The bZIP Transcription Factor ATFx Binds Human T-Cell Leukemia Virus Type 1 (HTLV-1) Tax and Represses HTLV-1 Long Terminal Repeat-Mediated Transcription

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The human T-cell leukemia virus type 1 (HTLV-1) viral protein Tax is a transactivator of transcription driven by the cognate viral long terminal repeat (LTR). Tax exerts its effect through three nonidentical copies of the Tax-responsive element (TxRE), a member of the asymmetric cyclic AMP response element (CRE) family of enhancer sequences. Transactivation is mediated via interaction of Tax with members of the CREB/ATF family bound to TxRE. We have identified a cellular repressor of transcription, activating transcription factor x (ATFx), as a novel Tax-binding protein. In addition to binding directly to Tax we show by electrophoretic mobility shift assay that ATFx binds to the TxRE enhancer element via the bZIP domain. The functional impact of this bridging interaction results in repression of both basal and Tax-induced transcription from the HTLV-1 LTR. ATFx is unique among ATF family of proteins in that it is cell cycle regulated and exerts a tight repressive control over apoptotic signaling. We propose that recruitment of ATFx to the HTLV-1 LTR serves to link viral transcription with critical events in cellular homeostasis.

Human T-cell leukemia virus type-1 (HTLV-1) is the causative agent of adult T-cell leukemia, a malignancy of CD4⁺ T lymphocytes (39, 53) and HTLV-1-associated myelopathy/tropical spastic paraparesis (16, 31). Tax is a 40-kDa protein that activates the HTLV-1 transcription in *trans* through three imperfect copies of a 21-bp motif, termed the Tax response element (TxRE) (7, 10, 34, 40, 43). Tax does not directly bind the HTLV-1 long terminal repeat (LTR) and its interaction with the TxRE is facilitated by DNA-bound cellular transcription factors. Within each 21-bp repeat there is an asymmetric cyclic AMP (cAMP) response element (CRE) recognized by members of the CRE binding protein/activating transcription factor (CREB/ATF) family of proteins. Although several members of the ATF/CREB family bind to the viral CREs, CREB appears to play a crucial role in Tax mediated transactivation of HTLV-1 LTR (1, 3, 5, 11, 17, 50, 54, 55).

Although Tax has a discrete functional “activation” domain (42), the overall molecular mechanism of HTLV-1 transactivation by Tax has been established as involving a cooperative interaction of Tax with these DNA-bound factors. Studies have shown that Tax interacts with CRE bound CREB and stabilizes the Tax-CREB-CRE complex (5, 18, 32, 44, 49, 54), and in the absence of Tax the dissociation rate of CREB from CRE is increased (5). It is postulated that association of Tax with the bZip domain of CREB results in increased stability of the α -helical structure of the parallel bZip dimers, resulting in both enhanced DNA binding and dimerization of CREB (36). In addition, there is evidence suggesting that Tax, besides binding to CREB, makes additional contacts with the 5′-flanking G/C-rich sequences of the CRE (28).

The formation of this Tax-CREB complex serves as a high-affinity docking site for the binding of several multifunctional transcriptional coactivators including p300/CBP and PCAF (p300/CBP associated factor), which are known histone acetyltransferases (22, 25, 27, 30). In fact, both CBP/p300 and PCAF have been shown to interact with Tax in a histone acetyltransferase activator-enhancer complex and it has been proposed that p300/CBP and PCAF, upon their Tax-mediated recruitment to the viral enhancer, modify histones to transform the local HTLV-1 promoter/chromatin architecture allowing enhanced interactions of several transcription promoting cellular factors (22). Conversely, one study has shown that Tax physically and functionally interacts with histone deacetyltransferase 1 (HDAC-1) and HDAC-1 represses the transactivation function of Tax (9). Therefore, it can be stated that Tax-mediated up-regulation of the HTLV-1 LTR relies both on recruitment of basal transcription factors via an activation domain, as is typical of many transactivators, as well as the profile and nature of DNA-bound proteins with which it interacts.

