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Human T-Cell Lymphotropic Virus Type 1 Tax Mediates Enhanced Transcription in CD4$^+$ T Lymphocytes

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Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma and is associated with a variety of immunoregulatory disorders. HTLV-1 has been shown to bind to and infect a variety of hematopoietic and nonhematopoietic cells. However, both in vivo and in vitro, the provirus is mostly detected in and preferentially transforms CD4$^+$ T cells. The molecular mechanism that determines the CD4$^+$ T-cell tropism of HTLV-1 has not been determined. Using cocultures of purified CD4$^+$ and CD8$^+$ T cells with an HTLV-1-producing cell line, we measured viral transcription by using Northern (RNA) blot analysis, protein production by using a p24 antigen capture assay and flow cytometric analysis for viral envelope, and proviral integration by using DNA slot blot analysis. We further measured HTLV-1 long terminal repeat-directed transcription in purified CD4$^+$ and CD8$^+$ T cells by using transient transfection assays and in vitro transcription. We demonstrate a higher rate of viral transcription in primary CD4$^+$ T cells than in CD8$^+$ T cells. HTLV-1 protein production was 5- to 25-fold greater in CD4$^+$ cocultures and mRNA levels were 5-fold greater in these cultures than in the CD8$^+$ cocultures. Transient transfection and in vitro transcription indicated a modest increase in basal transcription in CD4$^+$ T cells, whereas there was a 20-fold increase in reporter gene activity in CD4$^+$ T cells cotransfected with *tax*. These data suggest that unique or activated transcription factors, particularly Tax-responsive factors in CD4$^+$ T cells, recognize regulatory sequences within the HTLV-1 long terminal repeat, and this mediates the observed enhanced viral transcription and ultimately the cell tropism and leukemogenic potential of the virus.

Human T-cell leukemia virus type 1 (HTLV-1), the first characterized human retrovirus, is the etiologic agent of adult T-cell leukemia/lymphoma, an aggressive and fatal malignancy of CD4$^+$ T cells (37, 54). The virus is also associated with a variety of immunoregulatory disorders, including the neurodegenerative disease HTLV-1-associated myelopathy/tropical spastic paraparesis (17). HTLV-1 preferentially targets and transforms CD4$^+$ T cells but also infects many other cell types in vivo and in vitro, including CD8$^+$ T cells, B cells, and monocytes (2, 15, 16, 21, 26).

The mechanism of the in vivo CD4$^+$ T-cell tropism of HTLV-1 has not been determined. Cell tropism of a retrovirus is primarily determined at two stages of the virus life cycle: adsorption-penetration, and proviral transcription. Studies of binding of HTLV-1 to cell lines or primary cells (22, 53) and pseudotype interference assays (49) indicate that the viral receptor is present on many mammalian lymphoid cell lines and appears to be up-regulated by mitogenic activation of primary human peripheral blood mononuclear cells (PBMCs) (22, 53). Therefore, unlike cell tropism of human immunodeficiency virus, which is primarily determined by the presence of the viral receptor (9, 48), cell tropism of HTLV-1 is most likely determined at the postpenetration stage of the virus life cycle.

Our approach of using primary cells is uncommon in viral transcription work. Most reports on the transcriptional regulation of HTLV-1, and other retroviruses, have used various cell lines because of the availability of the cell lines and the inherent technical difficulties of working with primary cells. However, the biological relevance of viral transcriptional regulation in cell lines, compared with primary cells, is questionable. Additionally, differences in levels or activation states of transcription factors between various cell lines and between various types of primary cells may affect the outcome of virus transcription experiments or gel shift experiments (51, 55). Recent advances in primary cell separation techniques have allowed us the opportunity to answer fundamental questions, such as explaining the cell tropism of HTLV-1, by using biologically relevant in vitro systems.

We demonstrate that the long terminal repeat (LTR) of HTLV-1, containing viral promoter and enhancer sequences, is a determinant of cell tropism by promoting a higher rate of viral transcription and protein production in primary CD4$^+$ T cells than in primary CD8$^+$ T cells. This increase is most notably observed in the presence of the viral transactivating protein Tax (14). The LTR-determined cell tropism of HTLV-1 is in direct contrast to the cell tropism of human immunodeficiency virus, in which the LTR is not a major determinant (39). However, precedence for LTR-mediated cell tropism has been reported for both avian and murine retroviruses. This has been most clearly demonstrated with murine retroviruses. Moloney murine leukemia virus (M-MuLV) induces T-cell lymphoma, whereas Friend murine leukemia virus (F-MuLV) induces erythroleukemia when injected into newborn NFS mice (34, 43). Chatis et al. (7, 8) constructed chimeric viruses by exchanging the LTR segments between the two viruses. When the chimeric viruses were injected into newborn NFS mice, tumors which corresponded to the LTR, not the parental virus, arose; i.e., F-MuLV containing the LTR of M-MuLV caused T-cell tumors, whereas M-MuLV containing

