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Human T-Cell Leukemia Virus Type 2 Tax Mutants That Selectively Abrogate NFκB or CREB/ATF Activation Fail To Transform Primary Human T Cells

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Human T-cell leukemia virus (HTLV) Tax protein has been implicated in the HTLV oncogenic process, primarily due to its pleiotropic effects on cellular genes involved in growth regulation and cell cycle control. To date, several approaches attempting to correlate Tax activation of the CREB/activating transcription factor (ATF) or NFκB/Rel transcriptional activation pathway to cellular transformation have yielded conflicting results. In this study, we use a unique HTLV-2 provirus ($HTLV_{c\text{-}enh}$) that replicates by a Tax-independent mechanism to directly assess the role of Tax transactivation in HTLV-mediated T-lymphocyte transformation. A panel of well-characterized *tax-2* mutations is utilized to correlate the respective roles of the CREB/ATF or NFκB/Rel signaling pathway. Our results demonstrate that viruses expressing *tax-2* mutations that selectively abrogate NFκB/Rel or CREB/ATF activation display distinct phenotypes but ultimately fail to transform primary human T lymphocytes. One conclusion consistent with our results is that the activation of NFκB/Rel provides a critical proliferative signal early in the cellular transformation process, whereas CREB/ATF activation is required to promote the fully transformed state. However, complete understanding will require correlation of Tax domains important in cellular transformation to those Tax domains important in the modulation of gene transcription, cell cycle control, induction of DNA damage, and other undefined activities.

The human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) are oncogenic retroviruses associated with human T-cell malignancies and degenerative neurological disorders (reviewed in reference 18). HTLV-mediated cellular transformation and disease is a multistep process facilitated by the pleiotropic effects of the viral Tax protein. Tax is a transcriptional activator of the HTLV long terminal repeat (LTR) as well as many cellular promoters. Tax interacts with transcription factors and/or their regulatory components to activate or modulate several major transcription factor pathways, including the cyclic-AMP response element and activating transcription factor (ATF) binding (CREB/ATF) proteins (1, 6–9, 22, 54, 59, 62), NFκB/Rel (10, 24–26, 28, 29, 31, 33, 34, 61), and serum response factor (15, 53). Tax activation of the CREB/ATF pathway is critical for efficient viral gene expression and replication (2, 4, 6, 56, 60, 64).

Tax displays oncogenic potential in several experimental systems and recently has been shown to be essential for HTLV-2-mediated cellular transformation of human T lymphocytes (46). The precise mechanism by which Tax initiates the malignant process is unclear but likely involves several points of transcriptional and posttranscriptional disregulation in the infected T lymphocyte. To this end, Tax has been shown to activate a number of cellular genes involved in the regulation of cell proliferation, including interleukin-2 (IL-2), IL-3, IL-2 receptor, proliferating cell nuclear antigen, c-*fos*, c-*sis*, G-CSF, and GM-CSF (5, 13, 14, 21, 27, 39, 40, 42, 55, 57). Furthermore, Tax interferes with cell cycle control by altering the activity of p53, the mitotic checkpoint regulator MAD1, cyclin D, cyclin-dependent kinase 4 (cdk4) and cdk6, and the cdk inhibitor $p16^{INK4A}$ (23, 30, 36, 38, 41, 47, 52). Complete understanding of the mechanism of T-lymphocyte transformation will require correlation of Tax domains important in modulating gene transcription, cell cycle control, and induction of DNA damage with those required for cellular transformation.

A number of mutants in both HTLV-1 Tax (Tax-1) and HTLV-2 Tax (Tax-2) have been described that selectively abrogate the ability of Tax to activate transcription through the CREB/ATF or NFκB/Rel signaling pathway (45, 48, 49, 58). The major functional regions or domains of Tax important for transactivation by the CREB/ATF or NFκB/Rel signaling pathway are similar but not identical in Tax-1 and Tax-2 (45). Several approaches have been used to evaluate the domains of Tax-1 used to immortalize or transform rodent cell lines or primary human T lymphocytes. These analyses have yielded conflicting results as to whether Tax-1 activation of the CREB/ATF or NFκB/Rel pathway correlates with cellular transformation (3, 20, 43, 44, 51). The transforming domains of Tax-2 have yet to be defined, and their elucidation may provide insight into the differential pathogenesis exhibited by HTLV-1 and HTLV-2.

We recently reported the generation and characterization of a chimeric HTLV-2 that replicates by a Tax-independent mechanism (46). The Tax response element (TRE) in the U3 region was replaced with the enhancer (c-enh) from the cytomegalovirus (CMV) immediate-early promoter. Transcription of the chimeric HTLV-2 ($HTLV_{c\text{-}enh}$) was efficiently directed by this heterologous promoter-enhancer. Also, this unique virus transformed primary human T lymphocytes with an effi-

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ciency similar to that of wild-type HTLV-2 (wtHTLV-2) (46). The functional $HTLV_{c\text{-}enh}$ provides the first opportunity to perform a *tax* mutational analysis in an infectious virus without compromising the ability of the mutant viruses to efficiently replicate. In this study, we examined the effects of select Tax-2 mutants on the ability of HTLV-2 to transform human T lymphocytes. In our study, transformation is defined as continuous growth in culture in the absence of exogenous IL-2. Our results indicate that viruses expressing *tax* mutants that selectively abrogate NFκB/Rel or CREB/ATF fail to induce IL-2-independent T-lymphocyte transformation. Our results suggest that activation of NFκB/Rel provides a critical proliferative signal early in the cellular transformation process, whereas CREB/ATF activation promotes sustained cell growth and IL-2-independent cellular transformation.

