$ g of CMV-p50, or both. Lanes 1 and 5 show basal transcription of LTR-CAT, lane 2 shows suppression of LTR activity with p50 alone or activated transcription with Tat (lanes 3 and 4). Lanes 6–8 show transfections with p50 vector and various Tat peptides (2  $\mu$ g/reaction). Lanes 9–12 are similar to lanes 6–8, except no p50 expression vector was added.

A)



B)

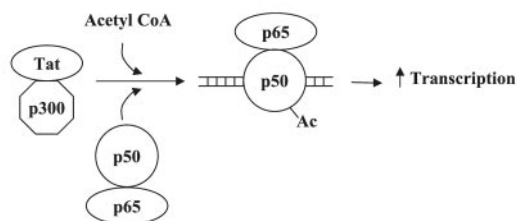


FIG. 6. **A model for activation of the DNA binding activity of p50 in p50/p65 complex by acetylation.** Panel A, sequence similarity among p50, p53, Tat, and ACTR on lysine residues that are the targets of the CBP/p300 HAT domain. Position 426 at the N terminus represents the amino acid number in human p50 protein. Panel B, p50 in the presence of Tat and CBP/p300 becomes acetylated and binds with higher affinity to DNA containing NF- $\kappa$ B binding sites. DNA binding by acetylated p50/p65 complex results in increased transcriptional activity of the promoter.

chinery through CBP/p300. Tat is known to bind CBP/p300 in the minimal HAT domain (1253–1710), and this binding is stable at high salt wash conditions. Moreover, Tat changes the conformation of CBP/p300 such that the altered molecule can bind to other transcription factors with higher affinity, implying that Tat influences the transcription machinery by aiding CBP/p300 to acquire new partners and increase its functional repertoire (25).

Acetylases are now known to modify a variety of proteins, including histones, transcription factors, nuclear import fac-

tors, and  $\alpha$ -tubulin (35). Acetylation regulates many diverse functions, including DNA recognition, RNA binding, protein-protein interaction, and protein stability. A number of non-histone proteins have been identified as substrates for CBP/p300. Many of these substrates are involved in the regulation of transcription and include p53 (36), E2F1 (37, 38), EKLF (39), TFIIE (40), TFIIF (40), TCF (41), GATA1 (42), HMG1(Y) (43), activin receptor type I (44), c-Myb (45), HIV-1 Tat (25, 46), SRC-1, and TIF2 (47).

In recent years it has become apparent that non-DNA-binding transcriptional coactivators, such as p300 and CBP, which were thought to function primarily as bridging proteins between DNA-bound transcription factors and the basal transcription complex, play a critical role as integrators of diverse signaling pathways in the selective induction of gene expression. Many examples of such phenomena are exemplified by the interaction of CBP/p300 with an array of transcription factors including sequence-specific DNA-binding proteins, such as NF- $\kappa$ B, CREB, or activator protein 1 (AP-1) family members, that interact with promoter and act as either enhancers or repressors of gene expression during cellular activation (25). The NF- $\kappa$ B that enters the nucleus from the cytoplasm in response to signaling is phosphorylated and associates specifically with CBP/p300. It is only the CBP/p300-associated NF- $\kappa$ B that is transcriptionally active (3, 32), leaving the possibility that NF- $\kappa$ B might not interact directly with general transcription factors, but instead use the CBP/p300 coactivator as a bridging molecule (33, 34).

Here, we have shown that NF- $\kappa$ B-p50 either alone or complexed with p65 could be acetylated by CBP/p300 HAT domain only when Tat (either wild type or peptides containing core and basic domains) is present in the system. This opens the possibility that Tat alters the specificity of CBP/p300 activity on a range of other associated factors. In fact, in an attempt to find other non-histone transcription factors that could serve as a substrate for Tat and p300 HAT complex, we utilized all known transcription factors affecting the HIV-1 transcription *in vitro* and have found that the human TBP can serve as a substrate

for acetylation *in vitro*.<sup>2</sup> The increased acetylation on TBP increased its DNA binding to HIV-1 TATA box. It remains to be seen whether TBP acetylation either alone or in the context of SL1, TFIID, or TFIIB could increase the rate of cellular pol I, II, and III transcription or simple reinitiation of HIV-1 or the transactivation response region independent promoters normally affected by Tat. Therefore, the increased in DNA binding of acetylated TBP to TATA box, or acetylated p53 to its target DNA (36), is consistent with the effect of acetylated p50 binding with higher affinity to the NF- $\kappa$ B DNA binding site.

Also, it is important to note here that we have performed *in vivo* labeling experiments, where we added either purified Tat or tumor necrosis factor- $\alpha$  to ACH2 (HIV-1-infected latent) cells, labeled with [<sup>3</sup>H]acetate, and immunoprecipitated the nuclear p50, and observed acetylation of NF- $\kappa$ B in these cells.<sup>3</sup> However, we have not been successful in obtaining relevant p50 mutant data because we find the K431A mutant to be very unstable in cells, and the K440A/K441A double mutant is unable to enter the nucleus (data not shown).