In addition to activating the HTLV-1 LTR, Tax also regulates gene expression via interaction with cellular promoters. Tax activates a variety of cellular genes through interaction with CRE-binding proteins for example those encoding interleukin-17 (8) and *c-fos* (2) as well as activating genes encoding interleukin-2, interleukin-2 receptor, granulocyte-macrophage colony-stimulating factor, and vimentin via induction of NF- κ B (51). Additionally, there is a class of cellular genes that are repressed by Tax such as the repair enzyme DNA polymerase β (24).

The mechanistic details of Tax-mediated repression of cellular genes are not clearly understood, although there is evidence that Tax mediates its repressive effect through interaction with cellular factors that bind E-box elements present in target gene promoters. For example, it has been suggested that Tax causes repression of p18INK4c (an inhibitor of CDK4) by

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interfering with the binding of coactivators CBP/p300 with E-box bound protein E47 (45, 46). In addition, Tax causes functional repression of the transcription factor *c-myc*, an E-box activator, by masking its N-terminal domain resulting in interference of normal transcriptional activity of *c-myc* (41). Therefore, Tax plays diverse transcriptional roles independent of DNA binding and the ability of Tax to interact with cellular proteins is a central theme in Tax function (14, 21, 33).

As part of our effort to identify Tax-interacting cellular factors, we have identified ATFX, a member of the activating transcription factor family, as a novel Tax-binding protein. We show that ATFX binds to Tax in vitro in the absence of the HTLV-1 LTR. Furthermore, we show that ATFX suppresses the Tax-mediated activation of HTLV-1 LTR and that ATFX binds to the HTLV-1 LTR via its C-terminal bZip domain. ATFX is unique among the CREB/ATF family in that expression is strictly regulated with cell cycle and function is intimately linked with apoptosis. The interaction between Tax and ATFX as a formal link between viral transcription and cellular homeostasis may have important implications in HTLV-1 biology.

MATERIALS AND METHODS

Yeast two-hybrid assay. For identification of HTLV-1 Tax binding proteins, a modified yeast two-hybrid system described earlier was used (47). A human leukocyte matchmaker cDNA library (BD Biosciences CLONTECH, Palo Alto, CA) was screened, using HTLV-1 Tax as bait. The yeast two-hybrid assays involved a primary positive screen for identifying Tax interacting proteins. Non-specific Tax binding proteins were eliminated in a secondary negative screen by using different Tax mutants. Positively identified cDNA clones were assessed for functional Tax binding properties by their ability to bind different Tax mutants.

Generation of ATFX clones. The C-terminal ATFX fragment identified in the yeast two-hybrid screen was subcloned into a pCDNA4 vector (Invitrogen, Carlsbad, CA) to generate pCDNA-ATFX-ΔN. Full-length ATFX was amplified from corresponding expressed sequence tag, EST BF52888, using primers 5'-GCA ACC GGA TCC ACA GCC ATG TCA CTC CTG-3' (ATFXfwBamHI) and 5'-GCA GAT ATC CCT GCC CTT CTA GCA GCT-3' (ATFXrevEcoRV) (Integrated DNA Technologies, Coralville, IA). The C-terminally truncated ATF mutant, containing amino acids 1 to 206 (ATFXΔC), lacking the bZIP domain was generated from the same EST clone using forward primer (ATFXfwBamHI) and reverse primer 5'-GCA GAT ATC AGG TCA GGT GGC AGG ATG TGG GTA-3' (ATFXΔC revEcoRV) (Integrated DNA Technologies, Coralville, IA).

The N-terminally truncated ATF deletion mutant, containing amino acids 81 to 283 (ATFXΔN), was amplified from a clone pulled out by yeast two-hybrid screen using Tax as bait. We used the Gateway cloning system (Invitrogen, Carlsbad, CA) to generate mammalian and prokaryotic expression clones for ATFX. In the first step the amplicons for ATFX full-length and truncated versions were subcloned into PENTR3C. Eukaryotic glutathione *S*-transferase (GST) expression clones (GSTATFXf, GSTATFX ΔN, and GSTATFXΔC), were made via recombination between the entry clones and PDEST27 eukaryotic GST expression clone. Similarly GFP expression clones were made via recombination between ATFX entry clones and pCDNA-DEST53 eukaryotic GFP expression clone. To make prokaryotic GST expression clones, recombination was performed between PENTR3CATFX (full-length, ΔC and ΔN) clones and PDEST15 prokaryotic GST expression vector.

