

## Transrepression of *lck* Gene Expression by Human T-Cell Leukemia Virus Type 1-Encoded p40<sup>tax</sup>

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**To understand the mechanism of p56<sup>lck</sup> protein downregulation observed in human T cells infected by human T-cell leukemia virus type 1 (HTLV-1), we have investigated the ability of the 3' end of the HTLV-1 genome as well as that of the *tax* and *rex* genes to modulate p56<sup>lck</sup> protein expression and p56<sup>lck</sup> mRNA synthesis. By using Jurkat T cells stably transfected with constructs that expressed either the 3' end of the HTLV-1 genome (JK C11-pMTEX), the *tax* gene (JK52-Tax) or the *rex* gene (JK9-Rex), we found that the expression of p40<sup>tax</sup> (Tax) was sufficient to modulate p56<sup>lck</sup> protein expression. Similarly, we found that the expression of the mRNA which encoded p56<sup>lck</sup> was repressed in Jurkat T cells which expressed Tax. This downregulation was shown to be proportional to the amount of *tax* mRNA found in the transfected cells, as evidenced by experiments that used cells (JPX-9) stably transfected with a *tax* gene driven by a cadmium-inducible promoter. Furthermore, cadmium induction of Tax in JPX-9 cells transiently transfected with a construct containing the chloramphenicol acetyltransferase (CAT) gene under control of the *lck* distal promoter (*lck* DP-CAT) resulted in the downregulation of CAT gene expression. In contrast, cadmium induction of Tax in JPX-9 cells transiently transfected with a CAT construct driven by a *lck* DP with a deletion extending from position -259 to -253 (a sequence corresponding to a putative E-Box) did not modulate CAT gene expression, suggesting that the effect of Tax on p56<sup>lck</sup> is mediated through an E-Box binding protein.**

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus associated with adult T-cell leukemia, an aggressive malignancy of mature T-helper lymphocytes in humans (42). In recent years, several research groups have reported evidence indicating that HTLV-1 induces the abnormal expression of several intracellular protein tyrosine kinases of the *src* family in infected cells. For example, the *lyn* gene, which is expressed in B lymphocytes but is only expressed at a very low level in normal T lymphocytes, is overtranscribed in T lymphocytes expressing HTLV-1 genes (51), whereas the *lck* gene, which is expressed at high levels in normal T lymphocytes, is not expressed in HTLV-1-infected T cells (29). Since protein tyrosine kinases participate in transmission or modulation of signals initiated at the cell surface, their dysfunction could contribute to leukemogenesis.

Because p56<sup>lck</sup> (the *lck* gene product) plays a major role in the regulation of T-cell activation (25, 45, 54), dysfunction of *lck* gene expression induced by HTLV-1 has become a focus of renewed interest. As already mentioned, several works (29, 31, 34) have indicated that the transcription of *lck* is blocked in the interleukin-2 (IL-2)-independent, HTLV-1-transformed T-cell lines (such as MT2 and MT4) but not in the IL-2-dependent ones or any others (such as CEM and HSB2). These observations have led to the hypothesis that either a transcriptional repressor which blocks the transcription of *lck* must be aberrantly induced in HTLV-1-infected cells or a factor which stimulates transcription of *lck* must be inhibited in HTLV-1-infected cells (29). Yet, the precise mechanism by which HTLV-1 modulates *lck* gene expression remains to be defined.

A possible candidate for the modulation of *lck* gene expression is the *tax* gene product. Indeed, HTLV-1 replication is strongly dependent upon expression of the virally encoded p40<sup>tax</sup> protein, a potent transcription activator (10, 14, 20, 47, 49). p40<sup>tax</sup> is highly pleiotropic, as it has been shown to transcriptionally activate a wide variety of cellular genes (reviewed in references 17 and 56). p40<sup>tax</sup> does not bind DNA directly (3, 41) but appears to stimulate transcription by acting on several structurally unrelated cellular transcriptional activator proteins. These include members of the NF- $\kappa$ B protein family, the activating transcription factor/cyclic AMP (cAMP)-responsive element-binding protein (ATF/CREB) family, and the serum response factor (5, 9, 16, 19, 44, 58). From an alternative perspective, p40<sup>tax</sup> may alter host cell metabolism through the repression of selected cellular genes. For example, the  $\beta$ -polymerase gene has been shown to be repressed by p40<sup>tax</sup> (26). The  $\beta$ -polymerase gene encodes a 39-kDa enzyme involved in DNA repair. p40<sup>tax</sup> inhibition of  $\beta$ -polymerase gene expression would likely result in the inability of the cell to properly repair damaged host cell DNA, possibly contributing to leukemogenesis. The cellular regulatory protein that mediates p40<sup>tax</sup> repression of  $\beta$ -polymerase expression has recently been identified as a member of the family of basic helix-loop-helix (bHLH) proteins (52). bHLH proteins bind to the E-Box consensus (CANNTG) DNA recognition sequence (36).

Another possible candidate for the modulation of *lck* gene expression is the *rex* gene product. Indeed, HTLV-1 uses an elaborate posttranscriptional regulatory system to control expression of viral structural and enzymatic proteins. The expression of *gag/pol* and *env* mRNAs, which are defective in stability, transport to cytoplasm, and polysomal loading, depends on the p27<sup>rex</sup> protein which acts by interacting with the Rex responsive element located on these viral mRNAs.

Here we demonstrate that the biological behavior of p56<sup>lck</sup> is

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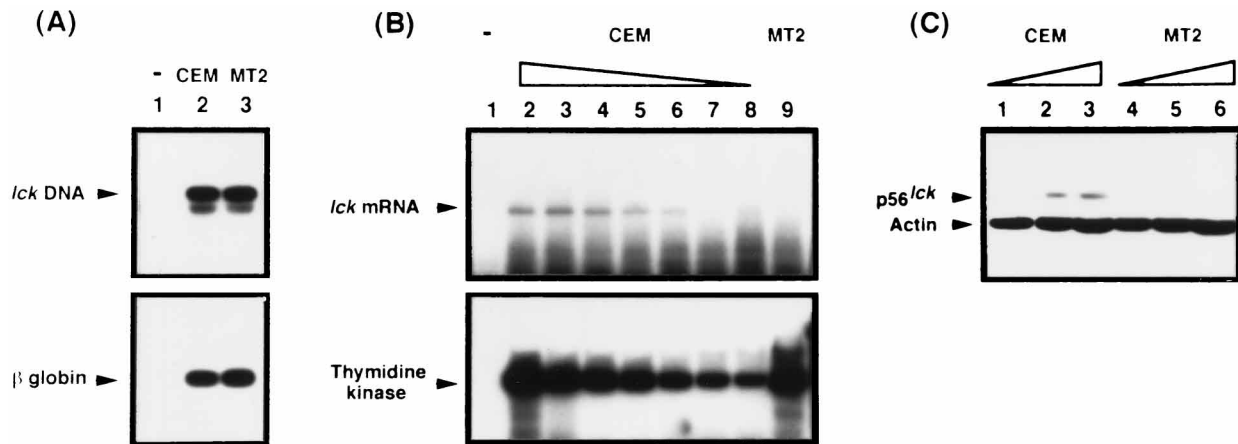