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the LTR of F-MuLV caused erythroid leukemias. It was later shown that particular enhancer sequences within the M-MuLV LTR were responsive to unique transcription factors present in T cells, making the combination of *cis* and *trans* transcription factors the prime determinants for cell tropism (5, 25). We demonstrate that HTLV-1 LTR-mediated transcription is enhanced in $CD4^+$ T cells, notably in the presence of Tax, and suggest that this enhanced rate of transcription explains both the cell tropism and the propensity of this virus to cause $CD4^+$ T-cell leukemias.

MATERIALS AND METHODS

Cells and coculture. PBMCs were obtained by leukophoresis from two HTLV-1-negative donors. The PBMCs were cultured for 4 days in RPMI 1640 supplemented with 10% fetal bovine serum, 0.3 mg of L-glutamine per ml, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 1 U of recombinant interleukin-2 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml (complete medium), and 2 μg of phytohemagglutinin P (PHA P) per ml in T75 flasks at 37°C in 7% CO_2. $CD4^+$ and $CD8^+$ T cells were separated from the cultured PBMCs by using a panning technique (Applied Immune Sciences Microsystems, Santa Clara, Calif.). Separation routinely yielded purities of greater than 95% for both the $CD4^+$ and $CD8^+$ populations, as measured by flow cytometric analysis using phycoerythrin-labeled monoclonal antibodies to CD2, CD4, CD8, CD3, CD20, and CD25 (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

Purified T-cell subsets were infected by coculturing 5×10^6 purified T cells with 5×10^5 gamma-irradiated (7,500 R) HuT 102 cells (12). Irradiated HuT 102 cells were also cultured alone as a control. Cultures were maintained in complete medium without PHA P. Cultures were centrifuged through Ficoll-Hypaque at 3 weeks postculture to remove cell debris and residual p24 capsid protein contributed by the irradiated cells.

Viral antigen detection. Viable cells were counted on a weekly basis, using trypan blue exclusion. Culture supernatant was also sampled weekly to measure soluble p24 capsid protein (HTLV-1,II Ag Assay; Coulter Corp., Hialeah, Fla.). Cell-associated gp46 envelope glycoprotein was measured at 6 weeks, using an indirectly labeled anti-HTLV-1 envelope monoclonal antibody (clone IC11; gift from T. Palker, Duke University, Raleigh, N.C.) with a fluorescein isothiocyanate-labeled sheep anti-mouse secondary antibody (Sigma Immunochemicals, St. Louis, Mo.) as previously described (23). Cells and data were analyzed by using a Coulter Elite cytometer and Coulter Immuno-4 analysis program (Coulter Corp.).

DNA slot blot. To compare proviral copy numbers in the infected $CD4^+$- and $CD8^+$-HuT 102 cocultures, total DNA was isolated from 5×10^6 cells (DNA STAT-60; Tel-Test Inc., Friendswood, Tex.) at the end of the 6-week culture period. DNA was quantitated by using a spectrophotometer, and equal amounts of DNA were applied to a positively charged nylon membrane through a slot blot manifold (6) and probed with α-^{32}P-labeled probes generated by using random primers (Ambicon, Austin, Tex.) from pMT-2, an HTLV-1 full-length clone (gift from F. Wong-Staal, University of California, San Diego, La Jolla). Controls included equal amounts of HuT 102 and HuT 78, an HTLV-1-negative T-cell line.

Northern (RNA) blot analysis. Irradiated HuT 102 cells (5×10^4) were cultured with 5×10^5 purified $CD4^+$ or $CD8^+$ T cells, or alone as a control, in six-well plates in complete medium for 7 days. At day 6, 5×10^5 lethally irradiated PBMCs were added to the cultures to provide adequate costimulation, and the cultures were subsequently stimulated with 2 μg of PHA P per ml. Eighteen hours poststimulation, total RNA was extracted by a guanidinium-based method (RNAzol B; Tel-Test) and Northern blot analysis was performed as previously described (6a), using α-^{32}P-labeled probes derived by random priming an HTLV-1 LTR-luciferase plasmid (described below). Amounts of RNA loaded were controlled by using an end-labeled glyceraldehyde-3-phosphate dehydrogenase probe (Clontech, Palo Alto, Calif.). Bands were quantitated densitometrically.