MATERIALS AND METHODS

Cells. The 729-6 B-cell line (hereafter called 729) and the human leukemic T-cell line JM4 (55) were maintained in Iscove's medium (Mediatech Inc.) supplemented with 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 the blood of healthy donors by centrifugation over Ficoll-Paque (Pharmacia). Cells were maintained in culture in RPMI 1640 medium supplemented with 20% FCS and antibiotics.

Plasmids. The wt LTR-2-chloramphenicol acetyltransferase (CAT) (wtLTR-CAT) reporter construct and the control vector SV2neo have been described elsewhere (12, 19). The $HTLV_{c\text{-}enh}$ proviral clone contains the enhancer (c-enh) from the CMV immediate-early promoter in place of the TRE in both the 5′ and 3′ LTRs and has been previously described (46). Previously characterized *tax-2* mutations (45) are designated by the wt amino acid single-letter code, the position in the Tax protein, followed by the mutated amino acid single-letter code or Term (termination or stop). These mutations were cloned into the $HTLV_{c\text{-}enh}$ proviral clone to generate $HTLV_{c\text{-}enh}$F4Term (ΔTax), $HTLV_{c\text{-}enh}$H3N, $HTLV_{c\text{-}enh}$C29S, $HTLV_{c\text{-}enh}$T113A, $HTLV_{c\text{-}enh}$S130A/L131F, $HTLV_{c\text{-}enh}$Y290Term, and $HTLV_{c\text{-}enh}$I319R/L320S.

Transfections and CAT assay. Plasmid DNA was introduced into cells by electroporation as previously described (11). Briefly, cells were washed with phosphate-buffered saline and resuspended (2×10^7 cells/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-mF charge; 250-V potential). Cells were transferred to 3 ml of medium and grown at 37°C for 48 h. Permanently transfected cells (stable transfectants) containing the wt or CMV enhancer-containing proviral clones were isolated following incubation in 24-well culture dishes (5×10^5 cell/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. Permanently transfected cells are designated "729," followed by the clone with which they were transfected.

Transfections for CAT assays included 5 μg of wtLTR-CAT, 15 μg of the proviral DNA clone, and 5 μg of pCMVβGal expression vector. Forty-eight hours posttransfection, cells were harvested and enumerated, and 10^6 cells were subjected to a β-galactosidase (β-Gal) colorimetric assay to normalize for transfection efficiency. Briefly, cells were lysed by sonication in 60 μl of 0.25 mM Tris (pH 7.8) and centrifuged for 15 min at 4°C; 30 μl of extract was incubated for 1 to 5 h at room temperature in 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, 66 mM $NaHPO_4$-Na_2PO_4, and 0.9 mg of *o*-nitrophenyl-β-D-galactopyranoside per ml. The reaction was terminated by the addition of Na_2CO_3, and the absorbance was quantitated at 410 nm. Extracts were made from the remainder of the cells and lysates, were normalized for transfection efficiency, and were subjected to CAT assays as described elsewhere (17). Percentages of ^{14}[C]chloramphenicol acetylation were quantified by the Molecular Dynamics Imaging System.

Metabolic labeling and immunoprecipitation. Permanently transfected 729 cell lines were metabolically labeled with [^{35}S]methionine cysteine (Trans-^{35}S-label, 100 mCi/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 $100{,}000 \times g$ (1 h at 4°C). Clarified extracts were immunoprecipitated with HTLV-2 patient antiserum containing antibody against p24 Gag in the presence of protein A-Sepharose (Pharmacia). Immunoreactive proteins were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Syncytium and transformation assays. Syncytium and transformation assays were performed as previously described (19). Briefly, 729 producer cells (5×10^5) were irradiated with 10,000 rads and then cocultivated either with 10^5 BJAB cells or 2×10^6 PBL (isolated from the blood of healthy donors by centrifugation over Ficoll-Paque) in 24-well culture plates. Syncytia were scored in BJAB cocultures 5 to 7 days postplating. Transformed T cells, defined as continuous growth in the absence of IL-2, grew out of PBL cocultures at 7 to 8 weeks postplating. In both cases, the presence of HTLV-2 expression was confirmed by detection of structural Gag protein in the culture supernatant by p19 Gag enzyme-linked immunosorbent assay (ELISA) (Cellular Products, Buffalo, N.Y.) with a detection sensitivity of 25 pg/ml.