FIG. 1. Comparative analysis of *lck* gene, *lck* mRNA and p56<sup>lck</sup> protein in CEM and MT2 cells. (A) (Upper) High-molecular-weight DNA was extracted from CEM and MT2 cells, and *lck* gene was monitored by PCR analysis using the p56-III/p56-IV oligonucleotide primer pair. PCRs were performed on total DNA extracts (1  $\mu$ g) prepared from CEM (lane 2) and MT2 (lane 3) cells. A control is shown (lane 1) in which a DNA-free sample was prepared for PCR and treated like the extracted samples. The amplified products were electrophoresed, blotted, and hybridized with an  $\alpha$ -<sup>32</sup>P-labelled *lck* probe. Labelled DNA products were visualized by autoradiography. (Lower) The product of PCR amplification using the  $\beta$  globin I/ $\beta$  globin II oligonucleotide primer pair, hybridized with an  $\alpha$ -<sup>32</sup>P-labelled  $\beta$ -globin probe, is shown as a control. (B) (Upper) Semiquantitative RT-PCR analysis of *lck* mRNA in CEM and MT2 cells. PCR analysis of AMV-retrotranscribed *lck* mRNA (2  $\mu$ g) in CEM cells (lanes 2 to 8 corresponding to 1:1, 1:2, 1:10, 1:20, 1:50, 1:100, and 1:200 dilutions of the sample, respectively) and MT2 cells (lane 9) was performed with the p56-I/p56-II oligonucleotide primer pair (p56-II was labelled with  $\gamma$ -<sup>32</sup>P), and visualized by autoradiography. A control is shown (lane 1) in which an RNA-free sample was prepared for PCR and treated like the extracted samples. (Lower) An autoradiogram of PCR amplification of retrotranscribed TK RNA is shown as a control. (C) Western blot analysis of p56<sup>lck</sup> in CEM and MT2 cells. Lysates from CEM (lanes 1 to 3) and MT2 (lanes 4 to 6) containing various amount of proteins (15  $\mu$ g, 30  $\mu$ g, and 60  $\mu$ g of total cellular protein extract, respectively) were electrophoresed onto an SDS-PAGE gel and blotted to a PVDF membrane. The membrane was treated as described in Materials and Methods, incubated with a mixture of anti-p56<sup>lck</sup> and anti-actin MAb, and then reacted with goat-anti-mouse immunoglobulin-peroxidase conjugate. Bound MAbs were detected by incubating the membrane with ECL reagent and exposure of membrane to hyperfilms-ECL.

modulated by the expression of the viral regulatory *tax* gene in HTLV-1-infected T cells through a mechanism of repression that involves the E-Box DNA recognition sequence encountered in the *lck* gene distal promoter (DP).

#### MATERIALS AND METHODS

**Plasmids.** LTR<sub>HTLV-1</sub>-CAT plasmid containing the chloramphenicol acetyltransferase (CAT) gene under the HTLV-1 long terminal repeat (LTR) promoter was provided by J. L. Virelizier (Institut Pasteur, Paris, France). Rex expression vector CMV-Rex was provided by W. Greene (Gladstone Institute of Virology, San Francisco, Calif.). Tax expression vector MT-Tax and pMTEX (also named pMTpX) plasmid containing the 3' end of HTLV-1 were previously described (22, 32). pGEX2T-*lck* containing the cDNA coding for the human p56<sup>lck</sup> was provided by S. Fischer (ICGM, Paris, France). A sequence of 1,434 bp extending from a *Stu*I site to a *Nco*I site was excised from pGEX2T-*lck* to be used as a probe for *lck* DNA and mRNA detection.

**Oligonucleotides.** The cellular oligonucleotide primers used in this study are as follows: TK I (5'-GAGTA CTCGG GTTCG TGAAC-3', nucleotides 24 to 43, sense mRNA), TK II (5'-GGTCA TGTGT GCAGA AGCTG-3', nucleotides 246 to 265, antisense mRNA),  $\beta$  globin I (5'-ACACA ACTGT GTTCA CTAG C-3', nucleotides 14 to 33, sense),  $\beta$  globin II (5'-CAACT TCATC CACGT TCACC-3', nucleotides 104 to 123, antisense), p56-I (5'-CCGAG CTCGT ACTGC CCTCT GTGGC CG-3', nucleotides 1591 to 1609, antisense mRNA), p56-II (5'-CCGAG CCGAG TACAC AGCCA GGGAG G-3', nucleotides 1271 to 1290, sense mRNA), p56-III (5'-GCACC GGTTC GGAGC TGG-3', nucleotides 7 to 24, sense), p56-IV (5'-CTTAC CGTGC CCTTG CC-3', nucleotides 283 to 299, antisense), *lck* DP-1 (5'-GAATT CGAAC TGTTG CC-3', nucleotides -730 to -714, sense), *lck* DP-2 (5'-GAAGA TCTGA GCTCC CTTCG-3', nucleotides 23 to 42, antisense), E-Box 1 (5'-TGAAT CTCTT GCCC CTGGA GGGCA G-3', nucleotides -272 to -239, sense), and E-Box 2 (5'-CTGCC CTCCA GGGGG CAAGA GATTC A-3', nucleotides -272 to -239, antisense). The HTLV-1 oligonucleotide primers are as follows: Tax 1 (5'-CCAAG ACCCG TCGGA GGCC-3', nucleotides 5128 to 5146, sense), Tax 2 (5'-CA GGC TGTTA GCGTG ACGG-3', nucleotides 7968 to 7986, antisense), Tax 3 (5'-TCCCA GGTGA TCTGA TGCTC-3', nucleotides 7448 to 7467, antisense), Tax 4 (5'-TGGTC TTAAT AGCCG CCAG-3', nucleotides 4998 to 5016, sense), TRL (5'-GTTGT ATGAG TGATT GGCGG GGTA-3', nucleotides 7566 to 7590, antisense), and TRU 2 (5'-TGTTT GGAGA CTGTG TACAA GGCG-3', nucleotides 7353 to 7376, sense). The oligonucleotide primer pair Tax 2/TRU 2 will detect retrotranscribed *tax* mRNA; the oligonucleotide primer pair Tax 3/Tax 4 will detect both *tax* and *rex* retrotranscribed mRNAs. The CAT oligonucleotide primers are CAT 1 (5'-CGCTC AGGAG CTAAG GAAGC-3', nucleotides 3111 to 3130, sense) and CAT 2 (5'-GCCAT TCATC GCCTT ATTAT

C-3', nucleotides 3852 to 3872, antisense). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium).