Northern blot analysis. Human multiple tissue Northern blots (BD Biosciences CLONTECH, Palo Alto, CA) were hybridized and washed according to the manufacturer's recommendation. The 800-bp full-length ATFX probe was generated by restriction digestion from the GST-ATFX construct. The probe was labeled with α -³²P dCTP (Perkin Elmer, Boston, MA) with a random-oligonucleotide priming kit (Invitrogen, Carlsbad, CA).

Western blot analysis. Cells were washed in PBS and lysed in M-PER protein lysis buffer (Pierce, Rockford, IL) containing the Complete Mini mixture of protease inhibitors (Roche Applied Science, Indianapolis, IN). The cell lysate was centrifuged, and the proteins were collected and quantified using the Bradford assay (Bio-Rad, Hercules, California). A total of 50 μ g of protein was separated on a 10% SDS-PAGE and transferred by semidry transfer method to

polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA). The membranes were then incubated in blocking solution for 1 h at room temperature then incubated overnight at 4°C in blocking (1X PBS, 0.1% Tween20, 5% nonfat milk) solution containing the appropriate primary antibody: anti-Tax (1:200 dilution) as described earlier (21), or anti-Xpress (1:2000) (Invitrogen, Carlsbad, CA), anti-GFP (green fluorescent protein) (1:1000) (Santa Cruz Biotech, Santa Cruz, CA), anti-GST (Sigma, St. Louis, MO) (1:3000). The membranes were washed once for 15 min and then four times for 5 min each in PBST (PBS plus 0.1% Tween 20). The blots were then incubated in blocking solution for 45 min at room temperature with the appropriate alkaline phosphatase-conjugated secondary antibody (Applied Biosystems, Foster City, CA) and washed in PBST as described above. The detection was performed using the Western Star Detection kit (Applied Biosystems, Foster City, CA).

Transfections and reporter assays. HeLa cells were seeded into six-well tissue culture plates to 50% confluence in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum, and penicillin-streptomycin. Transfections were done 24 h later by using the calcium phosphate coprecipitation method (20). For each transfection, 1.0 μ g of pU3RCAT was cotransfected with 1 to 3 μ g of Tax expressing plasmid and/or 1 to 3 μ g of ATFX expressing plasmid. The transfection medium was removed 16 h later and replaced with fresh medium. The cells were harvested 24 h later by scraping. Total cell extracts were made by freeze-thaw method, and the supernatants were used for chloramphenicol acetyltransferase (CAT) assays (19). For luciferase assays, a plasmid expressing Tax (1 μ g) and luciferase reporter plasmid HTLV-1-LTR-luciferase (1 μ g) were cotransfected in 293T cells with or without ATFX (2 μ g) expressing plasmid. Where ever indicated CREB and CREB2/ATF4 expressing plasmids were included in the transfection reactions. Total amount of DNA per transfection was normalized by using a blank vector DNA. Whole-cell protein extracts were made 36 h posttransfection and were normalized to amount of protein per assay. luciferase activity was detected as recommended by the manufacturer (Promega, Madison, WI) using a luminometer (Turner Design, Sunnyvale, CA).

Production of GST-tagged ATFX proteins and electrophoretic mobility shift assay. Plasmids expressing GST-tagged proteins were transformed into *E. coli*, strain BL-21. Cultures were grown overnight and diluted 1:10 with LB medium containing 100 μ g/ml ampicillin then allowed to grow at 25°C till it reached an OD of 0.5. L-Arabinose was added to a final concentration of 0.2 mM and the culture was allowed to grow for another 3 h. The bacterial cultures were harvested in cold PBS and lysed by sonication in PBS containing 1 mM dithiothreitol and protease inhibitor cocktail (Roche, Indianapolis, IN). The sonicated lysates were then centrifuged at 15000g for 30 min at 4°C.