RESULTS

Establishment of $HTLV_{c\text{-}enh}$ provirus-expressing cells with distinct Tax transactivation phenotypes. The chimeric proviral clone, termed $HTLV_{c\text{-}enh}$, contains the CMV enhancer in place of the TRE in both LTRs. Following introduction into human lymphoid cells, this clone produces infectious virus particles that replicate by a Tax-independent mechanism and are capable of infecting and transforming primary human T lymphocytes with an efficiency similar to wt HTLV-2 (46). This unique reagent allowed us to directly determine that Tax is essential for HTLV-2-mediated transformation of primary human T cells (46). We have recently generated and characterized a panel of Tax-2 mutants identifying regions within the 331-amino-acid protein important for activation of promoters through CREB/ATF or NFκB/Rel signaling (45). One of the next critical steps in understanding HTLV pathogenesis as well as providing insight into the origin of other T-cell leukemias and lymphomas is to correlate Tax activation of the CREB/ATF and NFκB/Rel signaling pathways with HTLV-mediated cellular transformation. Therefore, our *tax-2* point mutants, which display distinct transactivation phenotypes, were inserted into the functional $HTLV_{c\text{-}enh}$ proviral clone. The Tax transactivation phenotypes of the panel of Tax mutant proviral clones are expected to comprise four distinct groups. These include (i) activation of both CREB/ATF and NFκB/Rel (wt Tax and mutant H3N), (ii) activation of NFκB/Rel only (mutant C29S in the putative zinc binding domain, truncation mutant Y290Term, and mutant I319R/L320S, a point mutant similar to Tax-1 M47 [49]), (iii) activation of CREB/ATF only (mutant S130A/L131F, a point mutant similar to Tax-1 M22 [49]), and (iv) activation of neither CREB/ATF nor NFκB/Rel (truncation mutant F4Term and mutant T113A). We first confirmed the expected transactivation profiles of the *tax-2* mutants with respect to CREB/ATF and NFκB/Rel activation when expressed from the $HTLV_{c\text{-}enh}$ proviral clone. The DNA proviral clones were cotransfected with the CREB/ATF-dependent reporter plasmid, LTR-2-CAT, or the NFκB/Rel-dependent reporter plasmid, HIV-κB-CAT, into human JM4 T cells, and CAT activity was quantified. The results summarized in Table 1 confirm that the Tax mutants have the expected transactivation activity (45) when expressed from the chimeric $HTLV_{c\text{-}enh}$ proviral clone.

Our next goal was to determine the capacity of $HTLV_{c\text{-}enh}$ proviral clones expressing Tax mutants to synthesize viral proteins and direct viral replication. To this end, permanent 729 B-cell transfectants expressing wt Tax and mutant Tax $HTLV_{c\text{-}enh}$ proviral clones were isolated and further characterized. To monitor the production of viral proteins in these transfectants, cells were metabolically labeled, and lysates were subjected to immunoprecipitation (anti-p24Gag) and SDS-polyacrylamide gel electrophoresis analysis. Each of the transfectants chosen for this study produced similar levels of p24 Gag capsid protein (Fig. 1). Although each of the *tax* gene mutations was designed to maintain the integrity of the overlapping *rex* gene reading frame, the efficient expression of Gag protein confirms that Rex is fully functional. In addition, each transfectant was

TABLE 1. Phenotypic analysis of $HTLV_{c\text{-}enh}$ *tax* mutant clones[a]

Proviral plasmid	% Chloroamphenicol acetylation by reporter plasmid		
	pU3R-1-CAT (HTLV-1 LTR)	LTR-2-CAT (HTLV-2 LTR)	HIV-κB-CAT (HIV-κB-TATA)
Vector	<5	<5	<5
$HTLV_{c\text{-}enh}$(wtTax)	100	100	100
$HTLV_{c\text{-}enh}$H3N	92	75	105
$HTLV_{c\text{-}enh}$C29S	<5	<5	73
$HTLV_{c\text{-}enh}$T113A	<5	<5	<5
$HTLV_{c\text{-}enh}$S130A/L131F	59	79	<5
$HTLV_{c\text{-}enh}$F4Term(ΔTax)	<5	<5	<5
$HTLV_{c\text{-}enh}$Y290Term	<5	<5	57
$HTLV_{c\text{-}enh}$I319R/L320S	<5	<5	121

[a] JM4 T cells were cotransfected by electroporation with 5 μg of the indicated CAT reporter plasmid (pU3R-1-CAT, LTR-2-CAT, or HIV-κB-CAT), 5 μg pCMVβGal, and 15 μg of the indicated $HTLV_{c\text{-}enh}$ proviral plasmid encoding wt or mutant forms of Tax-2 or SV2neo vector control. After 48 h of growth, cells were harvested, normalized for transfection efficiency (β-Gal activity), and assayed for CAT activity. The numbers represent percent chloramphenicol acetylation values averaged over three independent experiments and normalized to the value for $HTLV_{c\text{-}enh}$(wtTax) (100%).

shown to contain full-length proviral genomes as assessed by PCR and Southern blotting (data not shown).

To assess whether the various transfectants continued to express Tax with the expected transactivation phenotype, cell clones were transfected with LTR-2-CAT or HIV-κB-CAT, and CAT activity was measured. The results, summarized in Table 2, indicate that the proviruses continue to display the expected Tax mutant transactivation phenotypes.

