JOURNAL OF VIROLOGY, Aug. 1996, p. 5194–5202 Vol. 70, No. 8
0022-538X/96/$04.00+0

The *tax* Gene of Human T-Cell Leukemia Virus Type 2 Is Essential for Transformation of Human T Lymphocytes

TED M. ROSS, SHERRIE M. PETTIFORD, AND PATRICK L. GREEN*

Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2363

Received 11 March 1996/Accepted 2 May 1996

The mechanism of human T-cell leukemia virus (HTLV)-mediated transformation and induction of malignancy is unknown; however, several studies have implicated the viral gene product, Tax. Conclusive evidence for the role of Tax in the HTLV malignant process has been impeded by the inability to mutate *tax* in the context of an infectious virus and dissociate viral replication from cellular transformation. To circumvent this problem we constructed a mutant of HTLV type 2 (HTLV-2) that replicates by a Tax-independent mechanism. For these studies, the Tax response element in the viral long terminal repeat was replaced with the cytomegalovirus immediate-early promoter enhancer (C-enh). Transcription of the chimeric HTLV-2 ($HTLV_{C\text{-}enh}$) was efficiently directed by this heterologous promoter. Also, the chimeric virus transformed primary human T lymphocytes with an efficiency similar to that of wild-type HTLV-2. A *tax*-knockout virus, termed $HTLV_{C\text{-}enh}\Delta Tax$, was constructed to directly assess the importance of Tax in cellular transformation. Transfection and infection studies indicated that $HTLV_{C\text{-}enh}\Delta Tax$ was replication competent; however, $HTLV_{C\text{-}enh}\Delta Tax$ failed to transform primary human T lymphocytes. We conclude that Tax is essential for HTLV-mediated transformation of human T lymphocytes. Furthermore, this chimeric HTLV, that replicates in the absence of Tax, should facilitate studies to determine the precise mechanism of T-lymphocyte transformation by HTLV.

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) are oncogenic retroviruses associated with human T-cell malignancies and degenerative neurological disorders. In addition to the essential retroviral genes *gag*, *pol*, and *env*, HTLV contains at least two *trans*-regulatory genes, *tax* and *rex*. Both Tax and Rex modulate viral gene expression and play important roles in viral pathogenesis (12, 14, 54). Tax is a *trans*-acting transcriptional activator of viral transcription and is essential for efficient viral replication (12, 23, 56). Tax function depends on *cis*-acting sequences, termed the Tax response element (TRE), located within the U3 region of the viral long terminal repeat (LTR) (9, 19, 34, 52, 53, 60). Tax does not bind DNA directly, but Tax *trans* activation of the LTR is mediated by cellular proteins, which include cyclic AMP response element (CRE)- and activating transcription factor (ATF)-binding proteins (CREB/ATF) (34, 36, 67–69). These proteins bind to three copies of conserved 21-bp repeat within the TRE facilitating transcription initiation (34, 36, 68, 69).

In addition to regulating HTLV gene expression, Tax also activates the expression of cellular genes involved in T-cell growth and differentiation. Similar to its action on the HTLV promoter, Tax stimulates expression of c-*fos*, c-*jun*, and granulocyte colony-stimulating factor genes through CRE sequences (21, 35, 50, 51, 66). In addition, Tax activates members of the NF-κB/Rel family of transcription factors, which leads to the deregulated expression of many NF-κB-responsive genes. These genes include those encoding interleukin-2 (IL-2), IL-2 receptor α, IL-3, and granulocyte-macrophage colony-stimulating factor, as well as human immunodeficiency virus type 1 (4, 7, 17, 33, 36, 40, 42, 44, 50, 51, 58, 63). Also, Tax increases the expression of c-*fos*, *erg-1*, *erg-2*, and parathyroid hormone-related protein through binding sites for serum response factor (2, 3, 20, 22, 47, 62, 63), and Tax represses gene expression from the DNA-polymerase β promoter (37). Therefore, these observations indicate that HTLV Tax is an important modulator of both viral and cellular gene expression and profoundly influences the expression of genes governing cell growth and activation.

The precise mechanism by which HTLV transforms T lymphocytes and induces malignancy in humans is unclear. Several observations indicate that Tax has oncogenic potential. First, Tax transforms rodent fibroblasts, resulting in the ability of these cells to form colonies in soft agar and to induce tumors in nude mice (27, 64). Second, studies with recombinant herpesvirus samiri/*tax* expression vectors indicate that Tax immortalizes T cells derived from mitogen-activated human cord blood lymphocytes (25, 26). Third, *tax* is oncogenic when expressed as a transgene in mice (5, 31, 48, 49). Taken together, these observations support the hypothesis that Tax plays a central role in HTLV-induced neoplastic transformation. However, all of these studies failed to directly assess the role of Tax in HTLV-mediated transformation of primary human T lymphocytes, the natural target for HTLV-associated malignancy.

Tax is critical for viral replication, which in turn is a prerequisite for transformation of human T lymphocytes. Therefore, it has not been possible to mutate *tax* in the context of an infectious virus and dissociate viral replication from cellular transformation. In this report, we describe the construction and characterization of a chimeric HTLV-2 that contains a substitution of the *cis*-acting TRE in the U3 region of the viral LTR with the cytomegalovirus immediate-early promoter enhancer (C-enh). This recombinant virus, $HTLV_{C\text{-}enh}$, replicates by a Tax-independent mechanism and transforms primary human T lymphocytes with an efficiency similar to that of wild-type HTLV-2 (wtHTLV-2). More importantly, a chimeric virus deficient for Tax expression ($HTLV_{C\text{-}enh}\Delta Tax$) failed to transform primary human T lymphocytes. Our results indicate

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Vanderbilt University School of Medicine, 1161 21st Ave. South, AA5224 Medical Center North, Nashville, TN 37232-2363. Phone: (615) 343-1039. Fax: (615) 343-7392. Electronic mail address: patrick.green@mcmail.vanderbilt.edu.

that Tax is essential for HTLV-mediated transformation of human T lymphocytes.

MATERIALS AND METHODS

Cells. B-cell line 729-6 and the human leukemic T-cell lines Jurkat and JM4 (55) were maintained in Iscove's medium supplemented to contain 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine. BJAB cells, a Burkitt's lymphoma human B-cell line, were maintained in RPMI 1640 medium containing the same supplements. Peripheral blood lymphocytes (PBL) were isolated from blood of normal donors by centrifugation over Ficoll-Paque (Pharmacia). Cells were maintained in culture in RPMI 1640 medium supplemented with 20% FCS and antibiotics.

Plasmids. LTR-II-CAT (wild-type LTR [wtLTR]-chloramphenicol acetyltransferase [CAT]) reporter construct, BC20.2 *tax/rex* cDNA expression vector, and control vectors SV_2neo and BC12 were previously described (15, 57). The HTLV-2 proviral clone pH6neo was used to create three infectious proviral clones used in this study (15). The wtHTLV-2 proviral clone is identical to pH6neo except for the deletion of 5′ and 3′ cellular DNA flanking the provirus. The HTLV-2 chimeric LTR ($LTR_{C\text{-}enh}$) was constructed by inserting the C-enh in place of the TRE. Briefly, an *Xba*I site was created 5′ to the TATA and CAAT boxes in the HTLV-2 promoter by site-directed mutagenesis (−53 relative to the transcription initiation site). The HTLV fragment containing the three 21-bp repeats was removed by digestion with *Aat*II and *Xba*I (−215 to −53 relative to the transcription initiation site) and replaced with the *Hin*cII-*Nco*I fragment containing the C-enh (−598 to −218 relative to the transcription initiation site). Subsequently, $LTR_{C\text{-}enh}$ was cloned 5′ to the CAT gene ($LTR_{C\text{-}enh}$-CAT) or inserted into the wtHTLV-2 backbone replacing the 3′ LTR ($HTLV_{3'C\text{-}enh}$) or both the 5′ and the 3′ LTRs ($HTLV_{C\text{-}enh}$).

The wtHTLVΔTax proviral clone and the chimeric $HTLV_{C\text{-}enh}$ΔTax proviral clone each contain TC-to-AG point mutations in the HTLV-2 coding sequence (nucleotides [nt] 7220 and 7221) introduced by site-directed mutagenesis performed with a Sculptor in vitro mutagenesis kit (Amersham) and the oligonucleotide primer 5′-TCGGGTAATCGGTCCTAAACC-3′. This mutation introduces a termination codon at amino acid 4 of Tax while leaving the overlapping Rex amino acid sequence unchanged. BC20.2ΔTax contains the identical TC-to-AG point mutations in the *tax/rex* cDNA expression vector BC20.2

Transfections and CAT assay. Plasmid DNA was introduced into cells by electroporation as previously described (11). Briefly, cells were washed with phosphate-buffered saline (PBS) and resuspended (2×10^7 cells per ml) in RPMI 1640 medium supplemented with 20% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine. A total of 5×10^6 cells were electroporated with 25 μg of DNA (900-μF charge, 250-V potential). Cells were transferred to 3 ml of medium and grown at 37°C for 48 h. Stable transfectants containing the wild-type or C-enh-containing proviral clones were isolated following incubation in 24-well culture dishes (5×10^5 cell per ml) in medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM glutamine, and geneticin (1.0 mg/ml). Following a 4- to 5-week selection period, viable cells were expanded in culture for further analysis. Stable transfectants are designated "729" followed by the clone with which they were transfected.