The resultant supernatant was incubated with presoaked glutathione-sepharose beads (Amersham, Piscataway, NJ) on a rotator for 2 h. Nonspecific proteins were removed by washing the beads with cold PBS three times. GST-tagged ATFX proteins were eluted off the beads using an elution buffer containing 10 mM Glutathione and were stored at -70°C. Electrophoretic mobility shift assays were performed as described earlier (6). Bacterially expressed purified ATFX (full-length and deletion mutants) proteins were incubated with ³²P-labeled dsDNA probe, encompassing two proximal TxREs, in binding buffer for 30 min at room temperature. The protein-DNA complexes were separated in a non-denaturing 4% polyacrylamide gel. The gel was dried and exposed to X-ray film.

Immunoprecipitation. To analyze potential physical interaction between Tax and ATFX, 2.5 $\times$ 10⁶ cells were lysed in 1 ml of M-PER lysis buffer (Pierce, Rockford, IL) containing the Complete Mini mixture of protease inhibitors (Roche Applied Science, Indianapolis, IN) for 30 min at 4°C. Immunoprecipitation was carried out by incubating whole-cell lysate with the appropriate antibody overnight at 4°C on a rotator. Added to this mixture was 100 μ l of 30% slurry of protein A-Sepharose beads in lysis buffer and incubated for 3 h at 4°C. The immune complexes bound to beads were pelleted by centrifugation at 12,000 rpm for 5 min at 4°C. The beads were washed four times with lysis buffer. The bound proteins were eluted by heating in SDS gel sample buffer at 95°C for 5 min, placed on ice for 1 min, and further centrifuged at 12,000 rpm for 2 min. A portion, 30 μ l, of supernatant was loaded, separated by a 10% SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). All subsequent steps are the same as described for Western blotting.

RESULTS

Identification of ATFX as a Tax binding protein. In our ongoing efforts to map the Tax interactome we identified the bZIP transcriptional repressor ATFX as a Tax-binding protein. We employed a noncommercial yeast two-hybrid approach

FIG. 1. Comparison of structure of the CREB, CREB2/ATF-4 and ATFx proteins, and ATFx expression constructs. (A) Alignment and domain organization of CREB, CREB2/ATF4, and ATFx proteins. The protein domains were predicted by scanning the protein sequences using the Simple Modular Architecture Research Tool (SMART) program, available at <http://us.expasy.org/tools/>. The N-terminal region of ATFx contains a negatively charged and proline-rich domain. (B) Shown are the different ATFx proteins used in these studies; full-length ATFx, N-terminal deletion of ATFx with intact b-zip domain (ATFx-ΔN), and C-terminal deletion of ATFx with the b-zip domain deleted (ATFx-ΔC).

which has been modified to reduce background or false positive signal via deletion of sequences around the promoter (47). The initial BLAST search of the sequenced clone revealed a fragment of ATFx deleted at the N terminus. The complete ATFx is a 30.7 kDa protein belonging to the activating transcription factor/cAMP response element binding protein family. The protein was originally identified as a repressor of transcription for cAMP induced transcription (35).

We conducted a consensus domain analysis using the Simple Modular Architecture Research Tool (SMART). This analysis predicted that ATFx contains a putative C terminal bZip domain (amino acid 206 to 270) and a bipartite nuclear localization signal (amino acids 213 to 230) as shown in Fig. 1A. The N terminal region is predominated by sequence with little homology to known structural motifs although we did note a large proline rich region (amino acids 78 to 209). Additionally, murine ATFx has been predicted to have a negatively charged proline rich region which is suggestive of acidic activation domains found in bZip transcription factors (38). Comparison of amino acid sequences of human ATFx and murine ATFx, revealed approximately 87% similarity. Based on sequence similarities, human ATFx is also expected to have a similar N-terminal activation domain.

Tax and ATFx coimmunoprecipitate in mammalian cells.

