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Isabelle Clerc, Nicholas Polakowski, Charlotte André-Arpin, Pamela Cook, Benoit Barbeau, et al.. An Interaction between the Human T Cell Leukemia Virus Type 1 Basic Leucine Zipper Factor (HBZ) and the KIX Domain of p300/CBP Contributes to the Down-regulation of Tax-dependent Viral Transcription by HBZ.. *Journal of Biological Chemistry*, 2008, 283 (35), pp.23903-13. 10.1074/jbc.M803116200 . hal-00319711

HAL Id: hal-00319711

<https://hal.science/hal-00319711>

Submitted on 27 May 2021

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An Interaction between the Human T Cell Leukemia Virus Type 1 Basic Leucine Zipper Factor (HBZ) and the KIX Domain of p300/CBP Contributes to the Down-regulation of Tax-dependent Viral Transcription by HBZ*

Received for publication, April 23, 2008, and in revised form, July 2, 2008. Published, JBC Papers in Press, July 2, 2008, DOI 10.1074/jbc.M803116200

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Activation of human T cell leukemia virus type 1 (HTLV-1) transcription is established through the formation of protein complexes on the viral promoter that are essentially composed of the cellular basic leucine zipper (bZIP) transcription factor cAMP-response element-binding protein (CREB (or certain other members of the ATF/CREB family), the HTLV-1-encoded transactivator Tax, and the pleiotropic cellular coactivators p300/CBP. HTLV-1 bZIP factor (HBZ) is a protein encoded by HTLV-1 that contains a bZIP domain and functions to repress HTLV-1 transcription. HBZ has been shown to repress viral transcription by dimerizing with CREB, which occurs specifically through the bZIP domain in each protein, and preventing CREB from binding to the DNA. However, we previously found that HBZ causes only partial removal of CREB from a chromosomally integrated viral promoter, and more importantly, an HBZ mutant lacking the COOH-terminal bZIP domain retains the ability to repress viral transcription. These results suggest that an additional mechanism contributes to HBZ-mediated repression of HTLV-1 transcription. In this study, we show that HBZ binds directly to the p300 and CBP coactivators. Two LXXLL-like motifs located within the NH₂-terminal region of HBZ are important for this interaction and specifically mediate binding to the KIX domain of p300/CBP. We provide evidence that this interaction interferes with the ability of Tax to bind p300/CBP and thereby

inhibits the association of the coactivators with the viral promoter. Our findings demonstrate that HBZ utilizes a bipartite mechanism to repress viral transcription.

Human T-cell leukemia virus type 1 (HTLV-1)⁵ is a retrovirus that is the causative agent of adult T-cell leukemia and a neurodegenerative disorder termed tropical spastic paraparesis/HTLV-1-associated myelopathy (1, 2). Following its integration, the provirus utilizes the cellular RNA polymerase II transcription machinery for replication of the viral genome and expression of viral genes. These processes are dependent on the viral transactivator Tax, which is essential for strong activation of HTLV-1 transcription. Tax alone lacks DNA binding activity and is therefore recruited to the viral promoter as part of a complex with the cellular transcription factor CREB or other members of the activating transcription factor/cyclic AMP-responsive element (CRE)-binding protein (ATF/CREB) family (3–6). These proteins carry a basic leucine zipper (bZIP) domain that stimulates protein dimerization and subsequent DNA binding. Dimer formation is specifically mediated through the leucine zipper (ZIP) domain, whereas DNA binding involves the basic region of each binding partner directly contacting the DNA. The Tax-CREB complexes associate with three Tax-responsive elements called viral CREs (vCREs) within the viral promoter that encompasses the U3 region of the 5'-long terminal repeat of the provirus. Each vCRE contains a central sequence similar to that of a cellular CRE that is recognized by CREB and flanking GC-rich sequences that are directly contacted by Tax (7–9). Like CREB, Tax incorporates into the ternary complex as a dimer (10, 11). Formation of these complexes on the viral promoter serves as a binding site for the coactivators p300 and CREB-binding protein (CBP) (12–15), which are recruited to the HTLV-1 promoter, in part, through a direct interaction with Tax. Indeed, the viral protein has been

* This work was supported by a Research Development Grant Program Award from East Carolina University (to I. C.) and by institutional grants from CNRS and the Université Montpellier 1 (UM 1) and grants from the Association pour la Recherche sur le Cancer (Grant 3606), the Fondation Recherche Médicale (Comité Languedoc), and the Ligue Contre le Cancer (Comité de l'Hérault) (to J. M. M.). 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.

¹ Supported by a fellowship from the Agence Nationale de Recherche sur le SIDA (ANRS).

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⁵ The abbreviations used are: HTLV-1, human T cell leukemia virus type 1; CRE, cAMP-response element; CREB, CRE-binding protein; CBP, CREB-binding protein; ZIP, leucine zipper; bZIP, basic leucine zipper; vCRE, viral CRE; HAT, histone acetyltransferase; ChIP, chromatin immunoprecipitation; aa, amino acids; GST, glutathione S-transferase.

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shown to interact with multiple domains of the coactivators, including the C/H1 domain, the KIX domain, and a COOH-terminal domain encompassing amino acids 2003–2212 (13, 15–17). Current evidence suggests that, among these domains, the interaction with the KIX domain is the primary mediator of p300/CBP recruitment to the viral promoter (13).

p300 and CBP are homologous multifunctional coactivators that are involved in regulating transcription of many cellular genes. Consequently, these coactivators are believed to contribute to a number of diverse biological functions, including proliferation, cell cycle regulation, apoptosis, differentiation, and the DNA damage response (18, 19). These coactivators possess intrinsic histone acetyltransferase (HAT) activity, which frequently imparts a stimulatory effect on transcription through acetylation of transcription factors as well as the core histone components of the chromatin (18, 20, 21). Additionally, through interactions with a diverse range of transcriptional regulatory proteins, p300 and CBP contribute to stable promoter binding of the core transcription machinery (18). In a similar fashion, these coactivators also serve as scaffolds for the integration of other transcriptional regulatory proteins into promoter-bound complexes. With respect to their central roles in transcription, p300 and CBP are often targeted by transforming viral proteins, such as the adenoviral E1A protein, the human papilloma virus E6 protein, the simian virus 40 large T antigen, and the HTLV-1 Tax protein (15, 22–24). Although it has become increasingly clear that p300 and CBP serve distinct, nonoverlapping functions in certain physiological processes, in the context of HTLV-1 transcription, these coactivators are currently believed to act interchangeably (5, 25, 26).

In addition to Tax, other viral proteins have been shown to regulate HTLV-1 transcription, one of which is HTLV-1 bZIP factor (HBZ) (27–29). Interestingly, this protein is uniquely encoded by a gene on the negative strand of the provirus and transcribed from a promoter located within the 3'-long terminal repeat (30–32). Therefore, HBZ expression is believed to be subjected to regulatory controls distinct from those affecting transcription from the 5'-long terminal repeat. Although various HBZ transcripts have been detected, the most abundant HBZ isoform expressed in HTLV-1-infected T cells was found to correspond to the 206-amino acid protein produced from the major alternative splice variant (30, 32–34). Differences among isoforms are limited to a few amino acid alterations at the NH₂-terminal of each protein. Each HBZ isoform contains an NH₂-terminal domain that can mediate transcriptional activation when the viral protein is artificially tethered to the DNA (28), a central domain involved in its nuclear localization (35), and a COOH-terminal bZIP domain (36). This latter domain carries a basic region with an amino acid sequence that is distinct from corresponding regions of other bZIP factors. Interestingly, HBZ was recently shown to localize to the *hTERT* promoter and stimulate transcription of this gene. However, it is reported to be associated with the promoter via a protein-protein interaction rather than direct DNA contacts (37).