**Cells.** The CD4<sup>+</sup> lymphoblastoid CEM cell line was obtained from the American Type Culture Collection (Rockville, Md.). The HTLV-1-transformed MT2 and C8166-45 cell lines were previously described (46, 57). Several clones of Jurkat T cells stably producing Tax and/or Rex proteins have been used in this study. These clones were derived after transfection of 10<sup>7</sup> Jurkat cells with 20  $\mu$ g of linearized Tax and/or Rex expression vector and 2  $\mu$ g of linearized SV40-Neo plasmid by electroporation at 280 V and 1,500  $\mu$ Fd with a Celljet electroporator (Eurogentec). Cells cultured in complete medium for 24 h were then seeded in individual microtiter wells (96-well plate) at 10<sup>4</sup> cells per well in complete medium containing 1 mg of G418 (Geneticin, Gibco-BRL)/ml and incubated at 37°C. Three weeks later, growing cells harvested from several wells were cloned by limiting dilution. Only a very few clones isolated after growth in G418-containing medium were found to harbor the HTLV-1-related DNA, as revealed by PCR analysis. Thus, for example, the expression vector DNA was found to be present in 7 and 3 out of 100 clones obtained after transfection with pMTEX or MT-Tax, respectively. The following clones were selected: JK C9, a clone of Jurkat cells stably transfected by an SV40-Neo expression vector; JK C11-pMTEX, stably cotransfected by the pMTEX expression vector and an SV40-Neo expression vector and expressing Tax and Rex; JK4, a clone of Jurkat cells stably transfected by CMV-Rex and SV40-Neo expression vectors (this clone was resistant to neomycin but does not express Rex); JK9-Rex, stably transfected by a CMV-Rex expression vector and an SV40-Neo expression vector and expressing Rex; and JK42-Tax, JK50-Tax, and JK52-Tax, stably cotransfected by a MT-Tax expression vector and an SV40-Neo expression vector and expressing Tax. JPX-9 is a clone of Jurkat cells stably transfected by a Tax expression vector in which Tax expression is dependent on heavy metal ions (37); this clone was kindly provided by M. Nakamura (Tohoku University, Sendai, Japan). Cells were cultured in RPMI 1640 medium containing 1% penicillin-streptomycin antibiotic mixture, 1% glutamax (GIBCO-BRL, Eragny, France), and 10% fetal calf serum (GIBCO) to a density of 5  $\times$  10<sup>5</sup> cells per ml in a 5% CO<sub>2</sub> atmosphere. Culture medium of stably transfected Jurkat cells was supplemented with 1 mg of G418 (GIBCO)/ml.

**PCR of DNA and RNA.** PCR detection of retrotranscribed RNAs was performed according to a previously published procedure (11). Briefly, total RNA was extracted from 2  $\times$  10<sup>6</sup> cells. To reduce the amount of DNA originating from lysis, supernatants were treated with RNase-free DNase (10 U/ $\mu$ l, Boehringer) for 30 min at 20°C and then for 15 min at 65°C. To 2  $\mu$ g of RNA sample (10  $\mu$ l) 200 ng of oligo(dT) primer (1  $\mu$ l) was added for 10 min at 65°C. Each sample was made up with reaction buffer (50 mM Tris-HCl [pH 8.3], 30 mM KCl, 8 mM MgCl<sub>2</sub>, 9 mM dithiothreitol, 320 nM deoxynucleoside triphosphates) to a final volume of 25  $\mu$ l, supplemented with 20 U of RNase inhibitor (Boehringer) and 25 U of avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer), and then incubated for 90 min at 42°C. PCRs were carried out on 4- $\mu$ l samples