TABLE 2. Phenotype of virus expressed from permanent transfectants

Cell line	% Chloroamphenicol acetylation[a]		Syncytium induction in BJAB cells[c]
	LTR-2-CAT (HTLV-2 LTR)	HIV-κB-CAT (HIV-κB-TATA)	
729 (uninfected)	<5	<5	−
729-wtHTLV	ND[b]	ND[b]	+(100)
729-$HTLV_{c\text{-}enh}$(wtTax)	100	100	+(100)
729-$HTLV_{c\text{-}enh}$H3N	80	102	+(100)
729-$HTLV_{c\text{-}enh}$F4Term(ΔTax)	<5	<5	+(100)
729-$HTLV_{c\text{-}enh}$C29S	<5	79	+(100)
729-$HTLV_{c\text{-}enh}$T113A	<5	<5	+(100)
729-$HTLV_{c\text{-}enh}$S130A/L131F	81	<5	+(100)
729-$HTLV_{c\text{-}enh}$Y290Term	<5	56	+(100)
729-$HTLV_{c\text{-}enh}$I319R/L320S	<5	125	+(100)

[a] Permanently transfected 729 cells were cotransfected by electroporation with 5 μg of the indicated CAT reporter plasmid (LTR-2-CAT or HIV-κB-CAT) and 5 μg of pCMVβGal. After 48 h of growth, cells were harvested, lysates were made and normalized for transfection efficiency (β-Gal activity), and cells were assayed for CAT activity. The numbers represent percent chloramphenicol acetylation values averaged over three independent experiments and normalized to the value for 729-$HTLV_{c\text{-}enh}$(wtTax) (100%).

[b] ND, not determined.

[c] Permanently transfected 729 cells were irradiated with 10,000 rads, and 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. Cultures were scored positive (+) or negative (−) for syncytia at 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.

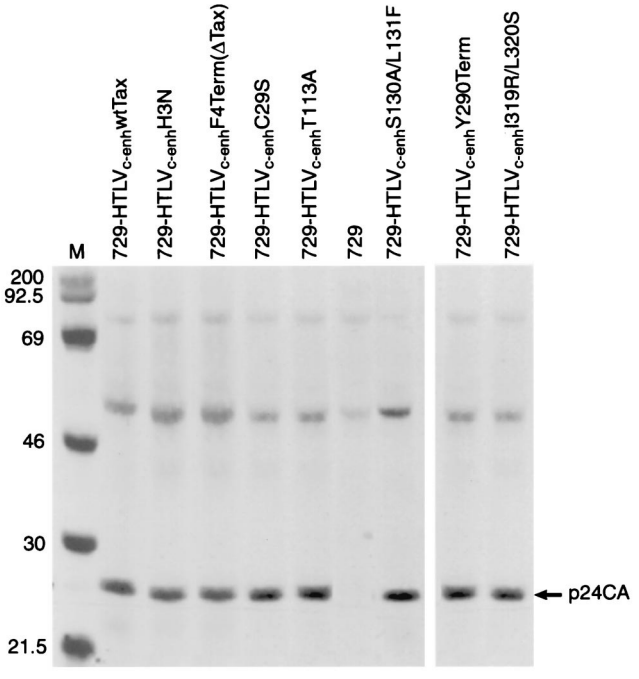

FIG. 1. Immunoprecipitation of [^{35}S]methionine-cysteine-labeled 729-$HTLV_{c\text{-}enh}$ producer cells. 729 cells (uninfected B-cell line) and 729 viral producer cell lines expressing viruses with various Tax mutants, as indicated, were metabolically labeled and cell lysates were prepared. Transfectant cell lysates were normalized by scintillation counting of trichloroacetic acid precipitates, and equivalent amounts were immunoprecipitated with human antiserum directed against the HTLV-2 p24 Gag capsid protein (CA). The sizes (in kilodaltons, indicated on the left) were determined by comparison to protein markers (Amersham) (lane M). Regardless of the Tax transactivation phenotype, each producer cell line expresses similar levels of p24 Gag capsid.

Infection and replication by $HTLV_{c\text{-}enh}$-expressing Tax mutants. To evaluate the capacity of the chimeric mutant viruses to produce infectious progeny virions, the transfectants were irradiated and cocultured with the human B-cell line BJAB. Productive infection of BJAB cells by HTLV-2 results in rapid induction of syncytia with cytopathicity (19, 46). Figure 2 shows the results of a representative assay comparing syncytium formation between irradiated uninfected 729 cells or irradiated 729-$HTLV_{c\text{-}enh}$F4Term(ΔTax) cells upon coculture with BJAB cells. Cocultivation of all 729-$HTLV_{c\text{-}enh}$ *tax* mutant cell clones with BJAB cells resulted in syncytium formation (Table 2). To

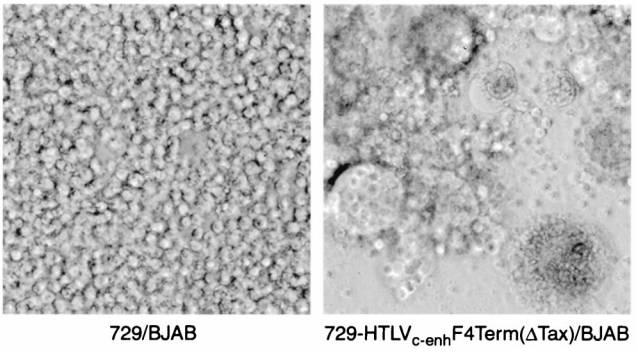

FIG. 2. A representative $HTLV_{c\text{-}enh}$ syncytium induction assay in BJAB cells. A total of 5×10^5 uninfected 729 cells or a representative stable transfectant, 729-$HTLV_{c\text{-}enh}$F4Term(ΔTax), were irradiated with 10,000 rads and cocultured with 10^5 BJAB cells. Syncytia were scored in BJAB cell cocultures microscopically 3 to 5 days postplating. Cells were photographed 3 days postplating.