Unlike Tax, HBZ functions to repress viral transcription. This effect may play a role in allowing infected T cells to escape the cytotoxic T-lymphocyte response by maintaining low levels of viral protein production (1, 38). In support of this model,

HBZ has been implicated in enhanced infectivity and persistence in HTLV-1-inoculated rabbits (27). At the molecular level, we have shown that the ZIP domain of HBZ contributes to its repressive function by mediating heterodimerization with CREB, CREB-2, CREM, and ATF-1 (28, 29). Since HBZ appears to lack the capacity to associate with the HTLV-1 promoter, formation of these heterodimers inhibits ATF/CREB factors from binding DNA and, consequently, prevents the recruitment of Tax to the vCREs. However, certain lines of evidence suggest that this mechanism does not fully account for the repressive effects of HBZ on HTLV-1 transcription. For example, using chromatin immunoprecipitation (ChIP) assays, we found that HBZ causes only a partial reduction in the level of CREB associated with the HTLV-1 promoter while producing a more dramatic decrease in transcription from the promoter (29). More importantly, we have shown that an HBZ mutant lacking the bZIP domain retains the ability to repress HTLV-1 transcription (29).

In this study, we provide evidence that an interaction between HBZ and p300/CBP contributes to the inhibitory effects of the viral protein on HTLV-1 transcription. We identified two LXXLL-like motifs in the NH₂-terminal domain of HBZ that are involved in this interaction specifically through the KIX domain of the coactivator. In line with this observation, we provide evidence that HBZ disrupts the interaction between Tax and p300/CBP, thereby inhibiting the association of the coactivator with the viral promoter. In conclusion, this mechanism may supplement the negative effect of HBZ on the formation of the Tax-CREB complex on the viral promoter to achieve sufficient repression of transcription for the maintenance of a persistent infection.

MATERIALS AND METHODS

Expression Vectors and Antibodies—The pBIND, pcDNA-HBZ-SP1-Myc, and pSG-Tax vectors were previously described (39, 40). HBZ fragments in pBIND were generated by PCR amplification of pcDNA-HBZ-SP1-Myc and cloned into the BamHI/XbaI sites of the vector. pcDNA-HBZ-ΔZIP-Myc (aa 1–161) was constructed by PCR amplification and cloned into the EcoRI/HindIII sites of pcDNA-MycHis (Invitrogen). Site-directed mutagenesis was performed, using the QuikChange site-directed mutagenesis kit (Stratagene) to produce the mutants S29A/S49A, S29D/S49D, L27A/L28A, L47A/L48A, and L27A/L28A/L47A/L48A. All constructs were sequenced to confirm that unintended mutations were not introduced during PCR amplification. The pGEX constructs expressing various regions of CBP fused to the *Schistosoma japonicum* glutathione S-transferase (GST) were described previously (16). Construction of the pGEX vector encoding GST-HBZ bZIP has been published (29). GST-HBZ and GST-HBZ-Mut(LXXAA)₂ were obtained by cloning PCR products into pGEX-2T (GE Healthcare) at the BamHI/EcoRI sites. The luciferase reporter plasmids K30-Luc and pG5luc and the reference plasmids PRL-TK-Luc and pcDNA-*lacZ* have been described (29, 41, 42).

Rabbit anti-p300 (N-15), anti-CBP (A-22), and anti-His₆ (H-15) and mouse anti-p300 (NM11) and anti-nucleolin (MS-3) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Myc (06-549) was pur-

chased from Millipore. The Tax monoclonal antibody (168B17-46-34) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The rabbit anti-HBZ antibody was previously described (28).

Cell Culture, Transfections, and Luciferase Assays—CHOK1-Luc, 293T, and CEM cells were cultured as described (29). The HTLV-1-associated cell line, ATL-2, obtained from M. Matsuo, and the HTLV-1 infected cell line, C8166/45, were cultured as described (16, 43). Vectors expressing wild type HBZ and truncated HBZ mutants fused with the DNA-binding domain of GAL4 of the pBIND vector were electroporated into CEM cells with the pG5luc luciferase reporter plasmid and pcDNA-*lacZ* reference plasmid as described (39). The 293T cells were transfected with K30-Luc, using the CalPhos mammalian transfection kit (Clontech BD Biosciences). Amounts of individual plasmids used in each transfection assay are indicated in the figure legends. The total amount of DNA in each transfection was equalized, using empty vectors as required. Cell extracts were prepared, equalized for protein content, and used for luciferase and β -galactosidase assays as described (39). Luciferase assays were performed in an automated luminometer with the Genofax A kit (Yelen Corp.). Luciferase activity was normalized to β -galactosidase activity. CHOK1-Luc cells were transfected with the plasmids indicated in the figure legends, using the LTX reagent (Invitrogen). Cells were harvested and lysed 24 h post-transfection, and luciferase activity was measured, using the dual luciferase reporter assay system (Promega) with a Turner Designs model TD 20-e luminometer. Luciferase activity was normalized to *Renilla* luciferase activity from the herpes simplex virus thymidine kinase promoter (pRL-TK; Promega).

Immunoprecipitation Assays—Vectors expressing wild type HBZ or HBZ mutants were electroporated with the GenePulser Xcell (Bio-Rad) into CHOK1 cells, and lysates were prepared in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 100 mM NaCl, 1 mM MgCl₂, 2 mM benzamide, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Antibody-bound beads were washed in radioimmunoprecipitation assay buffer, and 500 or 800 μ g of cell lysates were added to each antibody-bead suspension, incubated overnight, and washed several times in radioimmunoprecipitation assay buffer containing 300 mM NaCl. Bound proteins were analyzed on SDS-polyacrylamide gels and detected by Western blotting, as described (29). Immunoprecipitation assays with HTLV-1-infected cells were performed with 2.5 mg of cell extracts.

Expression and Purification of Recombinant Proteins and in Vitro Translation—*Escherichia coli* expression plasmids for GST, GST-C/H1-(302–451), GST-C/H1-KIX-(302–683), GST-KIX-(588–683), GST-C/H3-(1514–1894), GST-C1-(1894–2221), and GST-C2-(2212–2441) were transformed into BL21(DE3) pLysS *E. coli*. Proteins were expressed and purified by glutathione-agarose affinity chromatography, as described previously (16). The plasmid encoding GST-HAT-(1096–1757) was obtained from D. Thanos (Institute of Molecular Biology and Genetics, Athens, Greece). GST-HAT was similarly expressed and purified. Tax was expressed from the pTax-His₆ (44) expression plasmid in BL21(DE3) pLysS *E. coli*

and purified by Ni²⁺-nitrilotriacetic acid-agarose chromatography (Qiagen), as described previously (13). Purified proteins were dialyzed against TM buffer containing 50 mM Tris, pH 7.9, 12.5 mM MgCl₂, 100 mM KCl, 1 mM EDTA, pH 8.0, 1 mM dithiothreitol, 0.025% (v/v) Tween 20, and 20% (v/v) glycerol, aliquoted, and stored at -70°C . The dialysis buffer for Tax also included 20 μ M ZnSO₄. His₆-tagged p300 and FLAG-tagged CBP were expressed from recombinant baculoviruses (obtained from J. Kadonaga, University of California) in Sf9 cells and purified as previously described (45). *In vitro* translation of HBZ proteins was performed using the TNT translation system (Promega) in the presence of [³⁵S]methionine according to the manufacturer's protocol.

ChIP Assay—Cells (2×10^7) were electroporated with the GenePulser Xcell (Bio-Rad) in the presence of 20 μ g of total DNA. Cells were harvested 24 h later for luciferase and ChIP analyses. ChIP assays were performed essentially as described previously (5), using 5 μ g of p300 antibody for the immunoprecipitation.