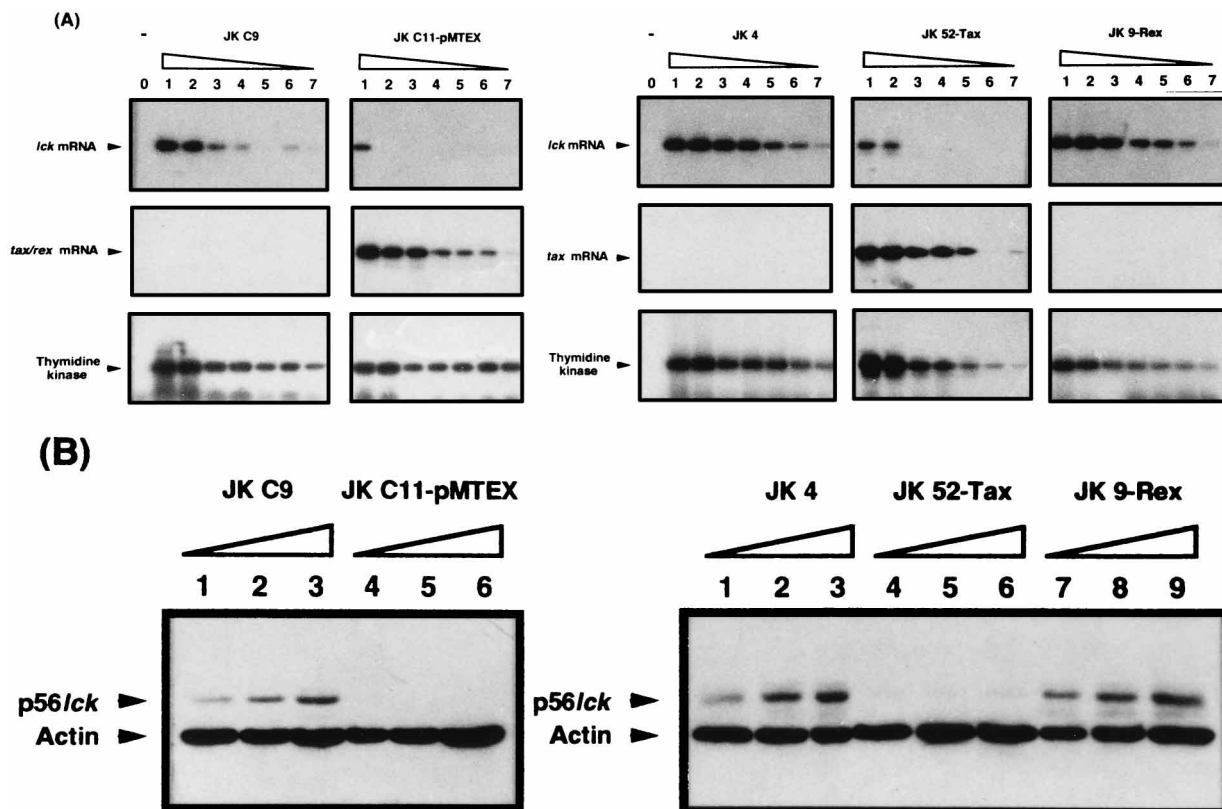


FIG. 2. Comparative analysis of *lck* mRNA and p56<sup>lck</sup> protein in Jurkat clones. (A) Semiquantitative RT-PCR analysis of *lck* mRNA expression in Jurkat JK C9 and JK C11-pMTEX clones (upper left panel) and in Jurkat JK4, JK52-Tax, and JK9-Rex clones (upper right panel). PCR analyses of AMV-retrotranscribed *lck* mRNA were performed with the p56-I/p56-II oligonucleotide primer pair. Different dilutions (1:1, 1:2, 1:10, 1:20, 1:50, 1:100, and 1:200; lanes 1 to 7, respectively) of the amplified products were electrophoresed, blotted, and hybridized with an  $\alpha$ -<sup>32</sup>P-labelled *lck* probe. Labelled DNA products were visualized by autoradiography. Autoradiograms of PCR amplifications of retrotranscribed viral mRNAs coding for Tax and Rex with the Tax 3/Tax 4 oligonucleotide primer pair (middle left panel) and of mRNAs coding for Tax only with the Tax 2/TRU 2 oligonucleotide primer pair (middle right panel), hybridized with an  $\alpha$ -<sup>32</sup>P-labelled *tax* probe, are shown as controls. Autoradiograms of PCR amplifications of retrotranscribed TK RNA are also shown (lower left and right panels). Lane 0 represents a control in which an RNA-free sample was treated like the extracted samples. (B) Western blot analysis of p56<sup>lck</sup> in JK C9 and JK C11-pMTEX clones (left panel). Lysates from JK C9 (lanes 1 to 3) and JK C11-pMTEX (lanes 4 to 6) containing increasing amounts of cellular proteins (15  $\mu$ g, 30  $\mu$ g, and 60  $\mu$ g, respectively) were electrophoresed onto an SDS-PAGE gel, blotted to a PVDF membrane, and the reactivity with anti-p56<sup>lck</sup> and anti-actin MAb was assayed by chemiluminescence (see the legend for Fig. 1C for details). Similarly, lysates from JK4 (lanes 1 to 3), JK52-Tax (lanes 4 to 6), and JK9-Rex (lanes 7 to 9) containing various amount of cellular proteins (15  $\mu$ g, 30  $\mu$ g, and 60  $\mu$ g, respectively) were assayed for reactivity with anti-p56<sup>lck</sup> and anti-actin MAb (right panel).

(or 4  $\mu$ l of serial dilutions of the sample) supplemented with an amplification mixture containing 20 pmol of each of the oligonucleotide primers and 2 U of *Taq* DNA polymerase. The amplification reaction was run in a PHC2 thermal cycler (Techne, Cambridge, United Kingdom). The amplified products were analyzed by electrophoresis in a 1% agarose gel, blotted for 2 h onto Hybond N<sup>+</sup> membrane (Amersham), and hybridized either with  $\alpha$ -<sup>32</sup>P-labelled p56<sup>lck</sup> probe prepared from pGEX2T-*lck* vector or with an appropriate probe (thymidine kinase [TK], *tax*-*rex*, *tax*, or CAT). Labelled DNA products were visualized by autoradiography (X-Omat AR films, Kodak). In some experiments (indicated in the figure legends), one of the primers was directly labelled with  $\gamma$ -<sup>32</sup>P before PCR.

**Cloning of the *lck* gene distal promoter.** Sequence of the human *lck* gene distal promoter (DP) has been previously reported (50). Cloning of the DP was performed by PCR as previously described (6). Briefly, a DNA fragment extending from position -730 to +42 was amplified from total cellular DNA extracted from CEM cells by PCR with the *lck* DP 1/*lck* DP 2 oligonucleotide primer pair (*lck* DP 2 contains a *Bgl*II cloning site). The amplified fragment was cloned in the PCR II plasmid by using the TA cloning kit (Invitrogen, Abingdon, United Kingdom). The insert was excised from the recombinant plasmid with *Bst*XI (*Bst*XI had been end blunted) and *Bgl*II digestion. This fragment was cloned at the *Bst*XI (end blunted) and *Bgl*II sites located 5' to the CAT gene of the pOPI3CAT plasmid (Stratagene, Cambridge, United Kingdom). The insert was sequenced by the chain-terminating inhibitors method by using the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) before the construct was used in functional assays. The cloned fragment showed no variation with the sequence previously reported for the human *lck* gene DP.

**Cloning of an E-Box-deleted *lck* gene DP.** Cloning of the *lck* DP deleted from the putative E-Box was performed by PCR site-directed mutagenesis. The

method used to construct the deletion mutant consisted of several rounds of PCR with four oligonucleotide primers, *lck* DP1, *lck* DP2, E-Box 1, and E-Box 2, two of which (E-Box 1 and E-Box 2) were used as mutagenic primers. Two short regions were separately amplified by two independent PCRs; the first PCR was performed with the *lck* DP1 and E-Box 2, and the second PCR was performed with the E-Box 1 and *lck* DP 2. The full-length mutated DNA fragment was produced in a third PCR performed on a template that consisted of fragments 1 and 2, which contain overlapping sequences, with the *lck* DP 1 and *lck* DP 2 primers. This fragment was cloned in the PCR II vector and then in the pOPI3CAT plasmid by using the cloning strategy described above.

**CAT mRNA assay.** JPX-9 cells were transiently transfected with *lck* DP-CAT or *lck* DP $\Delta$ E-Box-CAT plasmids according to the previously published procedure (7). Transfected cells were cultured in medium alone or in medium containing cadmium for 24 h at 37°C and then harvested and centrifuged at 2,000 rpm for 5 min, and total mRNAs were extracted with guanidine thiocyanate. After treatment with RNase-free DNase, the samples were treated for reverse transcription (RT)-PCR assay as described above.

**CAT assay.** JPX-9 cells transiently transfected with LTR<sub>(HTLV)</sub>-CAT were cultured in medium alone or in medium containing cadmium for 48 h at 37°C, and a CAT assay was performed according to the published method (39). Briefly, the method utilizes [<sup>3</sup>H]acetyl coenzyme A as acetyl donor and relies on the diffusion of labelled acetylchloramphenicol into a water-immiscible liquid scintillation containing cocktail. The quantity of [<sup>3</sup>H]acetylchloramphenicol produced was measured directly by counting the samples at selected time intervals.

**Western blot assay.** Cellular lysates were electrophoresed onto sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-12.5% PAGE) gels and blotted to polyvinylidene difluoride (PVDF) membranes (Millipore). The blot was then incubated for 1 h at room temperature with a blocking solution