The physical interaction between Tat and CBP/p300 HAT domain could potentially occur in the cytoplasm. This is especially true with cells treated with extracellular Tat, where similar to the enhanceosome affecting the interferon- $\beta$  promoter, CBP/p300 would modify a cytoplasmic transcription factor, such as p50/p65-I $\kappa$ B complex, and release the p50/p65 from its inactive form to an acetylated active form entering the nucleus. In fact, when looking at the p50/p65-I $\kappa$ B crystal structure and modeling for amino acid changes in the p50 protein, the C-terminal tail of p50 and its respective lysines (Lys-341, Lys-440, Lys-441), which are located outside of the DNA binding domain, allow for potential acetylation in p50 (51). Therefore, we predict that acetylation of p50 by the p300-Tat complex in the cytoplasm would release the p50 from its inhibitory I $\kappa$ B subunit and allow entry of active NF- $\kappa$ B into the nucleus, hence increase transcription.

Finally, it is interesting to note a sequence similarity among 50, p53, Tat, and activin receptor type I on lysines that are acetylated by CBP/p300 (Fig. 6A). Although not all sites are acetylated equally in these proteins, acetylation of these proteins controls either DNA, RNA, or protein binding activities, implying that acetylation is a critical step in transcriptional regulation. Collectively, we propose a model (Fig. 6B) in which acetylation of p50 by p300-Tat complex increases its DNA binding activity, which may recruit more p65 (transactivator subunit of NF- $\kappa$ B) and in turn increase the rate of transcription. Future experiments will define which promoters other than HIV-1 (*e.g.* interleukin-8) are affected by p50 acetylation and whether p50 DNA binding is regulated through the cell cycle.