Transfections. An HTLV-1 LTR-luciferase plasmid was created by directionally ligating the *Xho*I-*Hin*dIII fragment from an HTLV-1 LTR chloramphenicol acetyltransferase construct (44) into the polylinker of GL-2 Basic (Promega, Madison, Wis.). An HTLV-1 *tax*-expressing plasmid, under the control of the HTLV-1 LTR (32), was used for cotransfections.

Purified populations of $CD4^+$ and $CD8^+$ T cells were transfected by electroporation as previously described (4). The cells were transfected with a total of 10 μg of DNA consisting of 7.5 μg of the LTR-luciferase plasmid and 2.5 μg of pUC18 DNA or with 7.5 μg of the LTR-luciferase plasmid, 1.0 μg of the *tax* plasmid, and 1.5 μg of pUC18 DNA. Posttransfection, the primary cells were stimulated with PHA P (2 μg/ml) and were cultured in the presence of an equal number of PBMCs to provide adequate costimulation. Total proteins were harvested from the cells 48 h posttransfection, using a commercial cell lysis buffer (Promega), and luciferase activity was determined in a scintillation counter as specified by the manufacturer (Promega). Transfection efficiencies were controlled by using a DNA slot blot technique as previously described (1). Briefly, total DNA was extracted (DNA STAT-60; Tel-Test) from half of the volume of cell lysate. DNA was quantitated spectrophotometrically, and equal amounts were serially diluted onto a positively charged nitrocellulose membrane through a DNA slot blot manifold (6). DNA was probed with α-^{32}P-labeled probes derived by random priming the HTLV-1 LTR-luciferase and *tax* plasmids. Relative amounts of plasmid DNA were determined densitometrically, and then luciferase values were corrected for transfection efficiency.

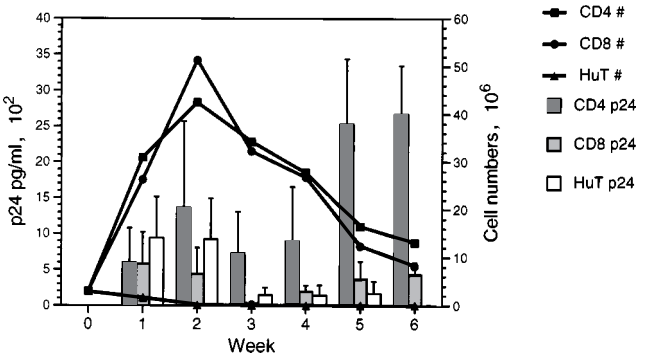

FIG. 1. $CD4^+$ T cells produce more viral antigen in a 6-week coculture assay. Purified populations of $CD4^+$ and $CD8^+$ T cells were cocultured with lethally irradiated HuT 102 cells. Irradiated HuT 102 cells were cultured alone as a control. The cultures were centrifuged through Ficoll-Hypague during week 3 to remove cell debris and residual antigen from the irradiated cells. Viable cell numbers (lines) and p24 capsid antigen concentration (bars) were determined weekly. The data represent the means of four trials, and the error bars represent standard deviation values. There is a 25-fold increase of p24 in the $CD4^+$ coculture, compared with the $CD8^+$ coculture at 3 weeks and a 5-fold increase at 6 weeks.

In vitro transcription. Whole cell extracts were prepared as previously described (27) from purified populations of mitogen-activated $CD4^+$ and $CD8^+$ cells. The protein was purified from the two T-cell subsets immediately following T-cell separation from PBMCs. In vitro transcription of the HTLV-1 LTR was performed as previously described (36), with the following modifications. Six micrograms of whole cell extract was incubated with 300 ng of *Hin*dIII-linearized HTLV-1 LTR-luciferase plasmid or *Bam*HI-linearized adenovirus major late promoter (36) for 30 min at 30°C in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, 3 mM $MgCl_2$, and 10% glycerol. Nucleotides (500 μM ATP, GTP, and CTP and 15 μCi of [^{32}P]UTP [ICN]) were added, and the reaction mixture was incubated for another 30 min at 30°C. A ^{32}P-end-labeled double-stranded DNA fragment, which served as a nucleic acid recovery control, was added to the buffer which stopped the reactions (20 mM Tris-HCl [pH 8.3], 150 mM NaCl, 0.2% sodium dodecyl sulfate). The HTLV-1 promoter directed a transcript of 278 bases, and the adenovirus promoter directed a transcript of 375 bases.