Cell extracts for CAT assays were harvested 48 h posttransfection as described previously (24). Three independent CAT assays were performed, and the mean for each sample was calculated. All CAT reactions were standardized for equivalent levels of protein, and incubation for a period of 1 h resulted in an enzymatic activity that was in the linear range. Percentages of [^{14}C]chloramphenicol acetylation were quantified by a Fuji Imaging System.

Metabolic labeling and immunoprecipitation. Stably transfected 729 cell lines, virally infected BJAB cells, or HTLV-2-transformed PBL were metabolically labeled with [^{35}S]methionine-cysteine (Trans-^{35}S-label; 100 μCi/ml; ICN Biochemicals, Inc.) in methionine-cysteine-free RPMI 1640 medium supplemented with 10% dialyzed FCS. Cells were lysed in radioimmunoprecipitation assay buffer (0.05 M Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 1.0% Triton X-100, 0.15 M NaCl, 2.0 mM phenylmethylsulfonyl fluoride), and lysates were clarified by centrifugation at 100,000 × *g* (1 h, 4°C). Clarified extracts were immunoprecipitated with antisera specific for HTLV-2 $p24^{Gag}$ in the presence of protein A-Sepharose (Pharmacia). Immunoreactive proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.

DNA transfer and hybridization. High-molecular-weight DNA was extracted from stably transfected 729 cells or from HTLV-2-infected BJAB cells and subjected to Southern blot analysis (61) as described elsewhere (28). The probe consisted of HTLV-2-specific ^{32}P-labeled oligonucleotide fragments. A 613-bp fragment (nt 7588 to 8200, based on the pH6neo HTLV-2 sequence) which hybridizes to the 3′ portion of the HTLV-2 genome was generated by PCR amplification (25 cycles) using HTLV-2-specific oligonucleotides and BC20.2 plasmid template. The 1,670-bp *Kpn*I fragment (nt 1702 to 3372, based on the pH6neo HTLV-2 sequence) hybridized to the 5′ portion of the HTLV-2 genome.

Syncytium and transformation assays. Syncytium and transformation assays were performed as previously described (29). Briefly, stable HTLV-2 producer cells (5×10^5) were irradiated with 10,000 rad and then cocultivated with either 5×10^5 BJAB cells or 10^6 PBL (isolated from blood of normal donors by centrifugation over Ficoll-Paque) in 24-well culture plates. Syncytia were scored in BJAB cocultures 5 to 7 days postplating. Long-term BJAB cocultures resulted in chronically infected BJAB producer cell lines, which were essentially syncytium free. Transformed T cells, defined as cells exhibiting continuous growth in the absence of IL-2, grew out of PBL cocultures 5 to 6 weeks postplating. In both cases, the presence of HTLV-2 was confirmed by detection of $p24^{Gag}$ capsid by radioimmunoprecipitation and SDS-PAGE analysis.

Prolonged proliferation of transformed cells was measured by [^{3}H]thymidine incorporation assays. Briefly, cells were incubated for 12 h with [^{3}H]thymidine (10 μCi/ml) and harvested onto glass fiber filters, and incorporated radioactivity was determined by liquid scintillation counting.

RESULTS

Construction and characterization of a chimeric LTR. The HTLV promoter region, which contains the TRE, directs transcription with high efficiency only in the presence of Tax. Within the TRE are three imperfectly conserved 21-bp repeat elements that are essential for Tax *trans* activation of the viral promoter (36, 69). Efficient HTLV gene expression in the absence of Tax required the construction of a chimeric LTR which maintained high transcriptional activity in human T lymphocytes, the target for HTLV cellular transformation. We replaced the *cis*-acting TRE of the wtLTR with the enhancer from the C-enh (8), creating $LTR_{C\text{-}enh}$ (Fig. 1A).

The transcriptional efficiency and Tax responsiveness of $LTR_{C\text{-}enh}$ was tested in a CAT transient assay. The initial LTR transcriptional analysis was performed in 729 B cells because of their high transfection efficiency by electroporation (11) and because established HTLV-2 replication and T-lymphocyte transformation assays use 729/HTLV-2 stable producer cells (13, 16). wtLTR-CAT or $LTR_{C\text{-}enh}$-CAT was cotransfected with a wtHTLV-2 *tax/rex* expression vector or parental vector alone into 729 B cells. As shown in Fig. 2A, the CAT reporter gene was transcribed very efficiently from $LTR_{C\text{-}enh}$ in the absence of Tax. In contrast, and as previously reported, CAT was not transcribed from the wtLTR over background levels in the absence of Tax (30, 54). Coexpression of Tax and Rex failed to affect the transcription efficiency of $LTR_{C\text{-}enh}$; however, coexpression of Tax and Rex significantly upregulated transcription from the wtLTR (Fig. 2A). These results with 729 B cells demonstrate that CAT gene transcriptional efficiency from $LTR_{C\text{-}enh}$ in the absence of Tax is similar to its transcriptional efficiency from the wtLTR in the presence of Tax.

Since the actual targets for HTLV transformation are T lymphocytes, the transcriptional efficiency and Tax responsiveness of $LTR_{C\text{-}enh}$-CAT was further tested in two human T-cell lines, JM4 and Jurkat. $LTR_{C\text{-}enh}$-CAT was cotransfected with a *tax/rex* expression vector or parental vector alone into JM4 T cells, and CAT activity was measured. Similar to the results in 729 B cells, the $LTR_{C\text{-}enh}$ was active in JM4 cells in the absence of Tax; however, $LTR_{C\text{-}enh}$ was activated three- to fourfold in the presence of Tax (Fig. 2B). The wtLTR was active only in the presence of Tax. A similar result was found with Jurkat T cells (data not shown). This increase in transcriptional activity of $LTR_{C\text{-}enh}$ in the presence of Tax is attributed to the activation state of NFκB. Although the cytomegalovirus immediate-early promoter is not dependent on NFκB for efficient transcription, it is NFκB responsive as a result of two NFκB motifs within the enhancer element. Therefore, in contrast to 729 cells, in which NF-κB is constitutively active (data not shown), NFκB is expressed as an inactive transcription factor in the cytoplasm of resting T cells, such as JM4 or Jurkat. In these cells, Tax activates the NFκB signaling pathway in a concentration-dependent manner, resulting in activation of NFκB-responsive genes (4, 6, 10, 17, 34, 40, 42, 45, 50, 59). Hence, in T cells, $LTR_{C\text{-}enh}$ is activated three- to fourfold in the presence of Tax, whereas $LTR_{C\text{-}enh}$ is maximally activated in 729 cells