To confirm interaction between Tax and ATFx we performed coimmunoprecipitation studies between recombinant Tax and ATFx. Plasmids expressing GFP-Tax and GST-ATFx-ΔN were cotransfected in 293T cells and whole-cell lysates were prepared. The cell lysates were subjected to coimmunoprecipitation with anti-GST or anti-Xpress tag antibody and were subjected to SDS-PAGE. The separated proteins were then analyzed by western immunoblotting using anti-GFP to detect GFP-Tax and GFP. As shown in Fig. 2, presence of Tax is clearly detected when whole-cell lysate containing GFP-Tax and GST-ATFx-ΔN proteins was subjected to immunoprecipitation using anti-GST antibody (Fig. 2, lane 1). As a negative control, the lysates containing GFP-Tax and GST-ATFx-ΔN were subjected to coimmunoprecipitation with control antibody (anti-Xpress). Only marginal amounts of GFP-Tax-containing complexes were detected with the control antibody. The GST-ATFx-ΔN construct used here does not have an Xpress tag and it is different from the ATFx-ΔN construct used in the experiment described for Fig. 4.

As a second negative control, the same whole-cell lysates were prepared from 293T cells following cotransfection with plasmids expressing GFP and GST-ATFx-ΔN. Immunopre-

FIG. 2. Coimmunoprecipitation of Tax and ATFx. Whole-cell lysates were prepared from cells transfected with plasmids expressing GFP-Tax and GST-ATFx-ΔN (lanes 1 to 3), GFP and GSTATFx-ΔN (lanes 4 and 5) or mock transfected (lane 6). The isolated cell lysates were either subjected to coimmunoprecipitation with either anti-GST (lanes 1 and 4) or anti-Xpress tag antibody (lane 2) or used without immunoprecipitation (lanes 3 and 4). The resulting precipitates were subjected to SDS-PAGE Western blot analysis using anti-GFP antibody for detection. For each lane, proteins present in whole-cell lysates and antibodies used for immunoprecipitation are labeled. The bands corresponding to GFP-Tax and GFP are shown by arrows.

cipitation with anti-GST antibody demonstrated that ATFx did not interact with GFP alone (Fig. 2, lane 4). These results indicate that Tax and ATFx specifically interact in mammalian cells. In addition, the truncation of the N-terminal 80 amino acids of GST-ATFx-ΔN did not appear to affect the binding to GFP-Tax, consistent with yeast two-hybrid screen which isolated a c-terminal clone. Together, these data indicate that the binding domain of ATFx which interacts with Tax is located in the C-terminal region.

ATFx is expressed in a variety of normal human tissues. We have examined the tissue specific distribution of ATFx to determine its expression levels in lymphatic tissues. Human multiple tissue blots (BD, CLONTECH) were hybridized with ³²P labeled full-length ATFx cDNA (Fig. 3). A message of approximately 2.2 Kb was detected for peripheral blood lymphocyte (PBL) as well as other tissues. The levels of ATFx expression were most abundant in testis, prostate and liver, and least abundant in brain, lung and kidney. A second species of message which is consistent with an alternative splice variant of approximately 1 kb was detected in skeletal muscle. The presented data demonstrates that ATFx is expressed in lymphatic tissues that are targets of HTLV-1 infection.

Expression of ATFx inhibits HTLV-1 LTR driven Tax expression. To explore the functional significance of ATFx expression we examined the effect of expression of ATFx on Tax-mediated transactivation of the viral LTR in 293T. Specifically, we examined the effect of ATFx on expression of Tax from a plasmid which expresses Tax via the HTLV-1 LTR (HPX). HPX was cotransfected with pCDNA4-ATFx-ΔN,

FIG. 3. Expression of ATFx in normal human tissue. Human multiple tissue RNA blots were hybridized with ³²P-labeled cDNA corresponding to the entire coding sequence of ATFx. The ≈2.2-kb message is shown by an arrow. It appears to be abundant in testis, prostate and liver. An alternative splice variant of ≈1kb is present in skeletal muscle.

which expresses Xpress-tagged ATFx-ΔN and whole-cell lysates were subjected to SDS-PAGE and Western blot analysis using an anti-Tax mouse monoclonal antibody.