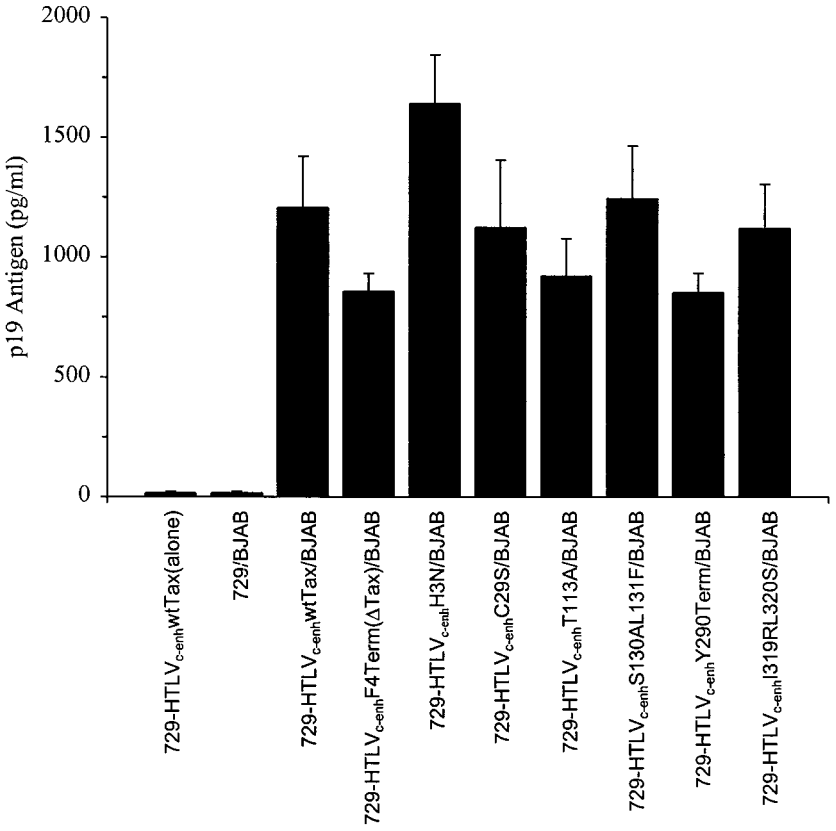

FIG. 3. p19 Gag expression in BJAB cells. Permanently transfected 729 cells or mock 729 cells were irradiated with 10,000 rads, and 100 cells were cocultivated with 5×10^5 BJAB cells in 24-well culture plates. Syncytia were microscopically visible in BJAB cocultures 5 days postplating. At day 7, viral particle production was estimated in the culture supernatants by p19 Gag antigen ELISA. The error bars indicate the standard deviation from three replicate wells. 729-HTLV$_{c\text{-}enh}$wtTax(alone), day 7 supernatant from 100 irradiated producer cells alone or without BJAB cells (the amount of p19 Gag detected in this sample was <25 pg/ml).

address the efficiency at which the viruses replicate and induce syncytia, 10-fold serial dilutions of irradiated producer cells were cocultured with BJAB cells. Syncytia were induced with as few as 100 irradiated producer cells (Table 2), and there was no apparent difference in the time course of syncytium induction by either the *tax* wild-type or *tax* mutant HTLV$_{c\text{-}enh}$ (data not shown). BJAB cells cocultured with 100 irradiated producer cells expressing the HTLV$_{c\text{-}enh}$ Tax mutants exhibit similar levels of Gag protein in the culture supernatant, as assessed by p19 Gag ELISA at 7 days postcoculture (Fig. 3). Taken together, these results demonstrate that HTLV$_{c\text{-}enh}$ replicates and spreads with similar efficiency regardless of *tax* or mutant *tax* genes.

Transformation of human T cells by HTLV$_{c\text{-}enh}$-expressing Tax mutants. Experiments were next performed to determine which Tax mutant phenotypes are required for HTLV-2-mediated transformation of primary human T lymphocytes. A typical transformation assay included irradiated 729 producer cells harboring either wtHTLV-2, HTLV$_{c\text{-}enh}$wtTax, or HTLV$_{c\text{-}enh}$mutantTax-2 proviral clones and freshly isolated human PBL in 24-well plates. In an attempt to provide an environment similar to that which occurs in vivo, the PBL were not previously activated with phytohemagglutinin and IL-2, nor did the media contain exogenous IL-2. Cell number and viability were monitored at approximately weekly intervals in order to follow cellular proliferation as a result of viral infection. Viral replication was confirmed by p19 Gag ELISA of culture supernatant at 3 weeks postcocultivation, a time point at which HTLV productively infected PBL would be expected to produce viral particles (as measured by p19 Gag), whereas particle production from residual irradiated viral producer cells would be expected to be low (Fig. 4). These results indicate that each virus, regardless of the Tax transactivation phenotype, is capable of productively infecting PBL. Therefore, Tax is not necessary for HTLV$_{c\text{-}enh}$ infection of PBL.