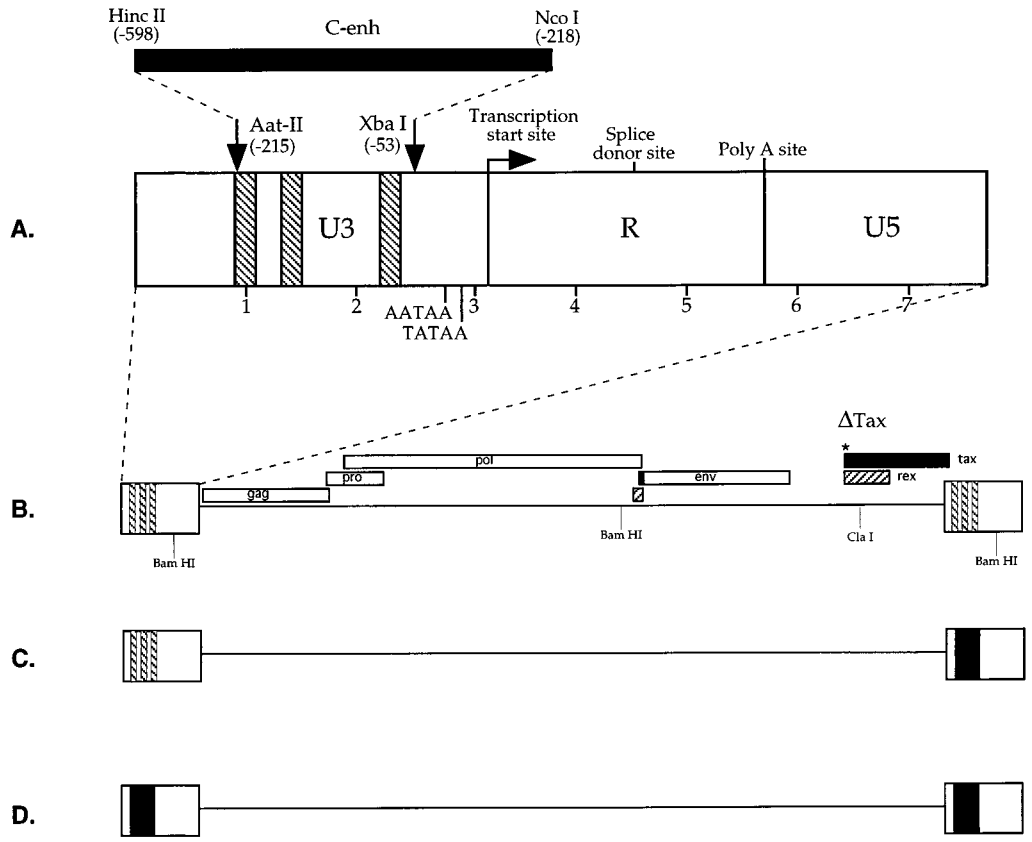

FIG. 1. Schematic representation of the HTLV-2 LTR and genome organization. (A) Diagram of the HTLV-2 LTR showing the locations of the U3, R, and U5 regions, transcription start site, splice donor site, and polyadenylation site. The three hatched boxes in the U3 region represent the three imperfectly conserved 21-bp repeats contained within the TRE. The location of the *Aat*II and *Xba*I restriction enzyme recognition sites (−215 and −53 relative to the start of transcription) are indicated. C-enh contained on a *Hin*cII-*Nco*I fragment (−598 and −218 relative to the start of transcription) is depicted above (black box). Numbers below the LTR represent bases (hundreds). (B) Diagram of the complete wild-type HTLV-2 proviral genome. Locations of the *gag*, *pro*, *pol*, *env*, *tax*, and *rex* genes and their corresponding reading frames are indicated. wtLTRs containing the three 21-bp repeats (hatched boxes), diagnostic restriction sites (*Bam*HI and *Cla*I), and ΔTax mutation (*) are indicated. (C) The proviral clone $HTLV_{3'C\text{-}enh}$ contains a 5′ wtLTR and a 3′ LTR with the C-enh heterologous sequences (black box) in place of the TRE. (D) The proviral clone $HTLV_{C\text{-}enh}$ contains both a 5′ and a 3′ chimeric $LTR_{C\text{-}enh}$.

and is not significantly modulated by Tax (Fig. 2A). These results indicate that in both T cells and B cells, $LTR_{C\text{-}enh}$ efficiently initiates transcription of the CAT reporter gene. It should be noted that in T cells, the relative levels of CAT activity of the wtLTR-CAT in the presence of Tax (29%) are similar to the levels of CAT activity of $LTR_{C\text{-}enh}$-CAT without Tax (25%). This finding suggests that gene expression from an HTLV-2 provirus containing $LTR_{C\text{-}enh}$ in the absence of Tax is similar to gene expression from a wtHTLV-2 provirus in the presence of Tax.

$HTLV_{C\text{-}enh}$ genome construction and transcriptional analysis. Having established that the chimeric LTR efficiently directs transcription of the CAT gene in the absence of Tax, we constructed proviral clones containing the chimeric LTR. The $HTLV_{C\text{-}enh}$ proviral clone contains both a 5′ and a 3′ chimeric LTR, whereas the $HTLV_{3'C\text{-}enh}$ proviral clone, used primarily as a control throughout these studies, contains a 5′ wtLTR and a 3′ chimeric LTR (Fig. 1C and D). Efficient gene expression from the $HTLV_{C\text{-}enh}$ proviral vector as well as viral progeny will be independent of Tax. In contrast, efficient gene expression from the $HTLV_{3'C\text{-}enh}$ proviral vector will initially be dependent on Tax; however, because of LTR duplication in the reverse transcription process, all progeny virus will replicate in a Tax-independent manner. Since transcription directed from the HTLV-2 wtLTR is responsive to Tax and Rex, previous studies have used the wtLTR-CAT to assay for Tax and Rex expression from proviral clones (29). The wtHTLV, $HTLV_{C\text{-}enh}$, and $HTLV_{3'C\text{-}enh}$ proviral clones were cotransfected with wtLTR-CAT into JM4 T cells, and functional levels of Tax and Rex were assessed by measuring CAT activity. Both $HTLV_{C\text{-}enh}$ and $HTLV_{3'C\text{-}enh}$ proviral clones produced functional Tax and Rex (Fig. 2C). Tax and Rex are expressed immediately from $HTLV_{C\text{-}enh}$, but expression is kinetically delayed from wtHTLV and $HTLV_{3'C\text{-}enh}$ because of the requirement of Tax to efficiently activate transcription from the wtLTR. Therefore, similar to the activity of $LTR_{C\text{-}enh}$-CAT, the $HTLV_{C\text{-}enh}$ clone had a transcriptional activity six- to sevenfold higher than that of either the wtHTLV-2 or the $HTLV_{3'C\text{-}enh}$ proviral clone. We conclude that $LTR_{C\text{-}enh}$ in the context of a complete proviral genome activates viral gene expression with an efficiency similar to that observed with reporter gene constructs.

Isolation of stable transfections. To determine the capacity of $HTLV_{C\text{-}enh}$ to synthesize viral proteins, direct viral replication, and induce cellular transformation, stable 729 cell transfectants containing the wtHTLV, $HTLV_{C\text{-}enh}$, or $HTLV_{3'C\text{-}enh}$ proviral clone were isolated. The presence of integrated HTLV-2 proviral DNA in the stable transfectants was determined by Southern blot analysis. Each of the stable transfectants analyzed contained complete copies of the provirus. The

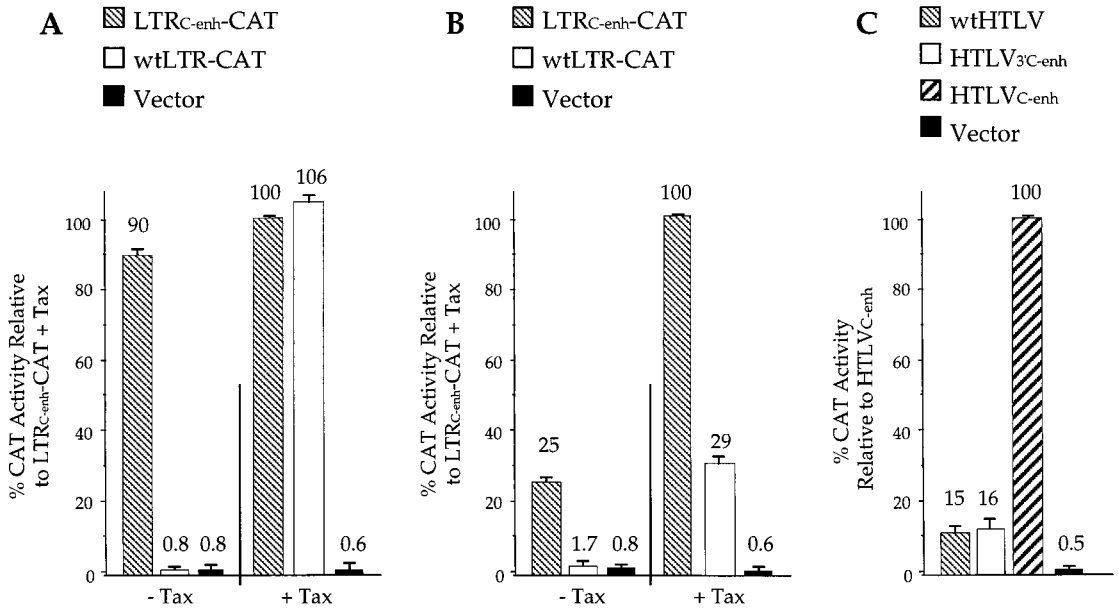

FIG. 2. Transcriptional efficiency of $LTR_{C\text{-}enh}$. (A) 729 B cells were cotransfected with $LTR_{C\text{-}enh}$-CAT or wtLTR-CAT and a wtHTLV-2 *tax/rex* expression vector, BC20.2 (+ Tax), or vector, BC12, alone (−Tax). Lysates were harvested 48 h posttransfection and subjected to CAT assay. Numbers above the bars are percent chloramphenicol acetylation values averaged over three experiments and normalized to the value for $LTR_{C\text{-}enh}$-CAT in the presence of Tax (100%). (B) JM4 T cells were cotransfected with $LTR_{C\text{-}enh}$-CAT or wtLTR-CAT and a wtHTLV-2 *tax/rex* expression vector (+ Tax) or vector alone (− Tax). Number above the bars are percent chloramphenicol acetylation values averaged over three experiments and normalized to the value for $LTR_{C\text{-}enh}$-CAT in the presence of Tax (100%). (C) JM4 T cells were cotransfected with wtLTR-CAT and either the wtHTLV-2 proviral clone, $HTLV_{3'C\text{-}enh}$ proviral clone, $HTLV_{C\text{-}enh}$ proviral clone, or vector (SV_2neo) alone. Numbers above the bars are percent chloramphenicol acetylation values averaged over three experiments and normalized to the value for $HTLV_{C\text{-}enh}$ (100%).

larger sizes of the hybridizing bands in both $HTLV_{C\text{-}enh}$ and $HTLV_{3'C\text{-}enh}$ confirmed the insertion of heterologous enhancer in the 3′ LTR (Fig. 3). Probes recognizing the 5′ end of the virus hybridize to a larger species in $HTLV_{C\text{-}enh}$, which allows distinction between cells harboring $HTLV_{3'C\text{-}enh}$ from $HTLV_{C\text{-}enh}$ genomes (data not shown). These results indicate that the stable transfectants have integrated proviral DNA with the heterologous enhancer.