**Acknowledgments**—We thank Dr. L. E. Harrison for CMV-p50 and J. Hiscott for GST-p65 purified protein.

#### REFERENCES

- Siebenlist, U., Franzoso, G., and Brown, K. (1994) *Annu. Rev. Cell Biol.* **10**, 405–455
- Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16**, 225–260
- Grilli, M., snf Memo, M. (1999) *Biochem. Pharmacol.* **57**, 1–7
- Gilmore, T. D. (1999) *Oncogene* **18**, 6842–6844
- Karin, M. (1999) *Oncogene* **18**, 6867–6874
- Perkins, N. D. (2000) *Trends Biochem. Sci.* **25**, 434–440
- Jacque, J. M., Fernandez, B., Arenzana-Seisdedos, F., Thomas, D., Baleux, F., Virelizier, J. L., and Bachelier, F. (1996) *J. Virol.* **70**, 2930–2938
- Karn, J. (1999) *J. Mol. Biol.* **293**, 235–254
- Demarch, F., d'Adda di Fagnagna, F., Falaschi, A., and Giacca, M. (1996) *J. Virol.* **70**, 4427–4437
- Nabel, G., and Baltimore, D. (1987) *Nature* **326**, 711–713
- Chen, B. K., Feinberg, M. B., and Baltimore, D. (1997) *J. Virol.* **71**, 5495–5504
- Fujita, T., Nolan, G. P., Ghosh, S., and Baltimore, D. (1992) *Genes Dev.* **6**, 775–787
- Kretzschmar, M., Meisterernst, M., Scheidereit, C., Li, G., and Roeder, R. G. (1992) *Genes Dev.* **6**, 761–774
- Lin, R., Gewert, D., Hiscott, J. (1995) *J. Biol. Chem.* **270**, 3123–3131
- Ross, E. K., Buckler-White, A. J., Rabson, A. B., Englund, G., and Martin, M. A. (1991) *J. Virol.* **65**, 4350–4358
- Kim, J. Y., Gonzalez-Scarano, F., Zeichner, S. L., and Alwine, J. C. (1993) *J. Virol.* **67**, 1658–1662
- Qian, J., Bours, V., Manischewitz, J., Blackburn, R., Siebenlist, U., and Golding, H. (1994) *J. Immunol.* **152**, 4183–4191
- Alcami, J., Lain de Lera, T., Folguez, L., Pedraza, M. A., Jacque, J. M., Bachelier, F., Noriega, A. R., Hay, R. T., Harrich, D., Gaynor, R. B., (1995) *EMBO J.* **14**, 1552–1560
- Pazin, M. J., Sheridan, P. L., Cannon, K., Cao, Z., Keck, J. G., Kadonaga, J. T., and Jones, K. A. (1996) *Genes Dev.* **10**, 37–49
- Goldman, P. S., Tran, V. K., and Goodman, R. H. (1997) *Rec. Prog. Horm. Res.* **52**, 103–120
- Blanco, J. C., Minucci, S., Lu, J., Yang, X. J., Walker, K. K., Chen, H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) *Genes Dev.* **12**, 1638–1651
- Chakravarti, D., Ogryzko, V., Kao, H. Y., Nash, A., Chen, H., Nakatani, Y., and Evans, R. M. (1999) *Cell* **96**, 393–403
- Shikama, N., Lyon, J., and La Thangue, N. B. (1997) *Trends Cell Biol.* **7**, 230–236
- Ott, M., Schnolzer, M., Garnica, J., Fischle, W., Emiliani, S., Rackwitz, H. R., and Verdin, E. (1999) *Curr. Biol.* **9**, 1489–1492
- Deng, L., de la Fuente, C., Fu, P., Wang, L., Donnelly, R., Wade, J. D., Lambert, P., Li, H., Lee, C. G., and Kashanchi, F. (2000) *Virology* **277**, 278–295
- Kashanchi, F., Duvall, J. F., and Brady, J. N. (1992) *Nucleic Acids Res.* **20**, 4673–4674
- Kashanchi, F., Piras, G., Radonovich, M. F., Duvall, J. F., Fattaey, A., Chiang, C. M., Roeder, R. G., and Brady, J. N. (1994) *Nature* **367**, 295–299
- Kashanchi, F., Shibata, R., Ross, E. K., Brady, J. N., and Martin, M. A. (1994) *J. Virol.* **68**, 3298–3307
- Hauber, J., Malim, M. H., and Cullen, B. R. (1989) *J. Virol.* **63**, 1181–1187
- Coleman, T. A., Huddleston, K. A., Ruben, S. M., Rosen, C. A., and Gentz, R. (1997) *Protein Expression Purif.* **9**, 40–48
- Lau, O. D., Kundu, T. K., Soccio, R. E., Khalil, E. M., Vassilev, A., Wolffe, A. P., Nakatani, Y., Roeder, R. G., and Cole, P. A. (2000) *Mol. Cell* **5**, 589–595
- Ghosh, S. (1999) *Immunol. Res.* **19**, 183–189
- Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) *Science* **275**, 523–527
- Perkins, N. D. (1997) *Int. J. Biochem. Cell Biol.* **29**, 1433–1448
- Kouzarides, T. (2000) *EMBO J.* **19**, 1176–1179
- Gu, W., and Roeder, R. G. (1997) *Cell* **90**, 595–606
- Martinez-Balbas, M. A., Bauer, U. M., Nielsen, S. J., Brehm, A., and Kouzarides, T. (2000) *EMBO J.* **19**, 662–671
- Marzio, G., Wagener, C., Gutierrez, M. I., Cartwright, P., Helin, K., and Giacca, M. (2000) *J. Biol. Chem.* **275**, 10887–10892
- Zhang, W., and Bieker, J. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9855–9860
- Imhof, A., Yang, X. J., Ogryzko, V. V., Nakatani, Y., Wolffe, A. P., and Ge, H. (1997) *Curr. Biol.* **7**, 689–692
- Waltzer, L., and Bienz, M. (1998) *Nature* **395**, 521–525
- Boyes, J., Byfield, P., Nakatani, Y., and Ogryzko, V. (1998) *Nature* **396**, 594–598
- Munshi, N., Merika, M., Yie, J., Senger, K., Chen, G., and Thanos, D. (1998) *Mol. Cell* **2**, 457–467
- Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90**, 569–580
- Tomita, A., Towatari, M., Tsuzuki, S., Hayakawa, F., Kosugi, H., Tamai, K., Miyazaki, T., Kinoshita, T., and Saito, H. (2000) *Oncogene* **19**, 444–451
- Kiernan, R. E., Vanhulle, C., Schiltz, L., Adam, E., Xiao, H., Maudoux, F., Calomme, C., Burny, A., Nakatani, Y., Jeang, K. T., Benkirane, M., and Van Lint, C. (1999) *EMBO J.* **18**, 6106–6118
- Chen, H., Lin, R. J., Xie, W., Wilpitz, D., and Evans, R. M. (1999) *Cell* **98**, 675–686
- Hottiger, M. O., Felzien, L. K., and Nabel, G. J. (1998) *EMBO J.* **17**, 3124–3134
- Weissman, J. D., Brown, J. A., Howcroft, T. K., Hwang, J., Chawla, A., Roche, P. A., Schiltz, L., Nakatani, Y., and Singer, D. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11601–11606
- Deng, L., Wang, D., de la Fuente, C., Wang, L., Li, H., Lee, C. G., Donnelly, R., and Kashanchi, F. (2001) *Virology* **289**, 312–326
- Huxford, T., Huang D. B., Malek, S., and Ghosh, G. (1998) *Cell* **95**, 759–770

<sup>2</sup> L. Deng and F. Kashanchi, unpublished results.

<sup>3</sup> L. Deng, unpublished results.