Multiple transformation assays were performed and established four distinct growth patterns, and a representative assay is presented in Fig. 5. Pattern 1 is represented by the negative control and presents the growth of PBL cocultured with irradiated uninfected 729 cells (Mock). A rapid decrease in viable cells was observed, and no viable cultures are produced. Pattern 2 is represented by PBL/729-wtHTLV-2, PBL/729-HTLV$_{c\text{-}enh}$wtTax, and PBL/729-HTLV$_{c\text{-}enh}$H3N cocultures, in which the characteristic transformation process is observed. Initially, there was a slight decrease in cell number, followed by a rapid expansion of cells for the duration of the assay. Flow cytometric analysis determined that these cells were primarily CD8$^+$ T cells (46 and data not shown). The expression of HTLV-2 was confirmed by the detection of p24Gag capsid protein in HTLV$_{c\text{-}enh}$wtTax-transformed cells (46) and HTLV$_{c\text{-}enh}$H3N mutant-transformed cells (data not shown). IL-2 was not added to the culture media, indicating that viability and growth of transformed cells is not dependent on exogenous IL-2. However, transformed cells did respond to exogenous IL-2 and the capacity to efficiently establish viable

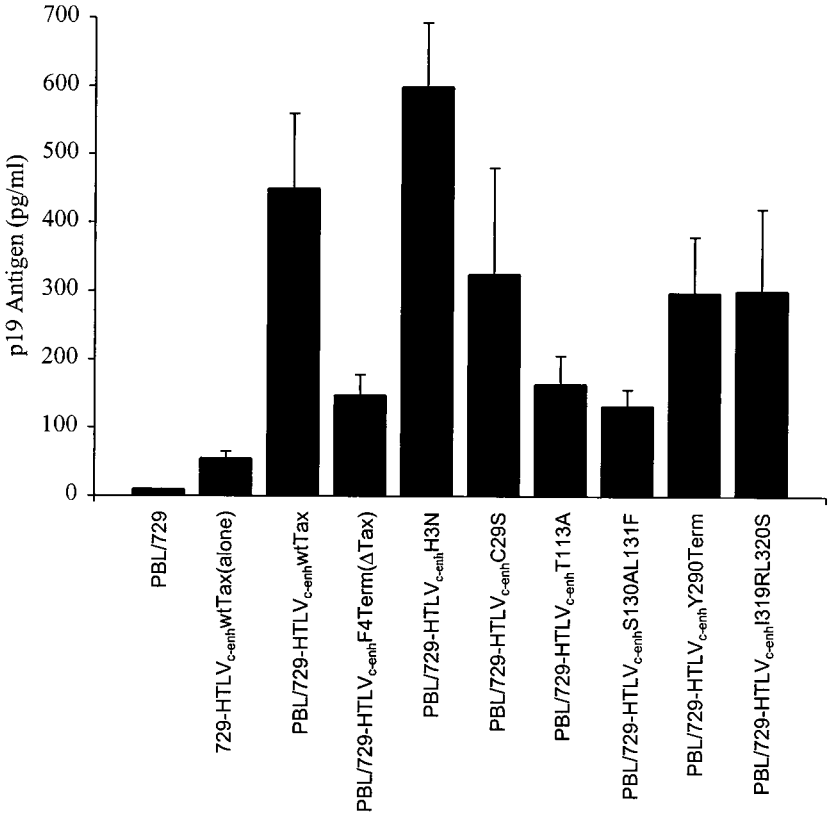

FIG. 4. p19 Gag expression in infected PBL. Cell supernatants were obtained at day 21, as described in the legend to Fig. 5. Viral particle production was estimated in the culture supernatant by p19 Gag antigen ELISA. The error bars indicate the standard deviation from three replicate wells. To control for particle production from 729 producer cells (background), culture supernatant from 10^6 irradiated cells, designated 729-HTLV$_{c\text{-}enh}$wtTax cells(alone), was measured after 3 weeks in culture.

IL-2-independent T-cell lines could be enhanced by providing IL-2 at 5 to 6 weeks following coculture (data not shown).

Pattern 3 is distinct and slightly shifted from the negative control and is represented by PBL/729-HTLV$_{c\text{-}enh}$F4Term (ΔTax), PBL/729-HTLV$_{c\text{-}enh}$S130A/L131F, and PBL/729-HTLV$_{c\text{-}enh}$T113A cocultures. A progressive loss of viable cells over time was seen, and by 36 days postcoculture, no viable cells remained. The slight positive shift of this curve compared to the negative control is attributed to the presence of replicating virus (Fig. 4); HTLV particles have been shown to be mitogenic for primary T cells (16, 63). The observation that HTLV$_{c\text{-}enh}$S130A/L131F fails to transform cells and displays a growth-inducing phenotype similar to HTLV$_{c\text{-}enh}$F4Term (ΔTax), which makes no Tax, suggests that Tax activation of the NFκB/Rel signaling pathway correlates with an initial phase of the cellular transformation process.