Gag protein expression by $HTLV_{C\text{-}enh}$. To monitor the production of proteins in these stable transfectants, cells were metabolically labeled with radioactive amino acids and immunoprecipitations were performed with cell lysates. Immunoprecipitation analysis of cell lysates with $p24^{Gag}$-specific antisera indicated that stable transfectants containing wtHTLV-2, $HTLV_{3'C\text{-}enh}$, and $HTLV_{C\text{-}enh}$ produced significant levels of $p24^{Gag}$ capsid protein (see Fig. 7A). These results demonstrate that $HTLV_{C\text{-}enh}$ is indistinguishable from wtHTLV-2 in the synthesis of $p24^{Gag}$ capsid protein.

Production of infectious virus and replication. To demonstrate the capacity of the chimeric viruses to produce infectious progeny virions, the stable transfectants were cocultured with the BJAB cell line. Productive infection of BJAB cells by HTLV-2 results in a rapid induction of syncytia and some cytopathicity (29). Syncytium formation is dependent on the efficient expression of viral Env. Syncytium formation was detected by microscopic evaluation of BJAB cells after cocultivation with irradiated stable transfectants. Cocultivation of 729-wtHTLV, 729-$HTLV_{3'C\text{-}enh}$, and 729-$HTLV_{C\text{-}enh}$ cells with BJAB cells resulted in syncytium formation (Table 1). We previously reported that the presence of infectious virus capable of spreading throughout the culture dramatically reduces the time required for syncytium induction and cytopathicity (29). To address the efficiency at which the chimeric virus replicates and induces syncytia, 10-fold serial dilutions of irradiated producer cells were cocultured with BJAB cells. Syncytia could be induced with as few as 100 irradiated producer cells, and there was no apparent difference in the time course of syncytium induction by either wtHTLV, $HTLV_{3'C\text{-}enh}$, or $HTLV_{C\text{-}enh}$. Long-term growth of these infected cultures resulted in syncytium-free BJAB cell lines expressing virus, as documented by new syncytium formation upon addition of fresh uninfected BJAB cells as previously reported (29). Nucleic acid hybridization and protein analysis of long-term BJAB cocultures revealed HTLV-2 proviral DNA with band sizes diagnostic for wtHTLV, $HTLV_{3'C\text{-}enh}$, or $HTLV_{C\text{-}enh}$ and $p24^{Gag}$ capsid protein expression, respectively (data not shown and Table 1). Taken together these results demonstrate that HTLV-2 containing this heterologous enhancer is stable and replicates in BJAB cells.

$HTLV_{C\text{-}enh}$ transforms human T lymphocytes. Having established that chimeric virus replicates efficiently, we next determined whether it has the capacity to transform primary T lymphocytes. Irradiated 729 producer cells were cocultured with freshly isolated primary PBL. The initiation of HTLV transformation is apparent within 4 to 5 weeks following coculture, as detected by expansion of cells from the PBL mixed cell population. $HTLV_{C\text{-}enh}$ and wtHTLV had the capacity to transform primary T cells with similar efficiencies under these experimental conditions (Table 1). Irradiated 729 cells cocultured with PBL or alone were used as controls and yielded no viable cultures. Flow cytometric analysis determined that cells transformed in these studies by both wtHTLV and $HTLV_{C\text{-}enh}$ were primarily $CD8^+$ T cells (data not shown). Viability and growth of the transformed cells were not dependent on exogenous IL-2. However, the capacity to efficiently establish viable IL-2-independent T-cell lines was enhanced by providing IL-2 5 to 6 weeks following coculture. Regardless of whether transformed cells arose following coculture of PBL with 729-wtHTLV,

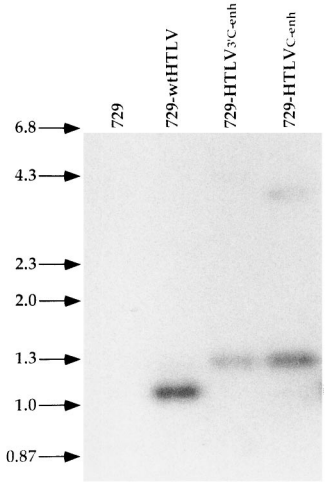

FIG. 3. Southern hybridization analysis of stable transfectant 729 B-cell DNA. Ten micrograms of high-molecular-weight cellular DNA was digested with *Cla*I and *Bam*HI (see Fig. 1 for locations), electrophoresed through a 0.7% agarose gel, blotted onto nitrocellulose paper, and hybridized with a ^{32}P-labeled HTLV-2-specific PCR-generated 613-bp fragment (nt 7588 to 8200). A 1.166-kb fragment is expected to be detected in 729-wtHTLV digested cell DNA, whereas a 1.376-kb fragment is expected in 729-HTLV$_{C\text{-}enh}$ digested cell DNA. The sizes (in kilobases, indicated on the left) were determined by comparison with *Hin*dIII-digested lambda DNA and *Hae*III-digested ϕX174 DNA.

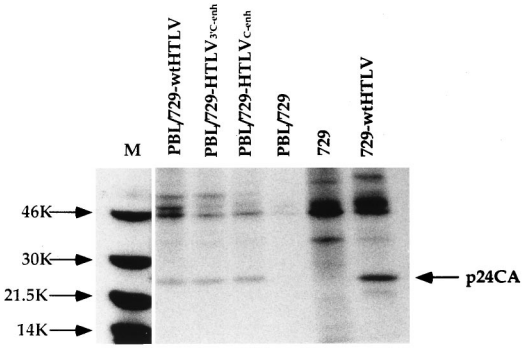

FIG. 4. Immunoprecipitation of [^{35}S]methionine-cysteine-labeled primary T-cell transformants. The indicated 729 viral producer cells were irradiated with 10,000 rad and cocultured with human primary PBL. Six to seven weeks post-coculture, at which time transformed T cells were present, 5×10^6 cells were metabolically labeled and cell lysates were prepared. Cell lysates normalized by scintillation counting of trichloroacetic acid precipitates were immunoprecipitated with human antisera directed against the HTLV-2 p24^{Gag} capsid protein. 729 cells (uninfected B-cell line) and 729-wtHTLV cells (chronically infected HTLV-2 producer cell line of 729 cell origin) are positive and negative controls, respectively. The sizes (in kilodaltons [K], indicated on the left) were determined by comparison with protein markers (Amersham) (lane M).

729-HTLV$_{3'C\text{-}enh}$, or 729-HTLV$_{C\text{-}enh}$ cells, the expression of HTLV-2 was confirmed by the detection of p24^{Gag} capsid protein in transformed cells (Fig. 4). These results indicate that the chimeric virus, HTLV$_{C\text{-}enh}$, replicates efficiently and maintains the capacity to productively infect and transform primary T lymphocytes.

Construction and characterization of HTLV$_{C\text{-}enh}$ *tax*-knockout mutation. To assess whether functional Tax is required for HTLV-mediated transformation of human T lymphocytes, a *tax* mutation eliminating Tax production was constructed. Tax and Rex are expressed from separate but overlapping reading frames, and the Tax initiator methionine codon is also the initiator methionine codon for the Env protein (15). Therefore, mutations introduced in the *tax* gene must maintain the *rex* reading frame and the Tax initiator ATG cannot be mutated. A mutation in the *tax* reading frame, termed Δ*tax*, which introduces a termination codon at position four (Phe to termination) was constructed and then cloned into the *tax*/*rex* cDNA expression vector, the wtHTLV-2 proviral clone, and the HTLV$_{C\text{-}enh}$ proviral clone. Tax functional analysis was performed with the wtLTR-CAT *trans*-activation assay in JM4 T cells. Irrespective of the expression vector, the Δ*tax* mutation ablated transcription of the CAT reporter gene (Fig. 5). In addition, Western blot (immunoblot) analysis failed to detect wild-type Tax (wtTax) in cells transfected with the Δ*tax*/*rex* cDNA expression vector; however, wild-type Rex (wtRex) protein expression and function were not affected (data not shown). Therefore, these results demonstrate that the Δ*tax* mutation completely eliminates Tax protein expression and Tax *trans*-activation function without affecting Rex expression and function.

TABLE 1. Infection and transformation of cells[a]

Stable transfectant	Syncytium induction in BJAB cells[b]	Primary T-cell transformation[c]	p24 production,[d] BJAB cells/PBL
729	−	−	−/−
729-wtHTLV	+(100)	+	+/+
729-HTLV$_{3'C\text{-}enh}$	+(100)	+	+/+
729-HTLV$_{C\text{-}enh}$	+(100)	+	+/+
729-HTLV$_{C\text{-}enh}$ΔTax	+(100)	−	+/ND[e]

[a] Stably transfected 729 cells were irradiated with 10,000 rad, and 5×10^5 cells were cocultivated with 10^6 PBL or serial 10-fold dilutions of irradiated cells were incubated with 5×10^5 BJAB cells in 24-well culture plates. Cells were fed twice a week with RPMI 1640 supplemented with 20% FCS and antibiotics.

[b] Syncytia were scored 3 to 7 days postplating. Numbers in parentheses indicate the minimum number of 729 producer cells required for syncytium induction following coculture with 5×10^5 BJAB cells.

[c] Transformation was scored as positive or negative 6 to 7 weeks following coculture of 729 producer cells with PBL.

[d] The presence of HTLV was confirmed by detection of HTLV p24 capsid by radioimmunoprecipitation and SDS-PAGE analysis using both types of cells.

[e] ND, not determined since there were no transformed primary cells to analyze.