Real Time PCR—Real time PCR was performed with an iCycler and the optical assembly unit (Bio-Rad). Reactions were done in triplicate using the iQ SYBR green Supermix (Bio-Rad) as described previously (5). The PCR primers for the HTLV-1 promoter are 5'-ATCATAAGCTCAGACCTCCGGGAA-3' and 5'-CCTGAGGACGGCTTGACAAACAT-3'. Standard curves were generated for this primer set using 10-fold serial dilutions of CHOK1-Luc input DNA (DNA purified from a fraction of the total chromatin prior to immunoprecipitation) from each ChIP assay and were included on each experimental plate. PCR efficiencies ranged from 75 to 86%, with correlation coefficients ranging from 0.97 to 1.0. Quantitation was done by comparing threshold cycle values for coimmunoprecipitated DNA to the threshold cycle value for the input DNA in each ChIP experiment, as described previously (46).

GST Pull-down Assays—All GST pull-down experiments were performed with 20 μ l of glutathione-agarose beads equilibrated in 0.5 \times Superdex buffer (12.5 mM HEPES, pH 7.9, 6.25 mM MgCl₂, 5 μ M ZnSO₄, 75 mM KCl, 20% (v/v) glycerol, 0.05% Nonidet P-40, 0.5 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). GST fusion proteins were incubated with glutathione-agarose beads for 1 h at 4 $^{\circ}\text{C}$ and then washed with 0.5 \times Superdex buffer. The second protein was then added to the washed beads and incubated overnight at 4 $^{\circ}\text{C}$. Beads were washed four times, and bound proteins were eluted with SDS sample dyes and resolved by electrophoresis on a 10% SDS-polyacrylamide gel. Radiolabeled HBZ proteins were detected by PhosphorImager analysis of dried gels. Tax was detected by Western blot analysis using the anti-His₆ antibody.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed with recombinant proteins, as previously described (29). Briefly, the quaternary complex was assembled using 80 fmol of CREB, 0.5 pmol of Tax, 0.5 pmol of GST-C/H1-KIX, and 7 fmol of ³²P-end-labeled double-stranded DNA probe in a 20- μ l reaction of 0.5 \times TM buffer with 5 ng of poly(dA)·poly(dT) DNA. Binding reactions were chilled on ice for 15 min and then supplemented with GST-HBZ or GST-HBZ-Mut(LXXAA)₂ (0.5, 1, and 2 pmol), as

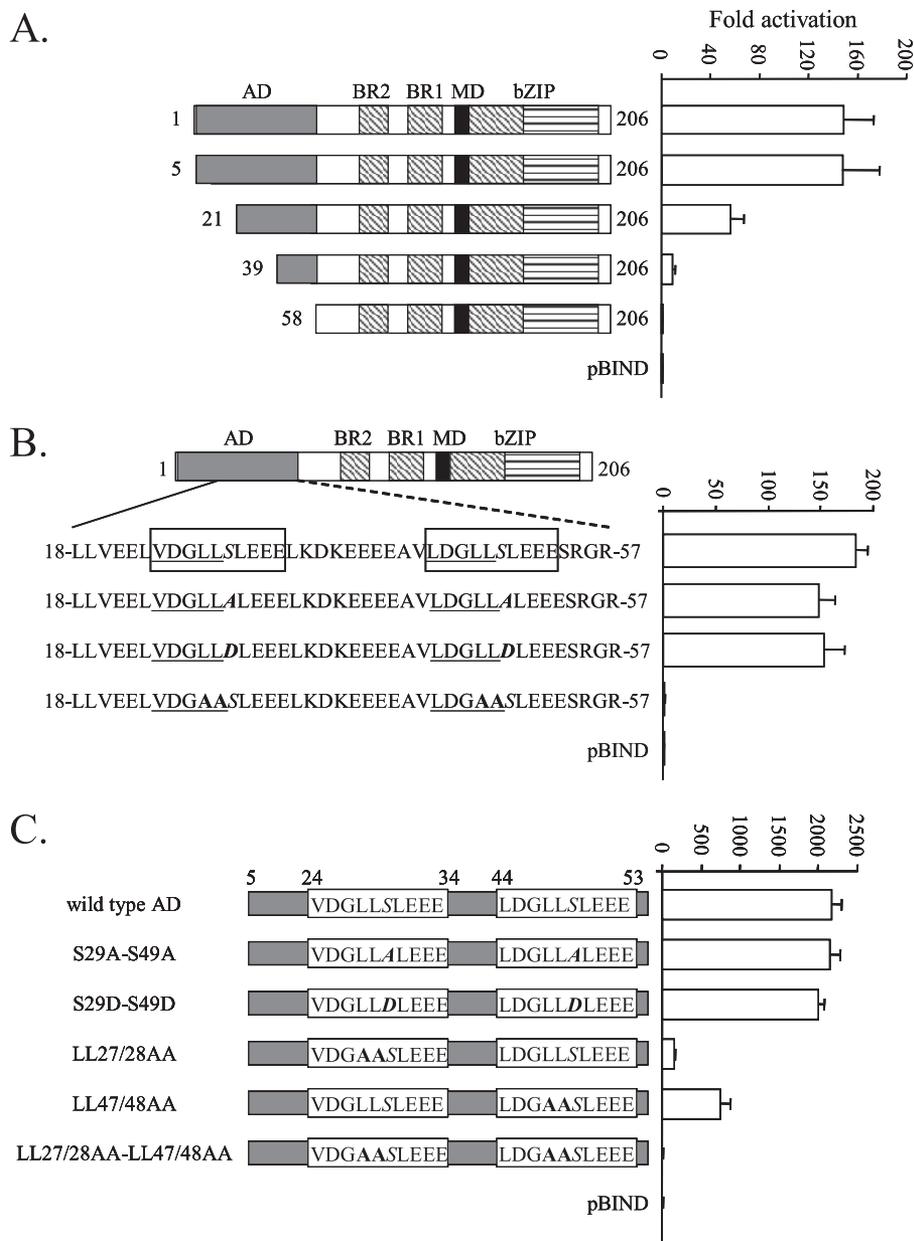


FIGURE 1. The activation domain of HBZ contains two LXXLL-like motifs involved in its transcriptional activity. *A*, HBZ carries an activation domain. The activation domain (AD), basic regions (BR1 and BR2), transcriptional modulatory domain (MD), and bZIP domain of HBZ are indicated. CEM cells were cotransfected with 5 μ g of pcDNA-*lacZ* (β -galactosidase internal control), 2 μ g of the pG5luc luciferase reporter vector, and 2 μ g of pBIND expressing the GAL4 DNA-binding domain alone or fused to either the full-length HBZ (206 amino acids) or the indicated HBZ NH₂-terminal truncation mutants. Luciferase values were normalized to β -galactosidase activity and are expressed as a -fold increase relative to that for cells transfected with pG5luc and pBIND only. The reported values are the average luminescence from three independent experiments, and the S.E. is indicated. *B*, the LXXLL-like motifs are required for the transcriptional activity of the HBZ activation domain. Experiments were performed as described in *A* with the full-length proteins. Mutations are indicated in the portion of the activation domain sequence shown. The repeat (V/L)DGLLSLEEE sequences are boxed, the LXXLL-like motifs are underlined, and the serine residues are italicized. Mutated residues are shown in boldface type. *C*, both LXXLL-like motifs are necessary for full transcriptional activity of HBZ activation domain. CEM cells were cotransfected with pG5luc and pBIND containing only the wild type or mutated activation domain of HBZ (from aa 5 to 57) as indicated. The mutated residues are in boldface type. Experiments were performed as described in *A*. Vectors used for transfection expressed the GAL4 DNA-binding domain fused to the activation domain of HBZ (aa 5–57) or the indicated activation domain mutants.

indicated. Reactions were chilled on ice for an additional 15 min and subsequently resolved on a 5% nondenaturing polyacrylamide gel. Gels were dried, and complexes were visualized by PhosphorImager analysis. The DNA probe used was the vCRE of the promoter-proximal 21-bp repeat.