As shown in Fig. 4A, Tax expression is not detected when HPX is cotransfected with pCDNA4-ATFx-ΔN expression plasmid (compare lanes 1 and 2) suggesting that ATFx suppresses the HTLV-1 LTR driven Tax expression in 293T cells. To confirm that these cells were transfected, the same cell lysate was assessed for expression of ATFx using anti-Xpress tag antibody (Fig. 4B). This experiment confirmed that these cells were transfected and capable of expressing introduced proteins. Furthermore, expression of a nonspecific protein, Chk2, was also evaluated for effect on HTLV-1 LTR driven expression of Tax by using similar to-transfection assays. Expression of Chk2 did not affect HTLV-1 LTR-mediated Tax expression (Fig. 4A, lane 3). These results suggest that ATFx negatively regulates the HTLV-1 LTR.

FIG. 4. Western analysis of Tax and ATFx-coexpressing cells. (A) Whole-cell lysates were prepared from 293T cells transiently transfected with plasmids expressing Tax (lane 1), Tax and ATFx-ΔN (lane 2), Tax and Chk2 (lane 3), and mock transfected (lane 4). Lysates were subjected to immunoblotting as described in the methods section and probed with anti-Tax antibody. (B) Cell lysate examined in lane 2 was also probed with anti-Xpress tag antibody to detect ATFx-ΔN expression (lane 5), and lane 6 is a mock-transfected negative control. For each lane, proteins present in whole-cell lysates are labeled. Bands corresponding to Tax and ATFx are shown by arrows.

FIG. 5. Effect of ATFx expression on the activation of the HTLV-1 LTR. (A) ATFx represses transcription from the HTLV-1-LTR. Activity was assessed by CAT reporter assays as described. Shown are the acetylated % conversions. HTLV-1-LTR-CAT (lanes 1 to 4) and CMV-CAT (lanes 5 and 6) were used as reporter plasmids. Plasmids expressing ATFx (lanes 1, 3, and 5) and HTLV-1 Tax (3 and 4) were used as shown. (B) Expression of CREB or CREB2 reverses ATFx repression of Tax-mediated activation of the HTLV-1-LTR. The effect of CREB and CREB2/ATF4 expression on ATFx-mediated repression of HTLV-1 LTR activation was assessed by luciferase assays. Increasing amounts of the respective plasmids, CREB and CREB2/ATF4, are indicated. Shown is fold activation of the resulting luciferase activity over the basal activity of HTLV-1-LTR-luciferase construct alone. The results shown are representative of three experimental replicates.

Effect of ATFx on activation of HTLV-1 LTR. To assess the mechanism of ATFx suppression of the HTLV-1 LTR, promoter reporter assays were performed. The HTLV-1 LTR (1 μ g) was used as the CAT reporter plasmid and was cotransfected with plasmid expressing ATFx (3 μ g) in the presence or absence of a Tax expressing plasmid (3 μ g). Total DNA levels were maintained at 7 μ g by the addition of a blank pCDNA vector (3 μ g) (Fig. 5, lane 1 to 4). Expression of ATFx alone repressed HTLV-1 LTR (Fig. 5, lanes 1 and 2) and it also repressed Tax-mediated activation of HTLV-1 LTR (Fig. 5, lanes 3 and 4). The effect of ATFx expression on a heterologous promoter, the CMV immediate early promoter was also examined. The results show that expression of ATFx did not seem to affect the activity of CMV promoter (Fig. 5, lanes 5 and 6), demonstrating that this effect was specific for the HTLV-1 LTR.

Since at least two other members of the CREB/ATF family, CREB and CREB2/ATF4, have been shown to positively influence Tax-mediated transactivation we examined the relationship between ATFx and these two family member proteins. The HTLV-1 LTR-luciferase (1 μ g) was used as the reporter plasmid and was cotransfected with Tax expressing plasmid (1 μ g) in presence and absence of ATFx expressing plasmid. As indicated, CREB (1 or 3 μ g) and CREB2/ATF4 (1 or 3 μ g) were included in the transfection reactions (Fig. 5 B). In this experiment ATFx again demonstrated a significant repression

of Tax-mediated transactivation of the HTLV-1-LTR. However, when CREB was coexpressed with ATFx the observed ATFx repression was reversed (Fig. 5B). Increasing the amount of expressed CREB resulted in enhanced Tax transactivation of the HTLV-1-LTR suggesting that the ATFx effect is competitive with the CREB effect. Similar results were obtained, in a dose-dependent manner, when CREB2/ATF4 was coexpressed with ATFx. Thus, activity of the HTLV-1-LTR may be regulated by the interaction of “positive” and “negative” transcriptional regulators of the CREB/ATF family.