Pattern 4 is represented by PBL/729-HTLV$_{c\text{-}enh}$C29S, PBL/729-HTLV$_{c\text{-}enh}$Y290Term, and PBL/729-HTLV$_{c\text{-}enh}$I319R/L320S cocultures; each virus encodes a distinct Tax mutant that maintains NFκB/Rel activation but is abrogated in CREB/ATF activation. Within the first 2 weeks postcoculture, a decline in cell number is observed that is similar to that in wtHTLV-2, HTLV$_{c\text{-}enh}$wtTax, or HTLV$_{c\text{-}enh}$H3N infection. Although these cells remain viable for up to 2 months postcoculture, they never enter an expansion phase, and at approximately 70 days postcoculture no viable cells remain. Consistent with the phenotype of wtHTLV-2-infected cells, flow cytometric analysis at 50 days postcoculture determined that these cells were primarily $CD8^+$ T-cells (data not shown). The simplest interpretation is that sustained viability of the cells is attributed to Tax activation of NFκB/Rel and is consistent with the reciprocal failure of HTLV$_{c\text{-}enh}$S130A/L131F to induce cell proliferation. Addition of IL-2 to the cultures at day 50 failed to result in immortalization or sustain cell proliferation (data not shown). These in vitro transformation assays of the wt and seven *tax* mutant HTLV$_{c\text{-}enh}$ viruses are consistent with the conclusion that Tax transactivation of NFκB/Rel correlates with cell proliferation and the initiation of the transformation process and that CREB/ATF signaling pathway promotes sustained cell growth and IL-2-independent cellular transformation.

DISCUSSION

In this study, seven *tax* mutants were analyzed in the context of a replication-competent HTLV provirus. The ultimate goal was to directly correlate the role of Tax activation of the CREB/ATF or NFκB/Rel signaling pathway with HTLV-mediated T-lymphocyte transformation. This is the first investigation into the role of Tax-2 activation of NFκB/Rel and CREB/ATF pathways in the transformation process. Our approach makes use of a well-characterized HTLV-2 (HTLV$_{c\text{-}enh}$) provirus that replicates by a Tax-independent mechanism. This unique virus eliminates the variation that independent *tax* mutations may have on viral transcription and replication efficiency. Our results indicate that Tax-2 mutant viruses that selectively abrogate NFκB/Rel or CREB/ATF fail to induce long-term growth of IL-2-independent T lymphocytes. Our results with HTLV$_{c\text{-}enh}$S130A/L131F are consistent with the hypothesis that Tax activation of NFκB/Rel provides an early

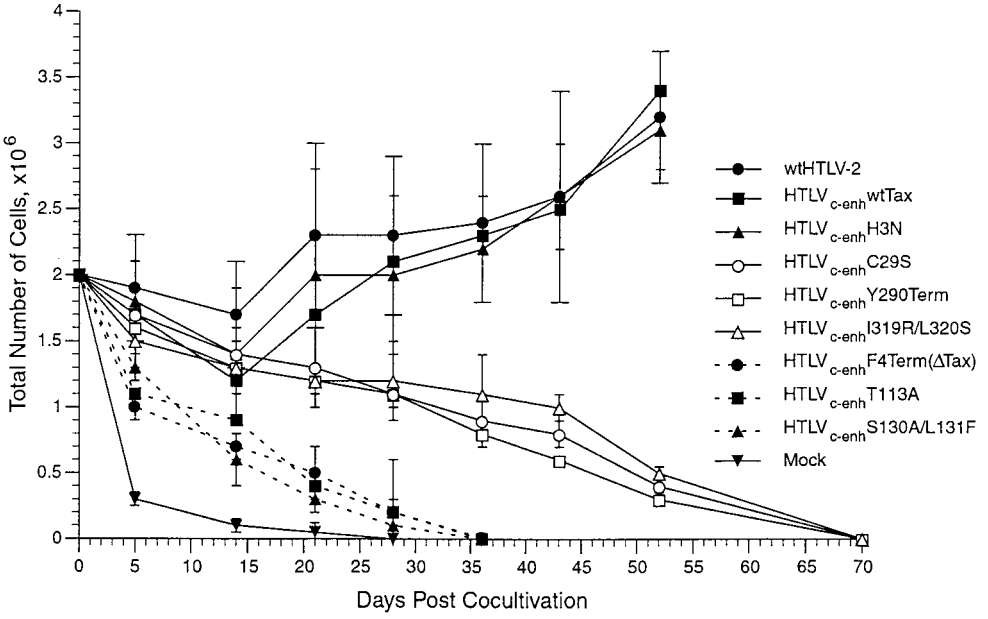

FIG. 5. Growth curve of $HTLV_{c\text{-}enh}$ T-lymphocyte transformation assay. Human PBL were isolated by Ficoll-Paque and cocultivated with irradiated (10,000 rads) 729 virus producer cells (described in Table 2) or 729 uninfected control cells. PBL (2×10^6) were cocultured with irradiated donor cells (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, 5, 14, 21, 28, 36, 43, 53, and 70 days postcocultivation. Four distinct growth patterns were observed. The mean and standard deviation was determined from three independent samples of each coculture: pattern 1 contains PBL/729 negative control (Mock) coculture; pattern 2 contains PBL/729-$HTLV_{c\text{-}enh}$F4Term(ΔTax), PBL/729-$HTLV_{c\text{-}enh}$T113A, and PBL/729-$HTLV_{c\text{-}enh}$S130A/L131F cocultures showing the critical importance of NFκB/Rel activation; pattern 3 contains PBL/729-$HTLV_{c\text{-}enh}$C29S, PBL/729-$HTLV_{c\text{-}enh}$Y290Term, and PBL/729-$HTLV_{c\text{-}enh}$I320R/L320S cocultures showing the requirement for activation of CREB/ATF in addition to NFκB/Rel; and pattern 4 contains PBL/729-wtHTLV-2, PBL/729-$HTLV_{c\text{-}enh}$wtTax, and PBL/729-$HTLV_{c\text{-}enh}$H3N or fully transformed cells.