RESULTS

Two NH₂-terminal LXXLL-like Motifs in HBZ Drive Transcriptional Activation—In correlation with its unique basic region, HBZ has not been shown to harbor DNA binding activity. However, by artificially tethering HBZ to DNA, we previously found that its NH₂-terminal domain is capable of activating transcription (28). Using this approach, we were interested in mapping the precise region within this domain that is responsible for the activation function. We therefore produced mammalian expression vectors for HBZ, carrying increasing NH₂-terminal truncations, fused to the yeast GAL4 DNA-binding domain. The transcriptional activity of these mutants was tested in CEM T cells in the presence of the pG5luc reporter vector that contains five GAL4-binding sites upstream of a core promoter and the luciferase gene. As shown in Fig. 1*A*, the region of HBZ involved in transcriptional activation was identified between amino acids 5 and 57, with numbering corresponding to the 206-amino acid primary HBZ isoform expressed in HTLV-1-infected T cells (32).

An analysis of this region revealed that it contains two identical subdomains rich in leucines and acidic residues, having the consensus sequence (V/L)DGLLSLEEE (Fig. 1*B*). Interestingly, the NH₂-terminal portion of this sequence resembles an LXXLL motif, which is well known to be a signature motif involved in recruitment of transcriptional coactivators (47–51). To test whether the LXXLL motifs of HBZ contributed to the transcriptional activity of the viral protein, we mutated the two last leucines to alanines in both motifs (LXXAA). Separate mutants were also constructed in which the serine residues adjacent to the LXXLL motifs (Ser²⁹ and Ser⁴⁹) were substituted with alanine or aspartate residues.

Mutants were fused to the GAL4 DNA-binding domain and tested for their ability to activate transcription from the pG5luc reporter plasmid in CEM cells. As shown in Fig. 1*B*, mutations of the serine residues produced very little effect on the transac-

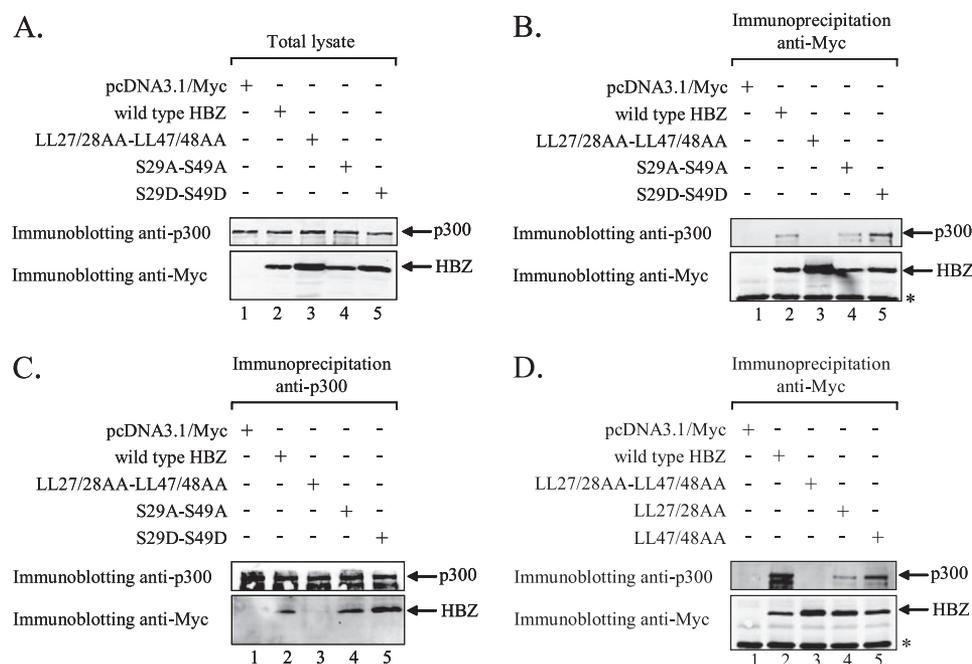


FIGURE 2. The LXXLL-like motifs of HBZ are involved in its interaction with p300 *in vivo*. CHOK1 cells were electroporated with 10 μ g of the empty vector pcDNA-MycHis or the vector encoding wild type HBZ or individual HBZ mutants (L27A/L28A/L47A/L48A (LL27/28AA-LL47/48AA)), S29A/S49A (S29A-S49A), S29D/S49D (S29D-S49D), L27A/L28A (LL27/28AA), and L47A/L48A (LL47/48AA)), as indicated. *A*, total lysates (50 μ g) were subjected to Western blot analysis using anti-Myc and anti-p300 antibodies, as indicated. *B*, proteins were immunoprecipitated with anti-Myc antibody and subjected to Western blot analysis using anti-p300 and anti-Myc antibodies, as indicated. The asterisk indicates the lower Ig chain. *C*, proteins were immunoprecipitated with anti-p300 antibody and subjected to Western blot analysis using anti-p300 and anti-Myc antibodies as indicated. *D*, proteins were immunoprecipitated with anti-Myc antibody and subjected to Western blot analysis using anti-p300 and anti-Myc antibodies, as indicated. The asterisk indicates the lower Ig chain.

tivational activity in comparison with the wild-type protein. However, transcriptional activity was completely abolished by mutations in both LXXLL motifs (Fig. 1B).

To determine whether a specific LXXLL motif in HBZ plays a primary role in transcriptional activation, each motif was separately mutated. The modified NH₂-terminal domains (amino acids 5–57) were then fused to the GAL4 DNA-binding domain to analyze transcriptional activation of the pG5luc reporter plasmid in CEM cells (Fig. 1C). Interestingly, removal of the COOH-terminal portion of HBZ resulted in greater overall activation than the full-length protein. This effect is potentially due to an increase in the accessibility of the LXXLL motifs in this context. We found that mutation of either motif resulted in partial loss of transcriptional activity, with mutation of the NH₂-terminal motif producing a more substantial reduction in activity (stimulation of about 150-fold) than the COOH-terminal motif (about 750-fold). Similar to results shown above, mutating both LXXLL-like motifs caused a complete loss in the activation function (Fig. 1C). Taken together, these data demonstrate that both LXXLL-like motifs within the NH₂-terminal domain of HBZ contribute to transcriptional activation.

HBZ Interacts with p300 *in Vivo*—The LXXLL motif is found in several transcription factors, where it serves as a direct binding site for the p300/CBP coactivators (47, 52–55). Given the prominent role of the dual LXXLL-like motifs in the transcriptional activity of HBZ, we were interested in determining whether HBZ could interact with p300/CBP *in vivo*. We therefore performed coimmunoprecipitation experiments using

CHOK1 cells transfected with either an expression vector for Myc-tagged, wild type HBZ or one of the three HBZ mutants analyzed above, all of which were also Myc-tagged (Fig. 2A, lanes 2–5). Using an anti-Myc-tag antibody for immunoprecipitation, p300 was detected in immunoprecipitates from cells expressing wild type HBZ (Fig. 2B, lane 2) or HBZ containing mutations in the serine residues (Ser²⁹ and Ser⁴⁹) (Fig. 2B, lanes 4 and 5). In contrast, p300 was not found in the immunoprecipitate from cells expressing HBZ with mutations in both LXXLL-like motifs (Fig. 2B, lane 3). Complementary results were obtained using an anti-p300 antibody for immunoprecipitation and the anti-Myc tag antibody to probe for HBZ and the mutants (Fig. 2C). The same results were obtained using human HeLa fibroblast cells in place of CHOK1 cells (data not shown). These results correlate the activation function of HBZ to an interaction with p300, which specifically requires the LXXLL motifs of the viral protein.

As shown in Fig. 1C, mutation of the NH₂-terminal LXXLL-like motif in HBZ resulted in a greater reduction in its transcriptional activity than mutation of the COOH-terminal motif. To determine whether this result reflects a dominant role of the NH₂-terminal motif in mediating the interaction with p300, we prepared expression vectors for Myc-tagged, full-length HBZ, in which each motif was mutated separately (L27A/L28A or L47A/L48A). Expression vectors were individually transfected into CHOK1 cells, and immunoprecipitation experiments were performed using an anti-Myc tag antibody. We found that mutation of the NH₂-terminal motif (L27A/L28A) in HBZ caused a greater reduction in amount of p300 in the immunoprecipitate than mutation of the COOH-terminal motif (L47A/L48A) (Fig. 2D, lanes 4 and 5). As in Fig. 2B, p300 was not immunoprecipitated with the double mutant (Fig. 2D, lane 3). These results suggested that the NH₂-terminal LXXLL-like motif in HBZ provides the major contribution to the interaction with p300.