Characterization of HTLV$_{C\text{-}enh}$ ΔTax proviral stable transfectants. To directly assess the role of Tax in cellular replication and HTLV-mediated transformation of primary T lymphocytes, 729 stable transfectants harboring wtHTLVΔTax or HTLV$_{C\text{-}enh}$ΔTax proviral DNA were isolated. As demonstrated by Southern blot analysis, each stable transfectant contained complete copies of the HTLV-2 provirus with hybridization bands of the predicted sizes (Fig. 6 and data not shown). The larger size of the hybridizing band in HTLV$_{C\text{-}enh}$ΔTax stable transfectant cell DNA confirm the insertion of the heterologous enhancer. The maintenance of the ΔTax mutation in the stable transfectants was confirmed by determining the nucleotide sequences of the PCR products of stable transfectant cell DNA (data not shown). We next determined whether viral proteins were produced by stable transfectants harboring Tax-deficient proviral genomes. Cells were metabolically labeled with radioactive amino acids, and immunoprecipitation analysis of cell lysates with p24^{Gag}-specific antisera was performed. Stable transfectants containing HTLV$_{C\text{-}enh}$ΔTax produced levels of p24^{Gag} capsid protein similar to those produced by wtHTLV and HTLV$_{C\text{-}enh}$ transfectants (Fig. 7A). However, none of the 10 wtHTLVΔTax stable transfectants produced p24^{Gag} protein (data not shown). These results indicate that Tax is dispensable for the efficient production of viral proteins from HTLV$_{C\text{-}enh}$ΔTax, since transcription is stimulated exclu-

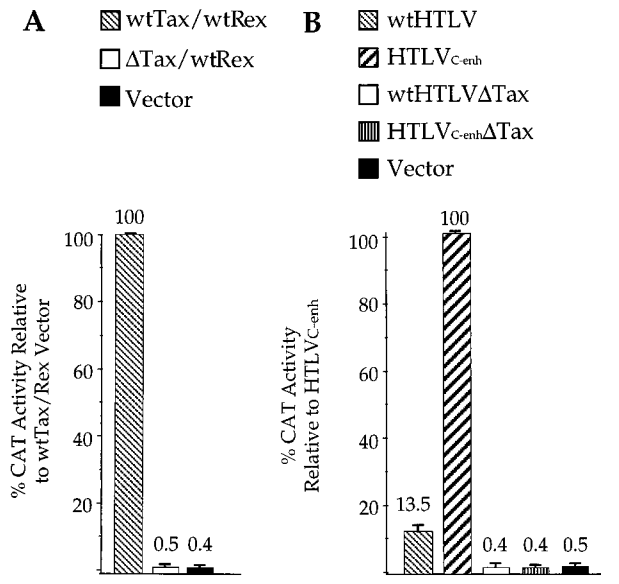

FIG. 5. ΔTax functional activity. (A) JM4 T cells were cotransfected with wtLTR-CAT and either wt*tax*/wt*rex* expression vector (BC20.2), Δ*tax*/wt*rex* expression vector (BC20.2-ΔTax), or vector alone. Numbers above the bars are percent chloramphenicol acetylation values averaged over three experiments and normalized to the value for wtTax/wtRex plus wtLTR-CAT (100%). (B) JM4 T cells were cotransfected with wtLTR-CAT and either the wtHTLV proviral clone, wtHTLVΔTax proviral clone, $HTLV_{C\text{-}enh}$ proviral clone, $HTLV_{C\text{-}enh}$ΔTax proviral clone, or vector (SV_2neo) alone. Number shown above the bars are percent chloramphenicol acetylation values averaged over three experiments and normalized to the value for $HTLV_{C\text{-}enh}$ plus wtLTR-CAT (100%).

sively by cellular transcription factors. In contrast, Tax is required for *trans* activation of the wtHTLV LTR, and therefore wtHTLVΔTax does not produce detectable HTLV proteins.

To demonstrate the capacity of $HTLV_{C\text{-}enh}$ΔTax to produce infectious progeny virions, the 729 stable transfectants were cocultured with the BJAB cell line. Cocultivation of 729-$HTLV_{C\text{-}enh}$ΔTax cells with BJAB cells resulted in syncytium formation, whereas no syncytia were detected in 729-wtHTLVΔTax/BJAB cocultures (Table 1). Syncytia could be induced with as few as 100 irradiated producer cells, and there was no apparent difference in the time course of syncytium induction by wtHTLV, $HTLV_{C\text{-}enh}$, or $HTLV_{C\text{-}enh}$ΔTax. Long-term syncytium-free BJAB cultures were expressing $HTLV_{C\text{-}enh}$ΔTax, as determined by $p24^{Gag}$ production (Fig. 7B). These results demonstrate that $HTLV_{C\text{-}enh}$ΔTax is replication competent and infectious for BJAB cells.

Tax is necessary for HTLV-2-mediated transformation of T lymphocytes. Experiments were performed next to determine whether Tax is required in HTLV-mediated transformation of human T lymphocytes. Irradiated 729 producer cells harboring either the wtHTLV, $HTLV_{C\text{-}enh}$, or $HTLV_{C\text{-}enh}$ΔTax proviral clone were cocultured with freshly isolated human PBL. A growth curve of a representative transformation assay indicated a progressive loss of viable cells over time in cocultures containing PBL and irradiated 729-$HTLV_{C\text{-}enh}$ΔTax producer cells (Fig. 8). In contrast, the transformation process was clearly apparent in PBL/729-wtHTLV and PBL/729-$HTLV_{C\text{-}enh}$ cocultures. The expression of HTLV-2 in these cells was confirmed by the detection of $p24^{Gag}$ capsid protein (Fig. 4). After 45 days of cocultivation, the responsiveness of the viable cell population to the addition of exogenous IL-2 was tested. After 3 days of IL-2 exposure, cells transformed by wtHTLV or $HTLV_{C\text{-}enh}$ had a rapid expansion in cell number, whereas there were no viable cells in PBL/729-$HTLV_{C\text{-}enh}$ΔTax cultures after 2 weeks of incubation with IL-2. Cells transformed by wtHTLV or $HTLV_{C\text{-}enh}$ have been maintained in culture in the absence of IL-2 for 140 days. Cells tested at 69 and 136 days still actively incorporate thymidine with a stimulation index 10- to 15-fold higher than that of matched irradiated controls (data not shown). We conclude from these results that Tax is essential for HTLV-mediated transformation of human primary T lymphocytes.

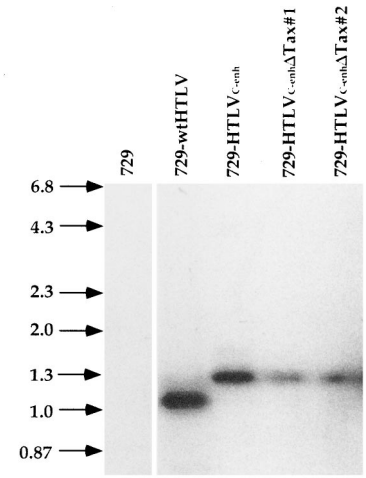

FIG. 6. Southern hybridization analysis of stable transfectant 729 B-cell DNA. Ten micrograms of high-molecular-weight cellular DNA was digested with *Cla*I and *Bam*HI (see Fig. 1 for site locations), electrophoresed through a 0.7% agarose gel, blotted onto nitrocellulose paper, and hybridized with a ^{32}P-labeled HTLV-2-specific PCR-generated 613-bp fragment (nt 7588 to 8200, based on the pH6neo HTLV-2 sequence). A 1.166-kb fragment is expected to be detected in 729-wtHTLV digested cell DNA, whereas a 1.376-kb fragment is expected in 729-$HTLV_{C\text{-}enh}$ and 729-$HTLV_{C\text{-}enh}$ΔTax digested cell DNA. Expected fragment sizes (indicated in kilobases on the left) are described in the legend to Fig. 3.

DISCUSSION

In this study, an infectious molecular clone of HTLV-2 was used to investigate the role of Tax in HTLV-mediated transformation of primary human T lymphocytes. The *tax* gene product clearly has oncogenic potential when tested in several assay systems. However, evidence that Tax is required for the transformation of primary human T lymphocytes within the context of HTLV infection has not been documented. Tax is critical for viral replication, which in turn is a prerequisite for the transformation of primary human T lymphocytes. Therefore, it has not been possible to mutate *tax* within the virus and dissociate viral replication from cellular transformation. We report the construction of a chimeric HTLV-2 that replicates by a Tax-independent mechanism. This chimeric HTLV-II ($HTLV_{C\text{-}enh}$) maintains the capacity to transform human T lymphocytes with an efficiency similar to that of wtHTLV-2. We constructed within the chimeric virus *tax*-knockout mutation that truncates the protein at amino acid 4, resulting in complete ablation of Tax function. Even in the absence of functional Tax, this *tax*-knockout virus ($HTLV_{C\text{-}enh}$ΔTax) remained replication competent and infectious but failed to transform primary human T lymphocytes. Taken together, the data lead us to conclude that the viral *tax* gene plays a necessary functional role in T-cell transformation mediated by HTLV-2.