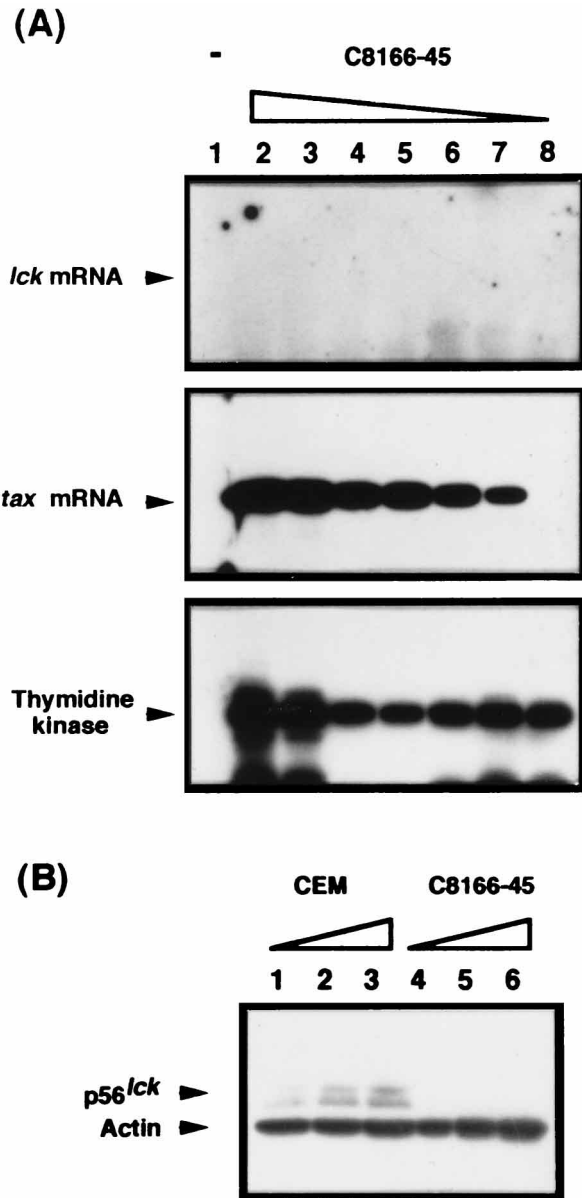


FIG. 3. Analysis of *lck* mRNA and p56<sup>lck</sup> protein in C8166-45 cells. (A) Semiquantitative RT-PCR analysis of *lck* mRNA in C8166-45 cells. PCR analysis of AMV-retrotranscribed *lck* mRNA (2  $\mu$ g) in C8166-45 cells was performed with the p56-I/p56-II oligonucleotide primer pair. Different dilutions (1:1, 1:2, 1:10, 1:20, 1:50, 1:100, and 1:200; lanes 2 to 8, respectively) of amplified products were electrophoresed, blotted, and hybridized with an  $\alpha$ -<sup>32</sup>P-labelled *lck* probe. Autoradiograms of PCR amplifications of retrotranscribed *tax* mRNA and TK RNA are shown as controls. (B) Western blot analysis of p56<sup>lck</sup> in CEM and C8166-45 cells. Lysates from CEM (lanes 1 to 3) and C8166-45 (lanes 4 to 6) containing various amounts of proteins (15  $\mu$ g, 30  $\mu$ g, and 60  $\mu$ g, respectively) were electrophoresed onto an SDS-PAGE gel, blotted to a PVDF membrane, and the reactivity with anti-p56<sup>lck</sup> and anti-actin MAb was assayed by chemiluminescence (see the legend for Fig. 1C for details).

(phosphate-buffered saline [PBS] containing 10% milk and 0.05% Tween 20) prior to addition of monoclonal antibody (MAb) (anti-p56<sup>lck</sup> MAb 3A5 was purchased from TEBU, Le Perray en Yvelines, France, and anti-actin MAb C4 was purchased from ICN Biomedicals Inc., Costa Mesa, Calif.). After 1 h at 20°C, the blot was washed three times with PBS-0.05% Tween 20 and incubated for 30 min with a 1:5,000 dilution of goat-anti-mouse immunoglobulin-peroxidase conjugate (Immunotech, Marseille, France). After three washes, bound MAb was detected by incubating the membrane for 1 min with enhanced chemilumines-

cence (ECL) reagent (Amersham). The membrane was then exposed for 0.5 to 5 min to hyperfilms-ECL (Amersham).

## RESULTS

**HTLV-1-infected human T-cell line MT2 lacks expression of p56<sup>lck</sup>.** The expression of p56<sup>lck</sup> message and p56<sup>lck</sup> protein was studied in MT2 cells, a human T-cell line infected with HTLV-1. As shown in Fig. 1A, PCR detection of the *lck* gene demonstrated the presence of this gene in MT2 cells as well as in the control CD4<sup>+</sup>/p56<sup>lck+</sup> CEM cell line. Although the *lck* gene was found in MT2 cells, no p56<sup>lck</sup> transcript could be detected in these cells. This is illustrated in Fig. 1B in which expression of the *lck* message in CEM and MT2 cells was analyzed using an RT-PCR assay. Using the same amount of retrotranscribed RNA (lanes 2 and 9, respectively) *lck* mRNA was detected in samples prepared from CEM but not from MT2 cells. *lck* mRNA was evidenced in CEM samples diluted 1:200 (lane 8), indicating the good sensitivity of the assay. Quantification of RNA amount used in the assay was performed with an internal RT-PCR control that amplified a fragment of the TK mRNA. The defect of MT2 cells to synthesize the *lck* mRNA was further evidenced at the protein level. As shown in Fig. 1C, immunoblotting analysis using an anti-p56<sup>lck</sup> MAb indicated that the p56<sup>lck</sup> protein was found in CEM cell lysates (lanes 1 to 3), but not in lysates from MT2 cells (lanes 4 to 6). The internal control of this experiment consisted of samples reacted with an anti-actin MAb, which demonstrated the presence of this antigen in both CEM and MT2 cell lysates.

Altogether, these results strongly suggested that HTLV-1 was involved in the downregulation of *lck* gene transcription in infected T cells, whereas this gene is usually transcribed in normal T cells and T-cell lines.

**Role of Tax in *lck* gene repression.** In order to provide direct evidence for the involvement of an HTLV-1 gene(s) in p56<sup>lck</sup> downregulation in infected T cells, CD4<sup>+</sup>/p56<sup>lck+</sup> Jurkat cells were stably transfected with an expression vector (pMTEX) that contained the 3' end of an HTLV-1 provirus. This construct was chosen because it contains a large fragment of the viral genome encompassing the *tax* and *rex* open reading frames. As a control, other cells were transfected with the vector that confers neomycin resistance. Expression of *lck*, TK, and *tax-rex* messages was compared in these cells. As shown in Fig. 2A (left panel), the *lck* mRNA was found in JK C11-pMTEX (a Jurkat clone that stably expressed pMTEX) only at a dilution of 1:1 (lane 1), whereas this message was detected in the control clone JK C9 at a dilution of up to 1:200, indicating that pMTEX downregulates *lck* gene expression. As expected, TK messages were detected in both clones. Moreover, the mRNAs that encode the Tax and Rex proteins were evidenced in JK C11-pMTEX. Finally, the study of p56<sup>lck</sup> protein by Western immunoblot provided further evidence that p56<sup>lck</sup> was undetectable in JK C11-pMTEX, whereas it was expressed in the parental cell line and JK C9 clone (Fig. 2B, left panel).

The above results indicated that a sequence present in the pMTEX construct mediated *lck* gene repression. Because this vector allows expression of both *tax* and *rex*, two viral regulatory genes known to influence transcription, we questioned whether the effect observed could be ascribed to one of these two genes or if it requires the synthesis of both proteins. The strategy used to address this question consisted of derivation of stably transfected Jurkat clones expressing either the *tax* or *rex* gene. As shown in Fig. 2A (right panel), the comparison of *lck* mRNA expression in Jurkat clones JK4, JK52-Tax (containing the Tax construct), and JK9-Rex (containing the Rex construct) indicated that *lck* gene expression was downregulated