Finally, to test whether endogenous cellular levels of HBZ and p300 are sufficient for this interaction, we performed coimmunoprecipitation experiments using extracts prepared from ATL-2 and C8166/45 cells. The ATL-2 cell line was derived from leukemic cells of an ATL patient (16, 43), and the C8166/45 cell line is an HTLV-1-infected T-cell line. As shown in Fig. 3 (lanes 3 and 6), p300 was coimmunoprecipitated with HBZ in both cell lines using an antibody directed against the bZIP domain of the viral protein (28). In negative control experiments, HBZ and p300 were not immunoprecipitated with pre-

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immune serum (Fig. 3, lanes 2 and 5). These results support the existence of the HBZ-p300 complex in virally infected cells.

The NH₂-terminal Domain of HBZ Contributes to Repression of Tax-dependent Viral Transcription in Vivo—HBZ was previously shown to repress HTLV-1 transcription (27, 29) by interacting with and thereby inhibiting the binding of ATF/CREB factors to the viral promoter (28, 29). This effect was found to be mediated through the bZIP domain of HBZ. Interestingly, we found that a truncated form of HBZ lacking the bZIP domain retained the capacity to down-regulate viral expression (29), suggesting that another domain of HBZ is also involved in the repression of HTLV-1 transcription. To determine whether the NH₂-terminal domain and, specifically, the LXXLL motifs fulfilled this function, we compared the effects of wild type HBZ and HBZ mutants on HTLV-1 transcription. The HBZ mutants used in these experiments included HBZ-ΔZIP (deleted of the ZIP domain), HBZ-Mut(LXXAA)₂

(mutated in both LXXLL-like motifs), and HBZ-ΔZIP-Mut(LXXAA)₂ (combined ZIP deletion and LXXLL mutations). Their effects were first tested in the context of a proviral DNA clone containing a luciferase reporter gene inserted in frame with the envelope amino acid sequence (41). This construct was derived from the K30 proviral DNA clone without altering the splice donor site for multiply spliced mRNA. The proviral DNA was cotransfected into 293T cells with the expression vector for HBZ, HBZ-ΔZIP, HBZ-ΔZIP-Mut(LXXAA)₂, or HBZ-Mut(LXXAA)₂, all of which were Myc-tagged. Luciferase assays were then performed to assess the effects of these HBZ mutations on transcription of the proviral DNA (Fig. 4A, top). As we previously showed (16), expression of wild type HBZ or HBZ-ΔZIP led to a reduction in luciferase activity (Fig. 4A, lanes 2 and 3). A similar reduction was also obtained with HBZ-Mut(LXXAA)₂ (Fig. 4A, lane 5), whereas in contrast, HBZ-ΔZIP-Mut(LXXAA)₂ did not significantly affect luciferase activity (Fig. 4A, lane 4). These results suggest that both the ZIP domain and the LXXLL-like motifs of HBZ negatively regulate the expression of K30-Luc. Protein levels of wild type HBZ and the mutants in transfected cells were determined by Western blot analysis (Fig. 4A, bottom). Point mutations in the LXXLL motifs did not significantly affect the migration of full-length HBZ (compare lanes 2 and 5) or the truncated form of the protein (compare lanes 3 and 4).

We were interested in determining whether the HBZ mutants affected Tax-mediated activation from chromosomally integrated HTLV-1 promoters that are packaged into physiological chromatin. Therefore, we used CHOK1-Luc cells that carry 2–4 genomically integrated copies of the HTLV-1 promoter cloned upstream of the luciferase gene (56). These cells were transfected with a Tax expression vector alone or in combination with wild type HBZ or individual HBZ mutants described above. In parallel with results obtained using the proviral clone, wild type HBZ and HBZ-ΔZIP each repressed Tax-mediated transcriptional activation, whereas HBZ-ΔZIP-Mut(LXXAA)₂ did not affect this process (Fig. 4B, lanes 3–5). HBZ-Mut(LXXAA)₂ also reduced luciferase activity, although less effectively than what was observed with the proviral clone (Fig. 4B, lane 6). Given that this mutant exhibits a deficiency in p300/CBP binding, this result correlates with studies demonstrating that the positive regulatory effects of the coactivators in HTLV-1 transcription are mainly observed in a chromatin context (25, 26, 56). Together, these data confirm that the LXXLL motifs of HBZ contribute to its repressive effects on Tax-dependent viral transcription.

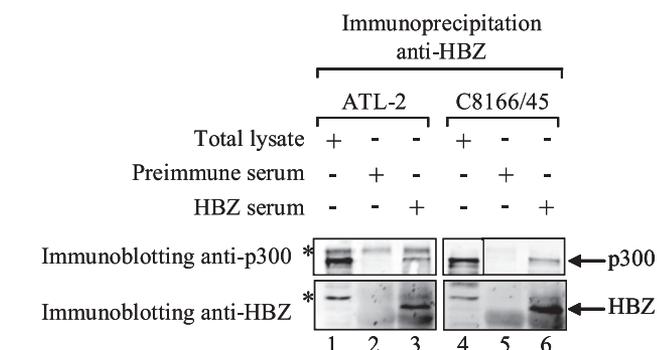


FIGURE 3. HBZ interacts with p300 in HTLV-1-infected cells. Proteins were immunoprecipitated from ATL-2 and C8166/45 cell extracts with anti-HBZ antibody and subjected to Western blot analysis using anti-p300 and anti-HBZ antibodies as indicated. The asterisks indicate cross-reactive bands.

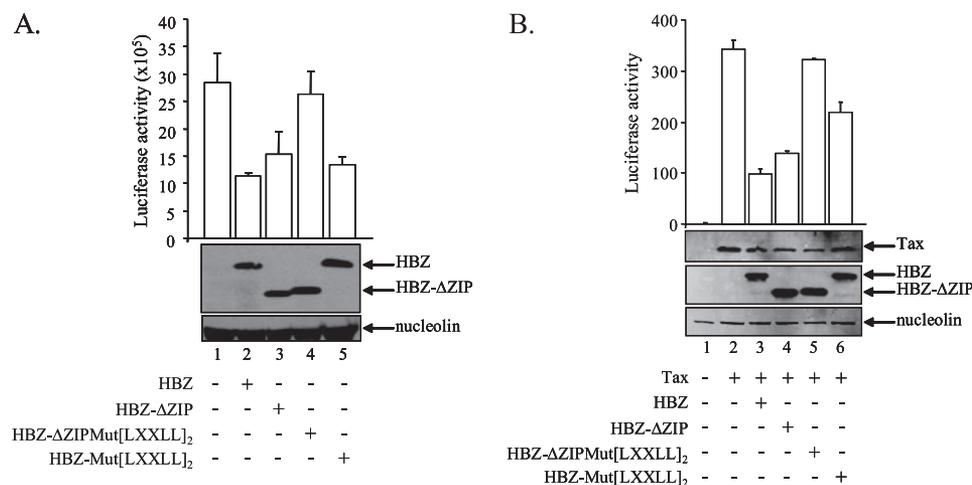


FIGURE 4. The LXXLL-like motifs of HBZ are involved in the repression of Tax-dependent viral transcription in vivo. *A*, the p300-interacting domain of HBZ is involved in the repression of proviral DNA expression. 293T cells were cotransfected with 2 μg of K30-Luc; 2 μg of pcDNA-MycHis expressing HBZ, HBZ-ΔZIP, HBZ-ΔZIP-Mut(LXXAA)₂, or HBZ-Mut(LXXAA)₂; and 1 μg of pcDNA-lacZ. Luciferase assays were performed 48 h after transfection, and reported values are the average luminescence ± S.E. from three experiments. *B*, the activation domain of HBZ is involved in the down-regulation of Tax-mediated transcriptional activation from the HTLV-1 promoter. CHOK1-Luc cells were transfected with pSG-Tax (100 ng) in the absence or presence of pcDNA-MycHis (200 ng) encoding wild type HBZ or the indicated mutants and 10 ng of pRL-TK-Luc. Luciferase assays were performed 24 h after transfection. The reported values are the average luminescence ± S.E. from three experiments. Expression of Tax and HBZ was determined by Western blot analysis using anti-Tax and anti-Myc antibodies, respectively. Nucleolin was probed as a loading control.