Although animal model systems for studying HTLV patho-

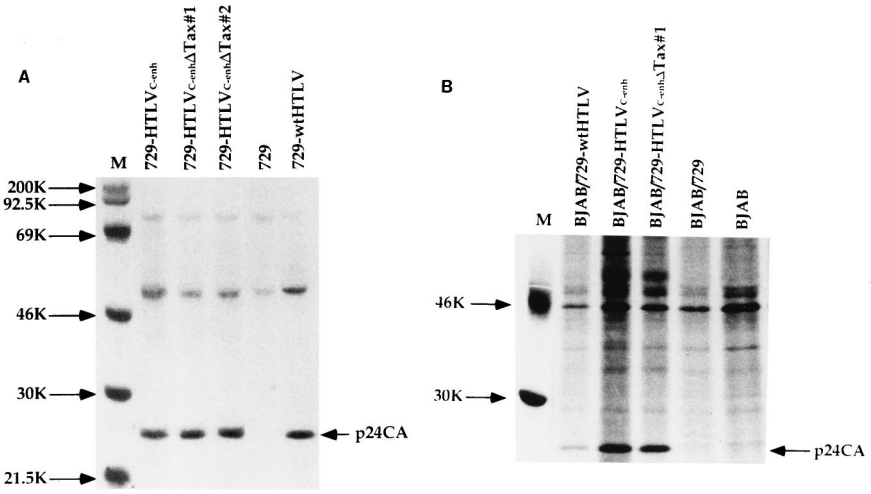

FIG. 7. Immunoprecipitation of [^{35}S]methionine-cysteine-labeled 729 producer cells and infected BJAB cells. (A) 729 producer cells (5×10^6) were metabolically labeled, and cell lysates were prepared. Stable transfectant cell lysates normalized by scintillation counting of trichloroacetic acid precipitates were immunoprecipitated with human antisera directed against the HTLV-2 p24^{Gag} capsid protein. Uninfected 729 cells and 729-wtHTLV cells are positive and negative controls, respectively. The sizes (in kilodaltons [K], indicated on the left) were determined by comparison with protein markers (Amersham) (lane M). The indicated 729 viral producer cells (5×10^5) were irradiated with 10,000 rad and cocultured with BJAB cells. Long-term chronically infected BJAB cultures (3 to 4 weeks postcoculture) were metabolically labeled and analyzed as for panel A. The sizes (in kilodaltons [K], indicated on the left) were determined by comparison with protein markers (Amersham) (lane M).

genesis are lacking, transformation of T lymphocytes in cell culture is a widely accepted experimental system for exploring the early events associated with viral pathogenesis (16, 29). Both HTLV-1 and HTLV-2 productively infect a limited number of human cell types, including lymphoid, fibroblast, and some epithelial cells. However, these oncogenic retroviruses exert their transforming effects exclusively on human T lymphocytes. Our transformation assays can be scored positive approximately 7 weeks following cocultivation of irradiated HTLV-2 producer cells with freshly isolated human PBL. These cultures proliferate independently of exogenous IL-2, but they are still IL-2 responsive, indicating the presence of T lymphocytes. wtHTLV and HTLV$_{C\text{-}enh}$ have the capacity to transform T cells with similar efficiencies, whereas HTLV$_{C\text{-}enh}$ΔTax was transformation defective. Cocultivation of PBL with HTLV$_{C\text{-}enh}$ΔTax producer cells showed a steady decrease in viable cell number over the cocultivation period. In fact, after 7 weeks of cocultivation, there was no proliferative response to exogenous IL-2. Although HTLV$_{C\text{-}enh}$ΔTax failed to transform T lymphocytes, it is interesting that cell viability decreased

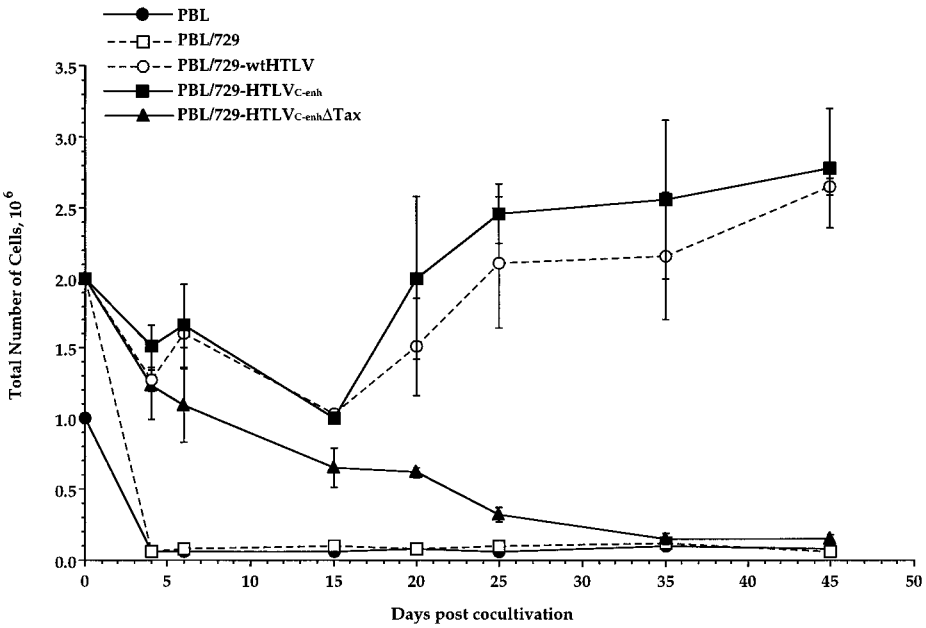

FIG. 8. Growth curve of HTLV-2 T-lymphocyte transformation assay. Human PBL were isolated by Ficoll-Paque and cocultivated with irradiated (10,000 rad) 729 producer cells (729-wtHTLV, 729-HTLV$_{C\text{-}enh}$, and 729-HTLV$_{C\text{-}enh}$ΔTax) or 729 uninfected control cells. PBL (10^6) were cocultured with irradiated donor cells (10^6) in 24-well plates. Cells were fed once per week with RPMI 1640 supplemented with 20% FCS. Cell viability was determined by trypan blue exclusion staining at 0, 6, 12, 15, 20, 25, 35, and 45 days postcocultivation. The mean and standard deviation of each time point was determined from three independent samples.

more slowly over time as than in uninfected control cocultures (Fig. 8). Previous studies have shown that the Env protein of HTLV, in conjunction with the CD2/LFA3 activation pathway, is mitogenic for T lymphocytes (18, 39, 43). The most likely explanation for the shift in the growth curve in this culture is that HTLV virions from virus producer cells and newly infected PBL are mitogenic for primary T cells. Without T-lymphocyte transformation, HTLV production declines, as a result of the death of irradiated 729 producer cells and the finite life span of infected lymphocytes, resulting in the eventual loss of viable cells.

Investigation of the in vivo cellular tropism of HTLV-1 and HTLV-2 has indicated that HTLV-1 is detected almost exclusively in $CD4^+$ T lymphocytes, whereas HTLV-2 has a preferential tropism for $CD8^+$ T lymphocytes (32, 41, 46). Our results are consistent with this observation, in that flow cytometric analysis determined that both wtHTLV and $HTLV_{C\text{-}enh}$ transformed primarily $CD8^+$ T cells. A selective infection of $CD8^+$ T cells by HTLV-2 could be a possible explanation for the low incidence of leukemia associated with this virus, since most HTLV-associated malignancies are of the $CD4^+$ T-cell phenotype and are primarily associated with HTLV-1 infection. HTLV-2-associated $CD8^+$ T-cell malignancies may be rare because of immune system interplay and tight regulation of $CD8^+$ T cells in vivo. It remains unclear which viral gene(s) is the determinant for differences in HTLV-2 and HTLV-1 tropism and pathogenesis.

The mechanism of HTLV-induced T-cell transformation remains unknown. Patients with HTLV-1 infection who develop adult T-cell leukemia do so after a 25- to 30-year latent period. HTLV viral load and Tax expression are low or absent in leukemic cells, suggesting that Tax is required to initiate transformation, but other secondary events are required to establish the malignant state. Our results demonstrate that the elimination of Tax from HTLV-2 results in the loss of transformation, suggesting that Tax is directly involved in the process. Tax stimulates the production of several cytokines involved in T-cell growth and differentiation, which has led to a cytokine/signal transduction model for HTLV transformation. *trans* activation of cellular genes by Tax is not clearly understood but appears to involve the CREB/ATF and Rel/NFκB family of transcription factors (1, 38, 65, 67). Deregulation of genes under the control of these host signalling pathways, such as those including IL-2, IL-2 receptor α, and granulocyte-macrophage colony-stimulating factor, may result in a constitutive autocrine or paracrine model of T-lymphocyte activation. Even though HTLV Env in conjunction with the CD2/LFA3 activation pathway is also mitogenic for T cells (18, 39, 43), this mitogenic property may be more important for retroviral integration since most retroviruses do not productively infect quiescent cells. Tax also represses expression of the polymerase β gene (37). This gene product is involved in DNA repair, suggesting a potential mechanism of Tax-induced genetic damage. Each of these different pathways, which is directly affected by Tax, may possibly converge, creating a unique set of circumstances required for T-lymphocyte transformation by HTLV.