ATFx interacts with HTLV-1 LTR. One mechanism by which ATFx may function to repress the HTLV-1 LTR is by direct binding to the TxRE elements. To assess this, we performed electrophoretic mobility shift assays. A 71-bp DNA sequence from HTLV-1 LTR region encompassing the two TATA-proximal TxRE elements was 32 P labeled and used as probe. GST-tagged full-length ATFx, the N-terminal truncation that retains the bZip domain (GST-ATFx- Δ N) and a mutant with the bZip domain missing (GST-ATFx- Δ C) were synthesized in bacteria and purified as described in Materials and Methods. The radiolabeled HTLV-1 LTR and ATFx proteins were incubated at room temperature for approximately 30 min and the protein/DNA complexes were separated on a native polyacrylamide gel followed by autoradiography. As evidenced from the shift of labeled probe DNA, the results clearly show that full-length ATFx and the N-terminal truncated ATFx deletion mutant

FIG. 6. Electrophoretic mobility shift assay to assess interaction between ATFx and the HTLV-1 LTR containing the TRE. Interaction of ATFx with the HTLV-1 LTR was examined by electrophoretic mobility shift assay. The probe used was a 71-bp region spanning TRE's A+B corresponding to the HTLV-1 LTR. Shown is an autoradiograph of EMSA with full-length ATFx (lane 1), b-zip domain deleted ATFx- Δ C (lane 2), and N-terminally deleted ATFx- Δ N (lane 3). Bands corresponding to the probe and ATFx complex are indicated.

(that retains the bZip domain) form a complex with the HTLV-1 LTR TxRE elements (Fig. 6, lanes 1 and 3). The presence of the bZip domain was crucial for ATFx and HTLV-1 LTR TxRE interaction as the C-terminal deleted mutant of ATFx failed to form a complex with the LTR as shown by the absence of the gel shift.

DISCUSSION

One of the key controlling events in HTLV-1 biology is transcription from the viral LTR-mediated by the HTLV-1 Tax protein. The trans-acting function of Tax, in addition to another HTLV-1 protein Rex, is essential for efficient replication of HTLV-1 (5, 52). Tax by itself does not bind to the viral LTR and does not possess an intrinsic enzymatic activity and it is thought that the viral transactivator functions via interaction with cellular transcription factors. It is also clear that in addition to the recruitment of basal transcription factors, Tax exerts influence upon viral transcription through interaction with cellular factors that are themselves either positive or negative regulators of transcription. Therefore, the ability of Tax to interact with cellular factors is a critical component of transcriptional regulation and thus HTLV-1 biology.

Transactivation by Tax via interaction with positive transcription factors is a complex process mediated via factor recruitment at one or more of the three 21-bp Tax response elements (TxREs) present in the U3 region of the HTLV-1 LTR (4, 12). The TxREs are composed of a central imperfect cyclic AMP response element (CRE). Similar to other CREs, TxRE binding transcription factors are globally members of the CREB/ATF family of proteins with a conserved C-terminal bZip domain which has been shown to be essential for interaction with Tax, and binding to TxRE. A significant number of studies have suggested that, in general, Tax-mediated transactivation of the LTR involves three mechanistic steps; Tax promotes homodimerization and recruitment of CREB/ATF proteins; Tax mediates stabilization of the LTR bound complexes; TxRE associated Tax recruits transcriptional coactivators like CBP/p300 (15, 22, 26-28, 48).