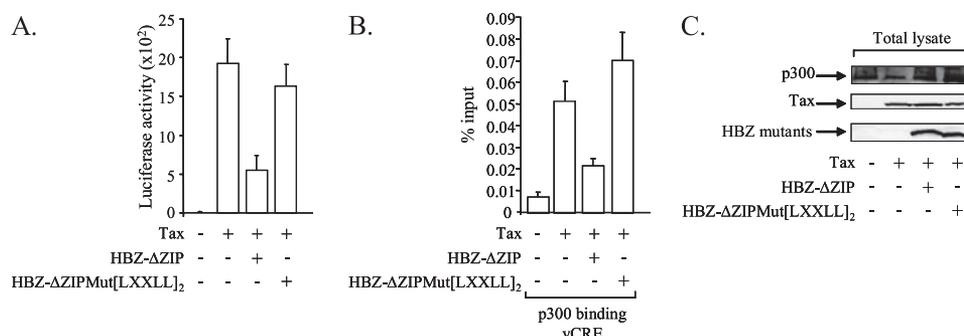


FIGURE 5. HBZ-ΔZIP displaces p300 from the HTLV-1 promoter *in vivo*. *A*, HBZ-ΔZIP represses Tax-mediated transcriptional activation from the HTLV-1 promoter. CHOK1-Luc cells were electroporated with pSG-Tax (6.5 μg) alone or in combination with pcDNA-MycHis (13.5 μg) encoding HBZ-ΔZIP or HBZ-ΔZIP-Mut(LXXAA)₂. Luciferase assays were performed 24 h after transfection. The reported values are the average luminescence ± S.E. from three experiments. *B*, HBZ-ΔZIP disrupts p300 binding at the HTLV-1 promoter through its LXXLL-like motifs. ChIP assays were performed 24 h post-transfection using the same transfected cells used in *A*. Real time PCR was used to quantify levels of p300-enrichment at the viral promoter by comparing amplification of the co-immunoprecipitated DNA with that of a fraction of the total input DNA. The graph is the average ± S.E. from three independent ChIP assays. *C*, Tax and HBZ-ΔZIP are expressed in transfected cells. Western blot analysis was performed at 24 h post-transfection with whole cell extracts from cells used in one of the experiments in *A* and *B*. The membrane was probed with anti-p300, anti-Tax, and anti-Myc antibodies.

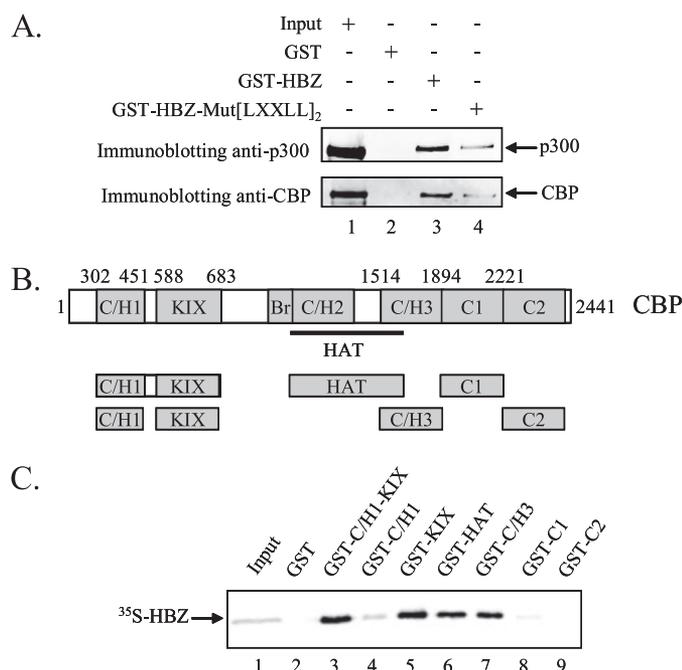


FIGURE 6. HBZ interacts with different domains of p300/CBP *in vitro*. *A*, HBZ interacts with p300 and CBP. Purified p300 or CBP (0.2 pmol) was incubated with 2.5 pmol of GST, GST-HBZ, or GST-HBZ-Mut(LXXAA)₂. Bound proteins were detected by Western blot analysis. A fraction of the input protein (20%) is shown in *lane 1*. *B*, schematic illustration of the GST-CBP fusion proteins used in this study. CBP domains include the cysteine-histidine-rich domains 1–3 (C/H1, C/H2, and C/H3), the kinase-inducible binding domain (KIX), the bromodomain (Br), the histone acetyltransferase domain (HAT), and the carboxyl-terminal regions (C1 and C2). *C*, HBZ binds to KIX, HAT, and C/H3 *in vitro*. Equal amounts of GST and GST fused to different CBP domains (10 pmol) were incubated with 2.5 μl of ³⁵S-labeled HBZ, and bound proteins were analyzed by SDS-PAGE and autoradiography. A fraction of the input protein (10%) is shown (*lane 1*).

the recruitment of p300 to the viral promoter. Using ChIP assays, we previously characterized the recruitment of Tax, CREB, p300, and other transcriptional regulatory proteins to the integrated HTLV-1 long terminal repeat (57). We also found that HBZ expression led to a modest decrease in the level of CREB at the viral promoter while producing a disproportionately larger

reduction in luciferase activity (29). The fact that the LXXLL motifs of HBZ mediate an interaction with p300 suggested that HBZ was inhibiting the recruitment of p300 to the viral promoter. To test this hypothesis, we performed ChIP assays using CHOK1-Luc cells. In these assays, DNA was coimmunoprecipitated using an antibody directed against p300, and the HTLV-1 promoter was amplified using real time PCR. As shown previously, transfection of these cells with the Tax expression vector increased p300 enrichment at the viral promoter (57) (Fig. 5*B*), correlating with an increase in luciferase activity (Fig. 5*A*). Strikingly, cotransfection with the HBZ-ΔZIP expression vector,

which encodes a truncated form of HBZ that is unable to interact with ATF/CREB factors (28, 29), significantly reduced the level of p300 at the promoter (Fig. 5*B*). However, cotransfection with the HBZ-ΔZIP-Mut(LXXAA)₂ expression vector did not cause a reduction in p300 enrichment (Fig. 5*B*). These results correlate HBZ-ΔZIP-mediated repression on Tax-dependent HTLV-1 transcription with a reduction in the level of p300 associated with the viral promoter. More specifically, they suggest that the LXXLL-like motifs in HBZ are involved in the interaction between the viral protein and p300 that leads to transcriptional repression by inhibiting the recruitment of the coactivator to the promoter.

The LXXLL-like Motifs Are Essential for Binding to the KIX Domain of p300/CBP—To better understand how the LXXLL-like motifs are involved in the down-regulation of viral transcription, we were interested in determining whether the interaction between HBZ and p300 was direct. We therefore performed GST pull-down assays using recombinant, purified proteins. In these experiments, wild type HBZ and HBZ-Mut(LXXAA)₂ were fused to GST. As shown in Fig. 6*A*, p300 and CBP were both found to bind specifically to HBZ (*lane 3*), since they did not interact with GST alone (*lane 2*). In contrast, mutations in the LXXLL-like motifs greatly decreased the interactions between HBZ and p300/CBP, although the binding of HBZ-Mut(LXXAA)₂ to p300/CBP was not completely abolished (Fig. 6*A*, *lane 4*).