The results obtained with the replication-competent $HTLV_{C\text{-}enh}\Delta$Tax do not eliminate the possibility that other viral gene products assist in the transformation process; rather, they suggest that without Tax, the initiation of HTLV-mediated transformation does not occur. Other genetic events are most likely necessary to lead to the full malignant state. Notwithstanding these uncertainties, the identification of a chimeric HTLV that replicates independently of Tax should facilitate studies addressing the precise mechanism by which Tax initiates the transformation process.

ACKNOWLEDGMENTS

We thank Alex Minella for technical assistance, Kyle Rybczyk and Katie Crumbo for venipuncture, and Chris Aiken, Dean Ballard, and Terry Dermody for critical comments.

This work was supported by grants from the National Institutes of Health (CA59581), Leukemia Society of America, and Mellon Foundation. P.L.G. is a scholar of the Leukemia Society of America.

REFERENCES

1. **Adya, N., L.-J. Zhao, W. Huang, I. Boros, and C.-Z. Giam.** 1994. Expression of CREB's DNA recognition specificity by Tax results from interaction with Ala-Ala-Arg at positions 282-284 near the conserved DNA-binding domain of CREB. Proc. Natl. Acad. Sci. USA **91:**5642–5646.
2. **Alexandre, C., P. Charnay, and B. Verrier.** 1991. Trans-activation of *Krox-20* and *Krox-24* promoters by the HTLV-I Tax protein through common regulatory elements. Oncogene **6:**1851–1857.
3. **Alexandre, C., and B. Verrier.** 1991. Four regulatory elements in the human c-fos promoter mediate transactivation by HTLV-I Tax protein. Oncogene **6:**543–551.
4. **Ballard, D. W., E. Böhnlein, J. W. Lowenthal, Y. Wano, B. R. Franza, and W. C. Greene.** 1988. HTLV-I *tax* induces cellular proteins that activate the κB element in the IL-2 receptor α gene. Science **241:**1652–1655.
5. **Bieberich, C. J., C. M. King, B. T. Tinkle, and G. Jay.** 1993. A transgenic model of transactivation by the Tax protein of HTLV-I. Virology **196:**309–318.
6. **Böhnlein, E., J. Lowenthal, M. Siekevitz, D. Ballard, B. R. Franza, and W. C. Greene.** 1988. The same inducible nuclear proteins regulate mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. Cell **53:**827–836.
7. **Böhnlein, E., M. Siekevitz, D. W. Ballard, J. W. Lowenthal, L. Rimsky, H. Bogerd, J. Hoffman, Y. Wano, B. R. Franza, and W. C. Greene.** 1989. Stimulation of the human immunodeficiency virus type 1 enhancer by the human T-cell leukemia virus type I *tax* gene product involves the action of inducible cellular proteins. J. Virol. **63:**1578–1586.
8. **Boshart, M., F. Weber, G. Jahn, H. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41:**521–530.
9. **Brady, J., K. T. Jeang, J. Durall, and G. Khoury.** 1987. Identification of the p40x-responsive regulatory sequences within the human T-cell leukemia virus type I long terminal repeat. J. Virol. **61:**2175–2181.
10. **Brockman, J. A., D. C. Scherer, T. A. McKinsey, S. M. Hall, X. Qi, W. Y. Lee, and D. W. Ballard.** 1995. Coupling of a signal response domain in IκBα to multiple pathway for NF-κB activation. Mol. Cell. Biol. **15:**2809–2818.
11. **Cann, A. J., Y. Koyanagi, and I. S. Y. Chen.** 1988. High efficiency transfection of primary human lymphocytes and studies of gene expression. Oncogene **3:**123–128.
12. **Cann, A. J., J. D. Rosenblatt, W. Wachsman, N. P. Shah, and I. S. Y. Chen.** 1985. Identification of the gene responsible for human T-cell leukemia virus transcriptional regulation. Nature (London) **318:**571–574.
13. **Chen, I. S. Y., A. J. Cann, J. P. Lugo, and J. D. Rosenblatt.** 1989. Models for mechanisms for transformation of the human T-cell leukemia viruses, p. 113–125. *In* M. E. Pullman, H. Hanafusa, and A. Printer (ed.), Retroviruses and disease. Academic Press Inc., New York.
14. **Chen, I. S. Y., A. J. Cann, N. P. Shah, and R. B. Gaynor.** 1985. Functional relationship of HTLV-II *x* and adenovirus E1A proteins in transcriptional activation. Science **230:**570–573.
15. **Chen, I. S. Y., J. McLaughlin, J. C. Gasson, S. C. Clark, and D. W. Golde.** 1983. Molecular characterization of genome of a novel human T-cell leukaemia virus. Nature (London) **305:**502–505.
16. **Chen, I. S. Y., S. G. Quan, and D. W. Golde.** 1983. Human T-cell leukemia virus type II transforms normal human lymphocytes. Proc. Natl. Acad. Sci. USA **80:**7006–7009.
17. **Cross, S. L., M. B. Feinberg, J. B. Wolf, N. J. Holbrook, F. Wong-Staal, and W. J. Leonard.** 1987. Regulation of the human interleukin-2 receptor alpha promoter: activation of a nonfunctional promoter by the transactivator gene of HTLV-I. Cell **49:**47–56.
18. **Duc Dodon, M., A. Bernard, and L. Gazzolo.** 1989. Peripheral T-lymphocyte activation by human T-cell leukemia virus type 1 interferes with the CD2 but not with the CD3/TCR pathway. J. Virol. **63:**5413–5419.
19. **Felber, B. K., H. Paskalis, C. Kleinman-Ewing, F. Wong-Staal, and G. N. Pavlakis.** 1985. The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. Science **229:**675–679.
20. **Fujii, M., T. Niki, T. Mori, T. Matsuda, M. Matsui, N. Nomura, and M. Seiki.** 1991. HTLV-I Tax induces expression of various immediate early serum responsive genes. Oncogene **6:**1023–1029.
21. **Fujii, M., P. Sassone-Corsi, and I. M. Verma.** 1988. c-*fos* promoter *trans*-activation by the *tax* I protein of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. USA **85:**8526–8530.
22. **Fujii, M., H. Tsuchiya, T. Chuhjo, T. Akizawa, and M. Seiki.** 1992. Interac-