Negative regulation of the viral LTR by cellular proteins is likely an important feature of the integrated virus that facilitates repression of virus replication and is believed to be necessary to escape the cytotoxic-T-lymphocyte response, allowing the virus to survive inside the host (23, 29). The b-ZIP protein CCAAT/enhancer binding protein β (C/EBP β), forms a heterodimeric complex with CREB2, which binds to the CRE site in TxREs and competes with the ability of Tax to recruit active complexes to the viral promoter (23). Further, the inducible cAMP early repressor (ICER), another member of CREB/ATF/CREM family, has been shown to repress Tax-mediated activation of the HTLV-1 LTR (29). Interestingly, the affinity for binding of ICER to the TxRE is increased in presence of Tax. Thus, Tax-mediated regulation of viral transcription is governed by multiple factors. Specifically, in addition to possessing an activation domain and recruiting basal transcription machinery, Tax-mediated transcription is influenced by interaction with transcriptional activating factors, and with transcriptional repressing factors.

We have identified ATFx, a member of the CREB/ATF factor family, as a novel repressor of HTLV-1 LTR transcription. ATFx is identical to human ATF5 and has 87% amino acid sequence similarity to mouse ATF5. Mouse ATF5 has also been named as ATF-7 (38). We observed specific repression of the HTLV-1 LTR that was not seen in other heterologous promoter and the repression was apparent in the presence of Tax. Therefore, the Tax-activated promoter was repressed by exogenously added ATFx. This suggested that the repression of ATFx is competitive with Tax-activation since Tax is able to reverse the repression of ATFx. The binding of ATFx to the viral LTR is mediated by the bZIP domain within ATFx and this same region is required for interaction with Tax. Thus, the interaction of ATFx with Tax would prevent ATFx repression of the HTLV-1-LTR.

The observation that the same domain within ATFx mediates a functionally competitive bridge is suggestive of several mechanistic models. One intriguing model is that the presence of ATFx on the viral promoter is involved in the recruitment of Tax to the promoter and that reversal of ATFx repression contributes to overall Tax activation. Thus, ATFx would be part of the molecular topology of the HTLV-1 promoter to which Tax binds. In light of the competitive relationship we observed between ATFx repression and CREB/CREB2 activation, it is reasonable to conclude that these positive and

negative regulatory mechanisms interact to achieve promoter function. This interaction could also extend to the cellular promoter targets of ATFx with which Tax may exert activation.

Unlike other members of the ATF family, ATFx is not known to affect cell cycle or proliferation and the known downstream targets for ATFx seem to be restricted to those genes that are involved in the regulation of apoptosis (37). Specifically, ATFx is an anti-apoptotic factor and the expression of ATFx is down-regulated in a variety of cell lines undergoing apoptosis following growth factor deprivation. The action of ATFx is believed to be conditionally related to apoptosis in that ATFx levels must be reduced to allow apoptosis to proceed. It has been established that the transcriptional activity of ATFx is required for cell survival, since a dominant negative mutant of ATFx (retaining the bZIP DNA-binding domain) suppresses the anti-apoptotic effects of ATFx.

Unique among the ATF family members, ATFx expression is cell cycle regulated and peaks in G₁/S and is undetectable in G₂/M. It is noteworthy that repression of ATFx is required in order for apoptotic signals to be effective and that presumably the requirement for ATFx changes with cell cycle. Specifically, ATFx is expressed highest during G₁/S, the most critical cell cycle period for initiation of apoptosis (37). Conversely, in G₂/M the levels of ATFx are very low perhaps indicating the cells are less susceptible to apoptosis. The consequence of ATFx repression of viral transcription to HTLV-1 infection is that transcription activity would increase during G₂/M and become suppressed at G₁/S.

It is interesting to speculate that HTLV-I may have evolved this mechanism of "monitoring" the levels of ATFx in order to regulate viral transcription around apoptotic signaling. We further note that we and others have shown that de novo Tax expression leads to G₂/M accumulation during which ATFx and suppression of the viral LTR would be lowest. One of the potential outcomes of Tax-induced G₂/M accumulation would be to insure viral transcription during a cell cycle period when the cell is least susceptible to apoptosis. Thus, the interplay between Tax and ATFx could provide a mechanism by which viral transcription is tied to cell homeostasis. Understanding the relationship between ATFx and Tax may help decipher the underlying mechanism that allows HTLV-1 infection to survive "dormancy" for so many years.

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