To determine which domains of the coactivator interact with HBZ, we performed GST pull-down assays using ³⁵S-labeled HBZ and different regions of CBP fused to GST. The specific domains of CBP that were analyzed included the C/H1-KIX (aa 302–683), HAT (aa 1096–1757), and C/H3 (aa 1514–1894) domains as well as two COOH-terminal domains (aa 1894–2221 and 2221–2441) (Fig. 6*B*). As shown in Fig. 6*C*, HBZ bound strongly and specifically to C/H1-KIX (*lane 3*), HAT (*lane 6*), and C/H3 (*lane 7*). The HAT and C/H3 domains partially overlap (Fig. 6*B*), suggesting that HBZ binds to the region common to both domains. Within the C/H1-KIX region, HBZ preferentially recognized the KIX domain, which consists of

HBZ Interacts with p300/CBP

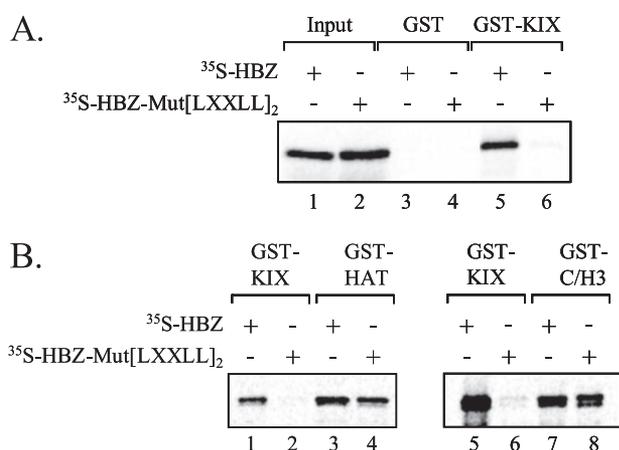


FIGURE 7. HBZ mutated at the LXXLL-like motifs lacks the ability to bind the KIX domain of CBP. *A*, HBZ-Mut(LXXAA)₂ does not bind the KIX domain. GST or GST-KIX (10 pmol) was incubated with 2.5 μ l of ³⁵S-labeled HBZ or HBZ-Mut(LXXAA)₂. Bound proteins were analyzed by SDS-PAGE and autoradiography. A fraction of the input protein (25%) is shown in lanes 1 and 2. *B*, HBZ-Mut(LXXAA)₂ binds to the HAT and C/H3 domain. GST-KIX, GST-HAT, or GST-C/H3 (10 pmol) was incubated with 2.5 μ l of ³⁵S-labeled HBZ or HBZ-Mut(LXXAA)₂. Bound proteins were analyzed by SDS-PAGE and autoradiography. The *odd* and *even* lanes correspond to the wild type and mutant proteins, respectively.

amino acids 588–683 of CBP (Fig. 6C, compare lanes 4 and 5). These results indicate that HBZ interacts with multiple domains of the coactivators.

We were therefore interested in defining which of the p300/CBP domains were essentially involved in the *in vivo* repression of viral transcription mediated through the LXXLL motifs of HBZ. Using GST pull-down assays, we compared the ability of ³⁵S-labeled wild type HBZ and HBZ-Mut(LXXAA)₂ to interact with the different coactivator domains (GST-KIX, GST-HAT, and GST-C/H3). As shown above, wild type HBZ interacted specifically with all three coactivator domains (Fig. 7). Interestingly, HBZ-Mut(LXXAA)₂ was defective for KIX binding (Fig. 7A) but retained the ability to interact with the HAT and C/H3 domains (Fig. 7B). These data show that the LXXLL-like motifs are directly involved in the interaction between HBZ and the KIX domain of p300/CBP.

HBZ Competes with Tax for Binding to the KIX Domain of p300/CBP—Since Tax recruits p300/CBP to the HTLV-1 promoter through a direct interaction with the coactivator C/H1-KIX domain (14, 16, 58), it is possible that HBZ competes with Tax for binding to the same site on the coactivator. To test this hypothesis, we performed GST pull-down assays using GST-C/H1-KIX with recombinant, purified Tax alone or in combination with increasing amounts of ³⁵S-labeled HBZ. As shown in Fig. 8A, the interaction between Tax and GST-C/H1-KIX was effectively reduced by HBZ, which, in turn, bound to GST-C/H1-KIX (compare lanes 4, 5, and 6).

To determine whether HBZ could displace KIX from the vCRE·Tax·CREB complex, we performed electrophoretic mobility shift assays in which we challenged the quaternary complex with increasing concentrations of GST-HBZ. The GST fusion protein was used due to the insolubility of *E. coli*-expressed, untagged HBZ. In binding reactions, a complex consisting of recombinant, purified Tax, CREB, and C/H1-KIX was formed on a radiolabeled DNA probe containing the proximal

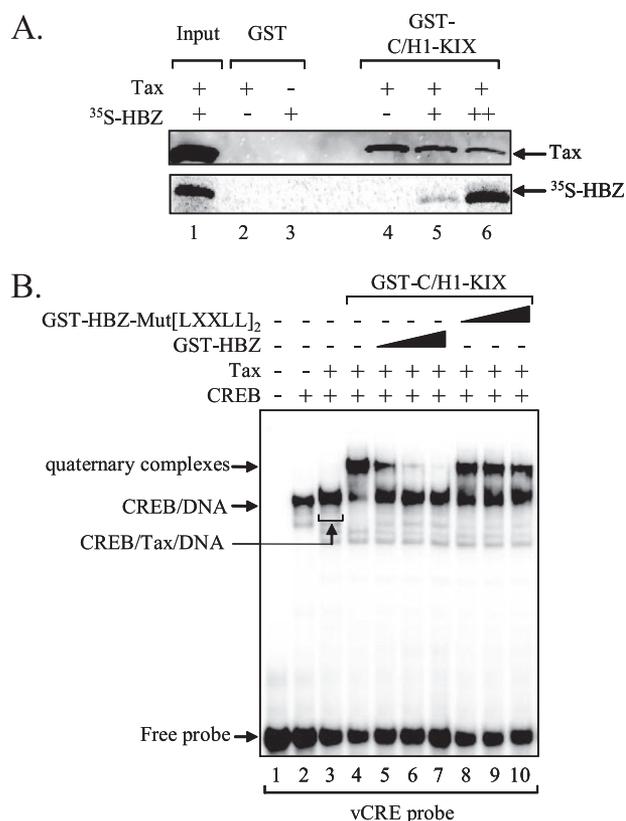


FIGURE 8. HBZ inhibits Tax-binding to the KIX domain of CBP. *A*, HBZ disrupts Tax binding to GST-C/H1-KIX. Ten pmol of GST (lanes 2 and 3) or GST-C/H1-KIX (lanes 4–6) was incubated with 1 pmol of purified Tax (lanes 2, 4, 5, and 6). Increasing amounts of ³⁵S-labeled HBZ (0.5 and 1 μ l) were added to lanes 5 and 6. Complexes were separated by SDS-PAGE, and bound Tax and HBZ were detected by Western blot and PhosphorImager analysis, respectively. A fraction of each input protein is shown in lane 1. *B*, HBZ inhibits KIX binding to the CREB-Tax-vCRE complex. Electrophoretic mobility shift assays were used to analyze protein-DNA interactions as described under “Materials and Methods.” Components added to each binding reaction and present in the shifted complexes are indicated. The quaternary complex is composed of CREB, Tax, and C/H1-KIX associated with the vCRE radiolabeled probe.

viral CRE sequence prior to the addition of GST-HBZ (Fig. 8B, lane 4). This step circumvented the inhibitory effects of HBZ on CREB, since the CREB homodimer is not affected by HBZ once it is bound to the DNA (29). These properties were also observed with another bZIP factor that is targeted by HBZ, c-Jun (59). In the current experiments, the addition of increasing amounts of GST-HBZ to binding reactions led to a dramatic loss of KIX from the complex (Fig. 8B, lanes 5–7). Indeed, a 1:1 molar ratio of HBZ to Tax was sufficient to displace more than 50% of the KIX from the complex. Unlike the wild type protein, GST-HBZ-Mut(LXXAA)₂ did not significantly affect the integrity of the quaternary complex (Fig. 8B, lanes 8–10). Identical results were obtained using KIX in place of C/H1-KIX (data not shown). Taken together, these results suggest that HBZ displaces p300/CBP from the HTLV-1 promoter by competing with Tax for the binding to the KIX domain of the coactivator. This effect contributes to the ability of HBZ to repress Tax-dependent viral transcription.