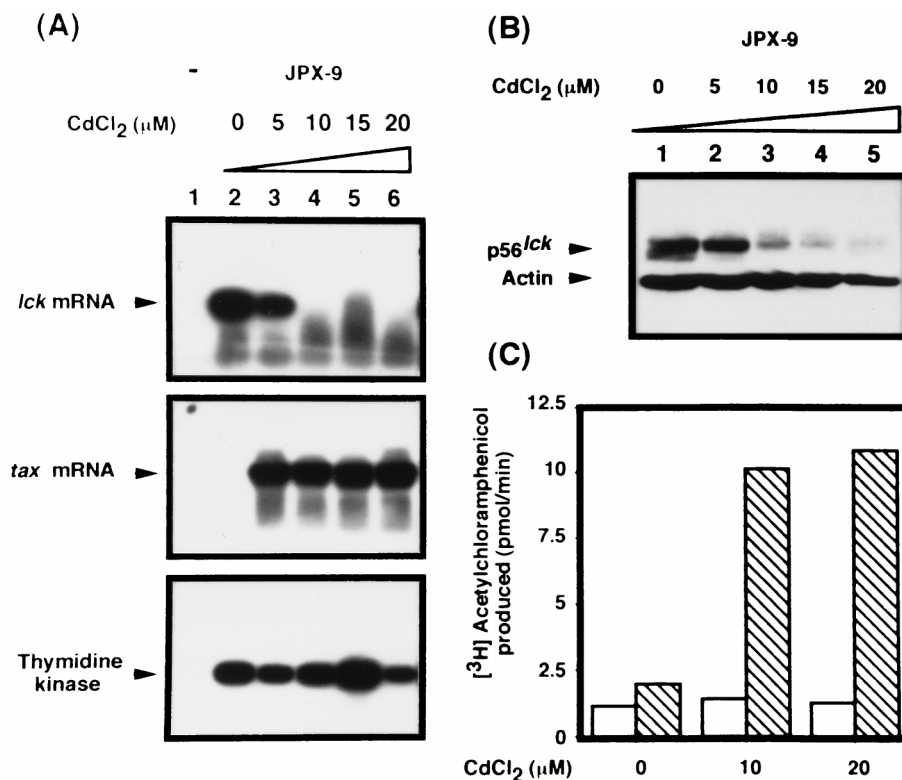


FIG. 4. *lck* mRNA and p56<sup>*lck*</sup> synthesis analysis in JPX-9 cells treated with various concentrations of CdCl<sub>2</sub>. (A) RT-PCR analysis of *lck* mRNA in JPX-9 cells treated with various concentrations of CdCl<sub>2</sub>. PCR analysis of AMV-retrotranscribed *lck* mRNA in JPX9 cells cultured in medium alone (lane 2) or medium containing 5 μM (lane 3), 10 μM (lane 4), 15 μM (lane 5), or 20 μM (lane 6) CdCl<sub>2</sub>, was performed with the p56-I/p56-II oligonucleotide primer pair. Amplified products were electrophoresed, blotted, and hybridized with an α-<sup>32</sup>P-labelled *lck* probe. Labelled DNA products were visualized by autoradiography. Autoradiograms of PCR amplification of retrotranscribed *tax* mRNA and TK RNA are shown as controls. Lane 0 represents a control in which an RNA-free sample was treated like the extracted samples. (B) p56<sup>*lck*</sup> was analyzed by Western blot in JPX-9 cells cultured in medium alone (lane 1) or medium containing 5 μM (lane 2), 10 μM (lane 3), 15 μM (lane 4), or 20 μM (lane 5) CdCl<sub>2</sub>. Cellular proteins (50 μg) were electrophoresed onto an SDS-PAGE gel, blotted to a PVDF membrane, and the reactivity with anti-p56<sup>*lck*</sup> was assayed by chemiluminescence (see the legend for Fig. 1C for details). As control, the membrane was also reacted with anti-actin Mab. (C) A CAT assay was also performed to control the expression of Tax protein in JPX-9 cells after induction by CdCl<sub>2</sub>. JPX-9 cells transiently transfected with PUC 18 (used as control plasmid) (□) or an LTR<sub>(HTLV)</sub>-CAT construct (▨) were cultured in medium alone or medium containing 10 μM or 20 μM CdCl<sub>2</sub>. The [<sup>3</sup>H]acetylchloramphenicol produced was measured as described in Materials and Methods.

only in the JK52-Tax clone suggesting that the *tax* gene was responsible for this effect. As expected, *tax* mRNA was found expressed in clone JK52-Tax only (Fig. 2A, right panel), and *rex* mRNA was found expressed in clone JK9-Rex (data not shown). The TK mRNA was found in the three clones. Finally, *lck* gene expression was also found to be downregulated in clones JK42-Tax and JK50-Tax, two other Tax-expressing clones (data not shown). The downregulation of *lck* gene expression in Tax-expressing cells was also supported by the results indicating that the p56<sup>*lck*</sup> protein could be detected only in clones JK4 and JK9-Rex (Fig. 2B, right panel).

To assess the physiological relevance of these observations, the expression of *lck* gene was evaluated in C8166-45 cells, an HTLV-1-transformed T-cell line which expresses high amounts of Tax but lacks expression of Rex (8). As shown in Fig. 3A, the *lck* message was not found in C8166-45 cells, and p56<sup>*lck*</sup> protein could not be detected in those cells (Fig. 3B).

These results indicate that Tax is directly involved in the downregulation of p56<sup>*lck*</sup> whereas Rex apparently played no role in the repression of the *lck* gene product.

**Repression of *lck* gene is dependent on Tax concentration in the cells.** To evaluate whether the repression of *lck* gene could be related to the amount of Tax protein found in transfected T cells, *lck* mRNA and protein were studied in the JPX-9 clone of Jurkat cells containing the *tax* gene under a promoter whose

expression is dependent on heavy metal ions (37). A CAT assay experiment was performed on JPX-9 cells transiently transfected with an LTR<sub>(HTLV)</sub>-CAT construct to estimate the expression of Tax after culture of the cells in a medium containing various concentrations of CdCl<sub>2</sub> (Fig. 4C); concentrations of 10 μM and 20 μM CdCl<sub>2</sub> stimulated CAT gene expression, indicating that these concentrations of CdCl<sub>2</sub> induce a significant expression of functionally active Tax protein. As shown in Fig. 4A, when cells were grown in the absence of cadmium chloride *lck* mRNA was clearly identified in these cells. At a concentration of 5 μM CdCl<sub>2</sub> the *lck* mRNA was still detected. The repression of *lck* gene became drastic at CdCl<sub>2</sub> concentrations equal to or greater than 10 μM. Similarly, downregulation of p56<sup>*lck*</sup> was evidenced at the highest concentrations (10 μM, 15 μM, and 20 μM) of CdCl<sub>2</sub> (Fig. 4B).

These results indicate that the extent of *lck* repression correlates with the level of Tax synthesis in *tax*-transfected cells.

**Effect of *tax* gene expression on *lck* promoter activity.** The human *lck* gene encodes two classes of transcripts (type I and II) containing different 5'-untranslated regions, which are expressed from two distinct promoters (50). The proximal *lck* promoter, positioned immediately adjacent to the *lck* gene, is active almost exclusively in the thymus (21). In contrast, the *lck* DP, which resides 34 kb upstream of the ATG of the *lck* gene, directs the expression of *lck* transcripts in both thymocytes and