tion of HTLV-1 Tax1 with p67SRF causes the aberrant induction of cellular immediate early genes through CArG boxes. Genes Dev. **6:**2066–2076.
23. **Fujisawa, J., M. Seiki, T. Kiyokawa, and M. Yoshida.** 1985. Functional activation of the long terminal repeat of human T-cell leukemia virus type I by a trans-acting factor. Proc. Natl. Acad. Sci. USA **82:**2277–2281.
24. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
25. **Grassmann, R., S. Berchtolds, I. Radant, M. Alt, B. Fleckenstein, J. G. Sodroski, W. A. Haseltine, and U. Ramstedt.** 1992. Role of the human T-cell leukemia virus type 1 X region proteins in immortalization of primary human lymphocytes in culture. J. Virol. **66:**4570–4575.
26. **Grassmann, R., C. Dengler, I. Muller-Fleckenstein, B. Fleckenstein, K. McGuire, M.-C. Dokhelar, J. G. Sodroski, and W. A. Haseltine.** 1989. Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a *Herpesvirus saimiri* vector. Proc. Natl. Acad. Sci. USA **86:**3351–3355.
27. **Green, J. E., G. Begley, D. K. Wagner, T. A. Waldmann, and G. Jay.** 1989. Transactivation of granulocyte-macrophage colony-stimulating factor and the interleukin-2 receptor in transgenic mice carrying the human T-cell leukemia virus type I *tax* gene. Mol. Cell. Biol. **9:**4731–4737.
28. **Green, P. L., D. A. Kaehler, L. M. Bennett, and R. Risser.** 1989. Multiple steps are required for the induction of tumors by Abelson murine leukemia virus. J. Virol. **63:**1989–1994.
29. **Green, P. L., T. M. Ross, I. S. Y. Chen, and S. Pettiford.** 1995. Human T-cell leukemia virus type II nucleotide sequences between *env* and the last exon of *tax/rex* are not required for viral replication of cellular transformation. J. Virol. **69:**387–394.
30. **Green, P. L., Y. Xie, and I. S. Y. Chen.** 1990. The internal methionine codons of the human T-cell leukemia virus type II *rex* gene are not required for $p24^{Rex}$ production or virus replication and transformation. J. Virol. **64:**4914–4921.
31. **Grossman, W. J., J. T. Kimata, F. H. Wong, M. Zutter, T. J. Ley, and L. Ratner.** 1995. Development of leukemia in mice transgenic for the *tax* gene of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. USA **92:**1057–1061.
32. **Ijichi, S., M. B. Ramundo, H. Takahashi, and W. W. Hall.** 1992. *In vivo* cellular tropism of human T cell leukemia virus type II (HTLV-II). J. Exp. Med. **176:**293–296.
33. **Inoue, J., M. Seiki, T. Taniguchi, S. Tsuru, and M. Yoshida.** 1986. Induction of interleukin-2 receptor gene by p40xI encoded by human T-cell leukemia virus type I. EMBO J. **5:**2883–2888.
34. **Jeang, K.-T., I. Boros, J. Brady, M. Radonovich, and G. Khoury.** 1988. Characterization of cellular factors that interact with the human T-cell leukemia virus type I p40x-responsive 21-base-pair sequence. J. Virol. **62:**4499–4509.
35. **Jeang, K.-T., R. Chiu, E. Santos, and S.-J. Kim.** 1991. Induction of the HTLV-I LTR by *jun* occurs through the Tax-responsive 21-bp elements. Virology **181:**218–227.
36. **Jeang, K.-T., P. R. Shank, and A. Kumar.** 1988. Transcriptional activation of homologous viral long terminal repeats by the human immunodeficiency virus type I or the human T-cell leukemia virus type I *tat* proteins occurs in the absence of *de novo* protein synthesis. Proc. Natl. Acad. Sci. USA **85:**8291–8295.
37. **Jeang, K. T., S. G. Widen, O. J. Semmes, and S. H. Wilson.** 1990. HTLV-I transactivator protein, Tax, is a *trans*-repressor of human β-polymerase gene. Science **247:**1082–1084.
38. **Kanno, T., K. Brown, G. Franzoso, and U. Siebenlist.** 1994. Kinetic analysis of human T-cell leukemia virus type I Tax-mediated activation of NF-κB. Mol. Cell. Biol. **14:**6443–6451.
39. **Kimata, J. T., T. J. Palker, and L. Ratner.** 1993. The mitogenic activity of human T-cell leukemia virus type I is T-cell associated and requires the CD2/LFA-3 activation pathway. J. Virol. **67:**3134–3141.
40. **Kimata, J. T., and L. Ratner.** 1991. Temporal regulation of viral and cellular gene expression during human T-lymphotropic virus type I-mediated lymphocyte immortalization. J. Virol. **65:**4398–4407.
41. **Lal, R. B., S. M. Owen, D. L. Rudolph, C. Dawson, and H. Prince.** 1995. In vivo cellular tropism of human T-lymphotrophic virus type II is not restricted to $CD8^+$ cells. Virology **210:**441–447.
42. **Leung, K., and G. J. Nabel.** 1988. HTLV-I transactivator induces interleukin-2 receptor expression through an NFκB-like factor. Nature (London) **333:**776–778.
43. **Maguer, V., H. Casse-Ripoll, L. Gazzolo, and M. Duc Dodon.** 1993. Human T-cell leukemia virus type I-induced proliferation of human immature $CD2^+$ $CD3^+$ thymocytes. J. Virol. **69:**5529–5537.
44. **Maruyama, M., H. Shibuya, H. Harada, M. Hatakeyama, M. Seiki, T. Fujita, J. Inoue, M. Yoshida, and T. Taniguchi.** 1987. Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-I-encoded p40xI and T3/Ti complex triggering. Cell **48:**343–350.
45. **McKinsey, T. A., J. A. Brockman, D. C. Scherer, S. W. Al-Murrani, P. L. Green, and D. W. Ballard.** 1996. Inactivation of IκBβ by human T-cell leukemia virus type 1 Tax: a potential mechanism for constitutive induction of NF-κB. Mol. Cell. Biol. **16:**2083–2090.
46. **Miyamoto, K., T. Kamiya, J. Minowada, N. Tomita, and K. Kitajima.** 1991. Transformation of $CD8^+$ T-cells producing a strong cytopathic effect on $CD4^+$ T-cells through syncytium formation by HTLV-II. Jpn. J. Cancer Res. **82:**1178–1183.
47. **Nagata, K., K. Ohtani, M. Nakamura, and K. Sugamura.** 1989. Activation of endogenous c-*fos* proto-oncogene expression by human T-cell leukemia virus type-I encoded p40x protein in human T-cell line, Jurkat. J. Virol. **63:**3220–3226.
48. **Nerenberg, M., S. H. Hinrichs, R. K. Reynolds, G. Khoury, and G. Jay.** 1987. The *tat* gene of human T-lymphotropic virus type I induces mesenchymal tumors in transgenic mice. Science **237:**1324–1329.
49. **Nerenberg, M. I., T. Minor, J. Price, D. E. Ernst, T. Shinohara, and H. Schwarz.** 1991. Transgenic thymocytes are refractory to transformation by human T-cell leukemia virus type I *tax* gene. J. Virol. **65:**3349–3353.
50. **Nimer, S. D.** 1991. Tax responsiveness of the GM-CSF promoter is mediated by mitogen-inducible sequences other that kappa B. New Biol. **3:**997–1004.
51. **Nimer, S. D., J. C. Gasson, K. Hu, I. Smalberg, I. S. Y. Chen, and J. D. Rosenblatt.** 1989. Activation of the GM-CSF promoter by HTLV-I and HTLV-II *tax* proteins. Oncogene **4:**671–676.
52. **Paskalis, H., B. K. Felber, and G. N. Pavlakis.** 1986. *Cis*-acting sequences responsible for the transcriptional activation of human T-cell leukemia virus type I constitute a conditional enhancer. Proc. Natl. Acad. Sci. USA **83:**6558–6562.
53. **Rosen, C. A., R. Park, J. G. Sodroski, and W. A. Haseltine.** 1987. Multiple sequence elements are required for regulation of human T-cell leukemia virus gene expression. Proc. Natl. Acad. Sci. USA **84:**4919–4923.
54. **Rosenblatt, J. D., A. J. Cann, D. J. Slamon, I. S. Smalberg, N. P. Shah, J. Fujii, W. Wachsman, and I. S. Y. Chen.** 1988. HTLV-II trans-activation is regulated by two overlapping nonstructural genes. Science **240:**916–919.
55. **Schneider, U., H. Schwenk, and G. Bornkamm.** 1977. Characterization of EBV genome-negative "null" and T cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkins lymphoma. Int. J. Cancer **19:**621–626.
56. **Seiki, M., J. Inoue, T. Takeda, and M. Yoshida.** 1986. Direct evidence that p40xI of human T-cell leukemia virus type I is a trans-acting transcriptional activator. EMBO J. **5:**561–565.
57. **Shimotohno, K., Y. Takahashi, N. Shimizu, T. Gojobori, I. S. Y. Chen, D. W. Golde, M. Miwa, and T. Sugimura.** 1985. Complete nucleotide sequence of an infectious clone of human T-cell leukemia virus type II: a new open reading frame for the protease gene. Proc. Natl. Acad. Sci. USA **82:**3101–3105.
58. **Siekevitz, M., F. B. Feinberg, N. Holbrook, F. Wong-Staal, and W. C. Greene.** 1987. Activation of interleukin 2 and interleukin 2 receptor (Tac) promoter expression by the trans-activator (tat) gene product of human T-cell leukemia virus, type I. Proc. Natl. Acad. Sci. USA **84:**5389–5393.
59. **Siekevitz, M., S. F. Josephs, M. Dukovich, N. Peffer, F. Wong-Staal, and W. C. Greene.** 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-I. Science **238:**1575–1578.
60. **Siomi, H., H. Shida, S. H. Nam, T. Nosaka, M. Maki, and M. Hatanaka.** 1988. Sequence requirements for nucleolar localization of human T-cell leukemia virus type I pX protein which regulates viral RNA processing. Cell **55:**197–209.
61. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
62. **Suzuki, T., J. I. Fujisawa, M. Toita, and M. Yoshida.** 1993. The transactivator tax of human T-cell leukemia virus type 1 (HTLV-I) interacts with cAMP-responsive element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-I. Proc. Natl. Acad. Sci. USA **90:**610–614.
63. **Suzuki, T., H. Hirai, J. I. Fujisawa, T. Fujita, and M. Yoshida.** 1993. A transactivator Tax of human T-cell leukemia virus type 1 binds to NF-kappa B p50 and serum response factor (SRF) and associates with enhancer DNAs of the NF-kappa B site and CArG box. Oncogene **93:**2391–2397.
64. **Tanaka, A., C. Takahashi, S. Yamaoka, T. Nosaka, M. Maki, and M. Hatanaka.** 1990. Oncogenic transformation by the *tax* gene of human T-cell leukemia virus type I in vitro. Proc. Natl. Acad. Sci. USA **87:**1071–1075.
65. **Wagner, S., and M. R. Green.** 1993. HTLV-I Tax protein stimulation of DNA binding of bZIP proteins by enhancing dimerization. Science **262:**395–399.
66. **Wano, Y., M. Feinberg, J. B. Hosking, H. Bogerd, and W. C. Greene.** 1988. Stable expression of the *tax* gene of type I human T-cell leukemia virus in human T cells activates specific cellular genes involved in growth. Proc. Natl. Acad. Sci. USA **85:**9733–9737.
67. **Yin, M.-J., E. J. Paulssen, J. S. Seeler, and R. B. Gaynor.** 1995. Protein domains involved in both in vivo and in vitro interactions between human T-cell leukemia virus type I Tax and CREB. J. Virol. **69:**3420–3432.
68. **Zhao, L.-J., and C.-Z. Giam.** 1992. Human T-cell lymphotrophic virus type I (HTLV-I) transcriptional activator, Tax, enhances CREB binding to the HTLV-I 21 base-pair repeats by protein-protein interactions. Proc. Natl. Acad. Sci. USA **89:**7070–7074.
69. **Zhao, L.-J., and C.-Z. Giam.** 1991. Interaction of the human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator Tax with cellular factors that bind specifically to the 21-base-pair repeats in the HTLV-I enhancer. Proc. Natl. Acad. Sci. USA **88:**11445–11449.