proliferative signal to HTLV-infected cells that is absolutely critical for the initiation of cell growth and the transformation process. One can equate this early step with maintaining cell growth and viability in culture by activating cellular growth genes, including IL-2 and IL-2 receptor genes. This hypothesis is supported further by the growth of primary cells infected with $HTLV_{c\text{-}enh}$C29S, $HTLV_{c\text{-}enh}$Y290Term, and $HTLV_{c\text{-}enh}$ I319R/L320S. These viruses, which encode a mutant Tax protein that activates NFκB/Rel but not CREB/ATF, facilitate cell viability for up to 2 months. However, the capacity of Tax to activate CREB/ATF is also critical to the transformation process, since PBL infected with these $HTLV_{c\text{-}enh}$ Tax mutants fail to exhibit the transformation profile observed with wt Tax-expressing virus. Although activation of both the NFκB/Rel and CREB/ATF pathways are implicated in the in vitro transformation process, we cannot rule out the possible contribution of other mechanisms critical for Tax transformation.

The role of CREB/ATF or NFκB/Rel pathways in Tax-1-mediated transformation has been previously studied using several different experimental systems, and the results have been controversial. One study indicated that Tax-1 activation of NFκB/Rel was dispensable but that activation of the CREB/ATF pathway was critical for the morphological transformation of Rat-2 cells (50). Two similar studies using Rat-1 cells implicated the NFκB/Rel pathway in the morphological transformation process (32, 58). Studies using various delivery systems investigated the ability of Tax-1 mutants to immortalize primary human peripheral blood mononuclear cells (PBMCs), again with inconclusive results. One study using retroviral vectors indicated that Tax-1 activation of NFκB/Rel is sufficient to promote the growth response of PBL to IL-2. However, clonal expansion of $CD4^+$ T cells required activation of the CREB/ATF pathway (3). Another study indicated that a Tax-1 variant incapable of activating NFκB/Rel when expressed from a herpesvirus saimiri vector retained its immortalizing potential for primary human T cells (44). A more recent study has examined the role of Tax-1 in immortalization or transformation of PBMCs in the context of a full-length HTLV-1 (43). Their results indicated that the activation of the NFκB/Rel pathway by Tax-1 correlates with IL-2-dependent immortalization of PBMCs and that CREB/ATF activation was dispensable. It is likely that these conflicting observations may be due to substantial differences in experimental systems used or the Tax-1 mutations characterized.

Our study, distinct in approach and use of HTLV-2, more closely resembles the HTLV-1 immortalization report by Robek and Ratner (43) because both utilize full-length proviruses and primary human cells as transformation targets. Both attempt to mimic natural infection in vivo, but there are several major differences. First, we use irradiated virus producer cells, not transfection, to efficiently infect PBL. Second, the PBL are not previously activated with phytohemagglutinin and IL-2, nor is the culture media supplemented with exogenous IL-2. Therefore, the endpoint for our assay is IL-2-independent cell growth, not IL-2-dependent immortalization. Lastly, the functional $HTLV_{c\text{-}enh}$ replicates by a Tax-independent mechanism. Thus, *tax* mutations do not have a compromising effect on viral gene transcription and the ability of the mutant viruses to efficiently replicate. Our results are in agreement with Robek and Ratner in that NFκB/Rel activation directly correlates with cellular transformation. However, the importance of Tax activation of the CREB/ATF pathway in the transformation process cannot be directly compared because of differences between our systems. Our results indicate that CREB/ATF activation correlates with the emergence of fully transformed cells that proliferate independently of IL-2. This possibility was

not addressed in previous IL-2-dependent immortalization assays.

HTLV-1 and HTLV-2 have distinct pathogenic properties but transform primary human T lymphocytes with similar efficiencies. These distinct pathogenic properties may be attributable to differences in Tax-1- and Tax-2-mediated transformation. The major functional regions or domains of Tax that are important for transactivation by the CREB/ATF or NFκB/Rel signaling pathway are similar but not identical in Tax-1 and Tax-2 (45). In addition, HTLV-1-transformed cells are associated with the constitutive activation of the Jak/STAT pathway, whereas in HTLV-2-transformed cells Jak/STAT is not activated, suggesting that the mechanism of in vitro transformation is different (35, 37).

A complete understanding of the mechanism of T-lymphocyte transformation will require correlation of Tax domains important in cellular transformation with those domains important in modulating gene transcription, cell cycle control, induction of DNA damage, and other undefined modes of action (7, 23, 30, 36, 38, 41, 47, 52). The HTLV-2 experimental system described in this study as well as the similar HTLV-1 system in development are useful for the dissection of Tax domains in the transformation process. Future comparisons between HTLV-1 and HTLV-2 will be critical for our understanding of the pathogenesis of HTLV infection.

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