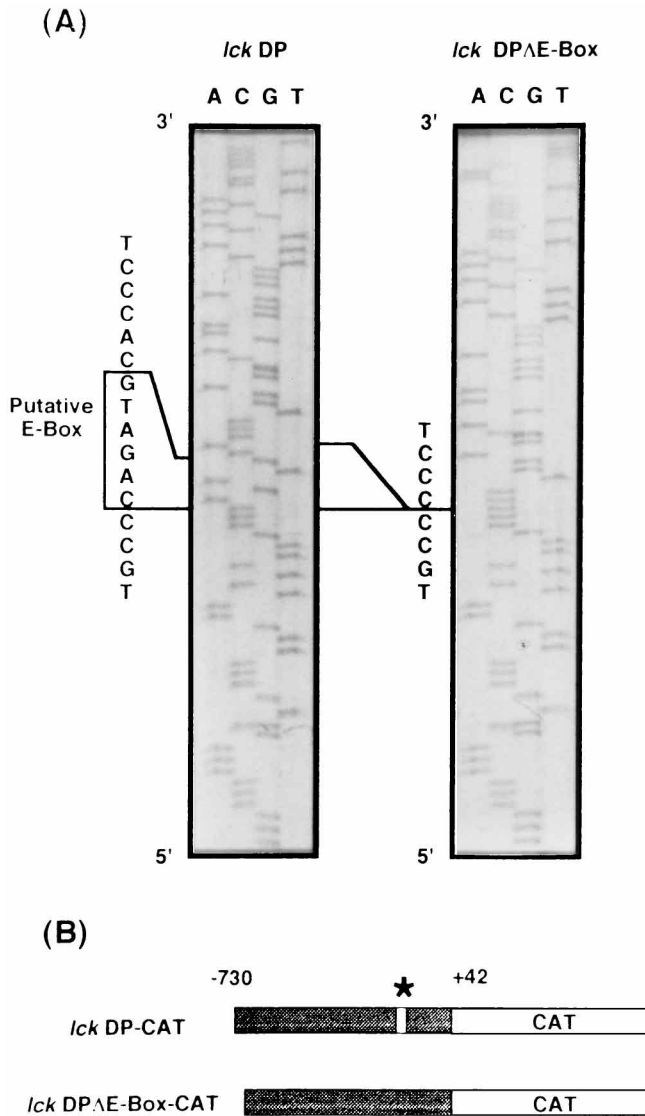


FIG. 5. Construction of *lck* DP-CAT and *lck* DP $\Delta$ E-Box-CAT. (A) Partial sequence of the *lck* DP and *lck* DP $\Delta$ E-Box that shows the putative E-Box (5'-CAGATG-3') in *lck* DP and its deletion in *lck* DP $\Delta$ E-Box. The deletion includes two additional bases at the 3' end of the putative E-Box (5'-CAGATGCA-3'). (B) Schematic representation of *lck* DP-CAT and *lck* DP $\Delta$ E-Box-CAT. \*, location of the putative E-Box in *lck* DP.

mature T cells (55). It has been previously reported (38) that the disappearance of the *lck* mRNA in HTLV-1-transformed cell lines correlates with the downregulation of the *lck* DP. In addition, our present observations suggest that Tax is involved in this action. To verify this hypothesis, we cloned the *lck* DP 5' to the CAT gene in a pOPI3CAT vector (Fig. 5). This construct, named *lck* DP-CAT, was then transiently transfected in JPX-9 cells, and CAT mRNA was evaluated by RT-PCR performed on total mRNA extracted from cells after 24-h culture in medium containing various concentrations of CdCl<sub>2</sub>. As shown in Fig. 6 (left panel), CAT transcription was evidenced in cells cultured in medium alone but was downregulated in JPX-9 cells treated with medium containing 15  $\mu$ M or 20  $\mu$ M CdCl<sub>2</sub>, indicating that expression of Tax modulates the *lck* DP activity in a dose-dependent manner.

These results confirm that the disappearance of the *lck* mRNA in HTLV-1-transformed cell lines correlates with the

downregulation of the *lck* DP activity and demonstrate that Tax is involved in this process by modulating the *lck* DP activity in a dose-dependent manner.

**Regulatory role played by the putative *lck* DP E-Box sequence in *lck* gene transrepression by Tax.** It has been previously reported that p40<sup>tax</sup>-mediated repression of  $\beta$ -polymerase expression is directed by a member of the family of bHLH proteins which bind to the E-Box consensus sequence (5'-CANNTG-3') (52). An analysis of the published sequence (50) of the human *lck* DP indicated the presence of a putative bHLH-binding site (5'-CAGATG-3') at position -259 to -253. To analyze the putative role of this sequence in the p40<sup>tax</sup>-mediated repression of the *lck* gene expression, *lck* DP was deleted from the E-Box (*lck* DP $\Delta$ E-Box) and cloned 5' to the CAT gene in a pOPI3CAT vector (Fig. 5). Transient transfections of this construct, named *lck* DP $\Delta$ E-Box-CAT, into JPX-9 cells were used to assess the role of the putative E-Box. As shown in Fig. 6 (right panel), the activity of this mutant promoter was not repressed in JPX-9 cells expressing Tax. To make sure that the signal observed could not be ascribed to DNA contamination during the preparation of mRNA samples, the same experiment was performed in the absence of AMV reverse transcriptase; under such conditions no CAT mRNA was detected.

These results indicate that Tax probably controls the human *lck* DP activity by acting on a bHLH protein which can bind to the E-Box present at position -259 to -253 in the *lck* DP.

## DISCUSSION

The main purpose of this work was to investigate which gene, among the several overlapping open reading frames present at the 3' end of the HTLV-1 genome, could encode for a protein implicated in the repression of *lck* gene expression in T cells. We clearly demonstrate that expression of Tax was necessary and sufficient to repress the *lck* gene in a concentration-dependent manner. Furthermore, we found that a potential bHLH recognition site (CAGATG) at position -259 to -253 of the *lck* DP probably contributes to the sensitivity of the *lck* promoter to Tax transrepression.

Several proteins encoded by HTLV-1 may modulate the expression of cellular genes or gene products. It has been previously shown that the p40<sup>tax</sup> activates a wide variety of cellular genes, such as the IL-2 and IL-2 $\alpha$  receptor chains, *c-fos*, Krox-20 and Krox-24, granulocyte-macrophage colony-stimulatory factor, vimentin, and SFA-1 (1, 2, 4, 18, 24, 32, 35, 48). Besides p40<sup>tax</sup>, it is also likely that p27<sup>rex</sup> could influence the expression of cellular gene products by conferring RNA stability to otherwise unstable mRNAs (59). Most recently, the p12<sup>i</sup> encoded by the pX region was shown to interact with cellular proteins that regulate T-cell activation (15, 30, 33). Among the different pX-encoded proteins that are known to modulate the expression of cellular genes or gene products, only p40<sup>tax</sup> has been reported to be able to repress the expression of cellular genes, namely the human  $\beta$ -polymerase gene (26), the p53 tumor suppressor gene (53), and the NF1 gene (13). However, it is worth noting that HTLV-1-mediated repression of p53 is controversial (43). In this report, we investigate which pX-encoded protein, or which combination of pX-encoded proteins, may be involved in the transcriptional repression of the *lck* gene in HTLV-1-infected cells (29, 31, 34). We found that *tax* gene expression was required to inhibit *lck* gene transcription (Fig. 1 and 2). Moreover, we found that expression of p40<sup>tax</sup> only was sufficient for such an inhibition to occur (Fig. 2 and 3).