DISCUSSION

HBZ was shown to repress Tax-mediated viral transcription by binding to cellular ATF/CREB factors and preventing them

from associating with the viral promoter (27–29). Interactions between HBZ and these transcription factors occur through the ZIP domains found in the proteins. However, we recently demonstrated that an HBZ mutant lacking this domain retains the ability to repress HTLV-1 transcription (29), suggesting that another domain in HBZ functions through an additional mechanism to facilitate down-regulation of HTLV-1 transcription. In the current study, we show that this mechanism depends on the binding of HBZ to the cellular coactivator p300/CBP. This interaction serves to inhibit recruitment of the coactivator to the HTLV-1 promoter, which is correlated with a reduction in viral transcription.

Two LXXLL-like motifs located in the NH₂-terminal domain of HBZ are the primary mediators of the HBZ-p300/CBP interaction. The LXXLL motif was originally identified in certain transcriptional coactivators as a sequence both necessary and sufficient for docking these proteins to nuclear receptors (48–51). Several viral and cellular proteins, such as Kaposi's sarcoma-associated herpesvirus ORF50, the chimeric oncogenic protein E2A-PBX1, and c-Myb have since been found to also carry LXXLL motifs that mediate interactions with p300/CBP (47, 52–55). In the case of HBZ, we found that mutation of LXXLL-like motifs dramatically reduced the interaction between the viral protein and p300/CBP *in vivo*. Furthermore, mutation of these LXXLL-like motifs and deletion of the ZIP domain completely restored p300 recruitment to the HTLV-1 promoter and concurrently abolished the repressive effects of HBZ on Tax-dependent viral transcription. It is interesting to note that the NH₂-terminal LXXLL-like motif in HBZ, VDGLL, is similar to the motif VDLIL that was identified as the optimal peptide sequence for binding to the coactivator KIX domain in the first round of a phage display analysis (52). We found that mutation of this motif led to a greater reduction in the activation function of HBZ than mutation of the COOH-terminal motif (Fig. 1C) and, consequently, a diminished interaction with p300 (Fig. 2D).

In the current study, we also demonstrate that the LXXLL-like motifs of HBZ are specifically required for a direct interaction between the viral protein and the KIX domain of CBP. The KIX domain is conserved between p300 and CBP and serves as a binding site for a variety of transcription factors, such as phospho-CREB, CREB-2, c-Jun, c-Myb, MyoD, p53, Stat1 α , and the sterol regulatory element-binding factors (52, 60). Interestingly, the KIX domain is also the principal region of p300/CBP that is recognized by the HTLV-1 Tax protein (13, 15–17). Indeed, the Tax-KIX interaction is believed to be essential for the stable association of the coactivators with the viral promoter (13). Therefore, HBZ-mediated repression of viral transcription may, in part, result from direct competition between HBZ and Tax for an overlapping region of p300/CBP. In support of this hypothesis, we found that HBZ could effectively compete with Tax for binding to a region of CBP that included both the KIX and C/H1 domains. The latter domain is also bound by Tax and may lend stability to the Tax-coactivator interaction (16).

We also show that HBZ displaces C/H1-KIX from a Tax-CREB complex bound to the vCRE, an event dependent on its LXXLL-like motifs. This effect is distinct from that targeting

CREB. Indeed, although HBZ inhibits CREB DNA binding activity in solution, it is unable to displace CREB from the ternary complex (29). In parallel, it is interesting to note that the LXXLL-like motifs provide a more substantial repressive effect on transcription from chromosomally integrated viral promoters than the ZIP domain (see Fig. 4B). Such promoters adopt a more physiological nucleosome structures than transiently transfected plasmid promoters (61), which is important for the full regulatory effects of p300/CBP on HTLV-1 transcription (25, 26). Together, these observations suggest that HBZ targets p300/CBP as the principle means of repressing HTLV-1 transcription, whereas inhibition of CREB recruitment produces an ancillary repressive effect.

In addition to the KIX domain, we found that HBZ binds directly to the HAT domain of CBP. This domain is conserved between CBP and p300 and is required for the acetylation activity of the coactivators. p300/CBP have been shown to acetylate histone and nonhistone proteins, such as CREB-2, c-Myb, E2F, GATA1, MyoD, p53, and p73 (19, 20, 60). Acetylation of the core histones serves to open the chromatin structure, thereby providing access of the transcription machinery to the DNA (18). Histone acetylation additionally helps to provide a docking site for specific transcriptional regulators on nucleosomes. In contrast, acetylation of transcription factors can modulate a number of functional properties, depending on which factor is modified. For example, transcription factor acetylation can affect such functions as sequence-specific DNA binding activity, protein-protein interactions, and nucleocytoplasmic shuttling (18). Because the LXXLL-like motifs of HBZ are not required for binding to the HAT domain, the significance of this interaction is unclear. It is possible that the interaction between HBZ and the HAT domain occurs following the initial binding of the viral protein to the coactivator KIX domain. Indeed, we show that the LXXLL-like motifs are required for the interaction with p300/CBP *in vivo* (Fig. 2). Whether HBZ is targeted for acetylation by p300/CBP or acts to inhibit the HAT activity of these coactivators warrants further investigation.

In showing that HBZ binds to p300/CBP, we have identified an important regulator of HTLV-1 transcription that is targeted by both Tax and HBZ. Other cellular proteins that interact with both viral proteins include CREB, CREB-2, ATF-1, and CREM (28, 29). It is interesting to speculate that, by competing with Tax for the binding of nuclear transcriptional regulators, HBZ affects the cellular localization of Tax and, therefore, influences how Tax functions in the cell. Indeed, the nuclear localization of some transcription factors has been found to depend on protein-protein interactions. Although predominantly found in the nucleus, a portion of Tax is present in the cytoplasm, where it targets proteins, such as IKK- γ (1, 2), tumor suppressors containing PDZ domains (62, 63), and centrosomal proteins (64, 65). The effects of these interactions are believed to contribute to cellular transformation. Further investigations will be necessary to analyze additional potential effects of HBZ on Tax functions.

We propose that HBZ has evolved a bipartite mechanism to repress Tax-dependent viral transcription, which may be important for evasion of host immune surveillance by HTLV-1-infected T cells. Through its COOH-terminal ZIP

domain, HBZ mediates loss of CREB from the viral promoter. However, our previous results indicated that HBZ eliminates only a small portion of CREB from the promoter *in vivo* (29), which alone may not produce a sufficient reduction in viral transcription to escape immunodetection. Consequently, HBZ additionally targets p300 and CBP and prevents the recruitment of these coactivators to the viral promoter. By targeting both CREB and p300/CBP, HBZ may effectively inhibit Tax-dependent viral transcription to allow HTLV-1-infected cells to persist in the peripheral blood over a long period of time.

Acknowledgments—We thank Dr. Matsuoka for the ATL-2 cells, Dr. Thanos for the GST-HAT domain, Dr. Kadonaga for the Sf9 viruses, and Dr. Jeang for the CHOK1-Luc cells.

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