The proximal *lck* promoter, positioned immediately adjacent

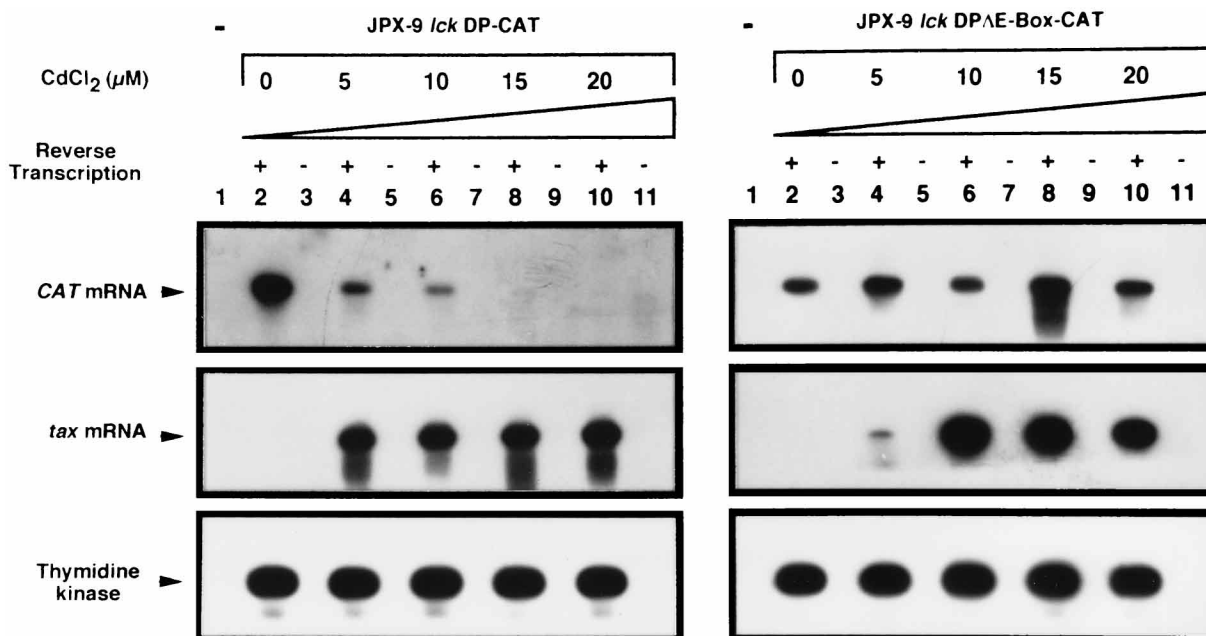


FIG. 6. Comparative analysis of *lck* DP and *lck* DPΔE-Box sensitivity to Tax. Modulation of *lck* DP- and *lck* DPΔE-Box promoter-driven CAT gene expression in JPX-9 cells induced by CdCl<sub>2</sub> to produce Tax was evaluated with the CAT 1/CAT 2 oligonucleotide primer pair. Cells transiently transfected with *lck* DP (left panel) or *lck* DPΔE-Box (right panel) were cultured in medium alone (lanes 2 and 3) or medium containing 5 μM (lanes 4 and 5), 10 μM (lanes 6 and 7), 15 μM (lanes 8 and 9), or 20 μM (lanes 10 and 11) CdCl<sub>2</sub>. Total mRNA was extracted and treated for 30 min at 37°C with an excess of DNase (70 U of DNase per μg of RNA). After heat inactivation of DNase, PCR amplifications were performed on mRNA samples either treated (lanes 2, 4, 6, 8, 10) or not (lanes 3, 5, 7, 9, 11) with reverse transcriptase. The amplified products were electrophoresed, blotted, and hybridized with an α-<sup>32</sup>P-labelled CAT probe. Labelled products were visualized by autoradiography. Lane 1 represents a control in which an RNA-free sample was treated like the extracted samples. Autoradiograms of PCR amplifications of *tax* mRNA and retrotranscribed *tax* mRNA and of TK RNA and retrotranscribed TK RNA are shown as controls.

to the *lck* gene, is active almost exclusively in the thymus (21), whereas the *lck* DP, which resides 34 kb upstream of the ATG of the *lck* gene, directs the expression of *lck* transcripts in both thymocytes and mature T cells (55). In the present study, we found that transient transfection of *lck* DP-CAT in JPX-9 cells resulted in CAT mRNA synthesis by these cells; in contrast, CAT mRNA synthesis driven by the *lck* DP was inhibited after induction of *tax* mRNA by cadmium treatment (Fig. 6). This result corroborates and extends previous observations by Nakamura and coworkers (38) who reported that the disappearance of the *lck* mRNA in HTLV-1-transformed cell lines is caused by a mechanism which downregulates the *lck* DP.

Proteins of the bHLH transcription factor family have been shown to function in the control of cell growth and differentiation (27), have been linked with certain leukemias (28, 40), and have been implicated in Tax-induced repression of β-polymerase gene expression (52), possibly resulting in a deficiency in DNA repair followed by progression to malignant transformation. Most recently, bHLH proteins have been implicated in Tax-induced repression of the p53 tumor suppressor gene (53), possibly preventing the p53-dependent cell growth arrest or apoptotic cell death triggered after DNA damage and resulting in a tendency to facilitate tumorigenesis (reviewed in reference 23). An analysis of the published sequence (50) of the human *lck* DP, indicated the presence of a putative bHLH binding site (5'-CAGATG-3') at position -259 to -253. It is worth noting that the central two nucleotides of the hexamer motif CANNTG known as the E-Box are usually either GC or CG (12) and that bHLH factors which are divided into class A, class B, and class C proteins bind preferentially to the CAG CTG or CACGTG motif. Yet, the CAGATG motif has already been shown to serve as a bHLH recognition site in the AKV

murine leukemia virus enhancer. Therefore, we hypothesized that bHLH proteins may be involved in Tax-mediated down-regulation of *lck* gene expression. Transfection experiments of JPX-9 cells with the *lck* DPΔE-Box-CAT construct (Fig. 6), suggested that the putative E-Box found in the human *lck* DP is involved in the transrepression of *lck* gene transcription by Tax, since *lck* DPΔE-Box-CAT seems to be insensitive to Tax expression. Although our data suggest that a bHLH protein appears likely to be involved in Tax repression of *lck* gene expression, the mechanism by which it could occur remains to be defined. Preliminary studies were performed using an electrophoretic mobility shift assay to analyze whether a double-stranded oligonucleotide containing the putative bHLH binding site from the *lck* DP (5'-TCTTG CCCAG ATGCA CCCTG GAGGG-3') was able to bind to nuclear extracts from the Tax-expressing JK52-Tax clone, the Tax-negative JK9-Rex clone, and the JK C9 clone. A similar shift in oligonucleotide migration was observed with all three nuclear extracts (data not shown), suggesting that Tax is probably unable to inhibit the formation of the protein-oligonucleotide complex that we have identified. If it turned out that the proteins found in the complex belong to the bHLH family, our results would corroborate the observations made by another research group who has studied the regulation of the β-polymerase promoter by bHLH (52) and claimed no inhibition of bHLH-E-Box interaction in Tax-expressing cells. It would be interesting to study the possibility that Tax may inhibit the transcriptional activation function of the bHLH by direct protein-protein interaction. This work is currently under progress.

In conclusion, our results highlight the highly pleiotropic transcriptional deregulation properties of Tax. Since p56<sup>lck</sup> is directly involved in regulation of T-cell activation, these results

may contribute to a further understanding of the mechanisms which form the basis for a direct link between the HTLV-1 infection of T cells and their oncogenic transformation.

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