RESULTS

$CD4^+$ T cells produce more HTLV-1 in culture. To investigate which of the two major T-cell subsets better supports HTLV-1 replication in vitro, we cocultured highly purified populations of either $CD4^+$ or $CD8^+$ T cells with lethally irradiated HuT 102 cells. Virus production was assayed in two ways: by flow cytometry to measure cell-associated viral envelope gp46, and by a p24 capsid antigen capture assay as a measure of soluble released virus. Although the numbers of viable $CD4^+$ and $CD8^+$ T cells remained relatively equal throughout the experiments, the cumulative p24 levels in the $CD4^+$ cultures ranged from 5- to 25-fold greater than in the $CD8^+$ cultures in weeks 3 to 6 (Fig. 1). To further compare levels of virus production in the two lymphocyte subsets, cell surface expression of HTLV-1 envelope gp46 was assessed by indirect immunofluorescence and flow cytometry. The $CD4^+$ cultures contained only 15% more gp46-expressing cells compared with the $CD8^+$ cultures (Fig. 2). Diluting DNA slot blot analysis indicated that the $CD4^+$ cultures contained only 35% more viral DNA compared with the $CD8^+$ culture (Fig. 3). Taken together, these data suggest that whereas both the num-

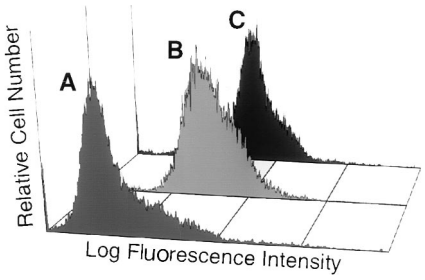

FIG. 2. Similar numbers of $CD4^+$ and $CD8^+$ cells exhibit HTLV-1 envelope gp46 antigen at 6 weeks postculture as measured by flow cytometry. After 6 weeks of coculture with irradiated HuT 102 cells, the purified $CD4^+$ and $CD8^+$ T cells were assessed for surface expression of the envelope glycoprotein gp46. The glycoprotein was indirectly labeled by using a mouse monoclonal antibody (IC11) and a fluorescein isothiocyanate-labeled sheep anti-mouse secondary antibody. (A) Isotype control; (B) $CD8^+$ coculture; (C) $CD4^+$ coculture. There is a 15% increase of gp46-expressing cells in the $CD4^+$ coculture (C) compared with the $CD8^+$ coculture (B).

bers of proviral copies and numbers of cells infected with and producing HTLV-1 are similar between the two cultures, the $CD4^+$ cultures appear be produce virus at a much greater rate.

$CD4^+$ T cells produce more viral mRNA in short-term coculture. To ascertain whether the fivefold differences in the viral protein levels observed in the $CD4^+$- and $CD8^+$-HuT 102 6-week cocultures were due to transcriptional or posttranscriptional events, total RNA was harvested from 1-week $CD4^+$- and $CD8^+$-HuT 102 cocultures and subjected to Northern blot analysis (Fig. 4). We harvested RNA at 7 days postcoculture because early differences in protein and DNA levels could not be ascertained as a result of the presence of the irradiated HuT 102 cells and to lessen any potential differences in rates of infection between the two cocultures. There was a fivefold increase in the amount of viral RNA in the $CD4^+$ culture compared with the $CD8^+$ culture (Fig. 4A). There was a negligible amount of viral RNA contributed from the irradiated HuT 102 cells to the two cocultures since virtually no RNA could be extracted from these cells, as demonstrated by the lack of any band detectable with the glyceraldehyde-3-phosphate dehydrogenase probe (Fig. 4A). These data suggest that the increased amount of viral protein produced in the 6-week $CD4^+$ cocultures was due, at least in part, to an increased rate of proviral transcription.

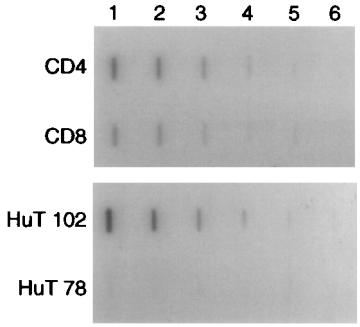

FIG. 3. The $CD4^+$ and $CD8^+$ cocultures contained similar amounts of HTLV-1 DNA. After 6 weeks of coculture of $CD4^+$ and $CD8^+$ T cells with irradiated HuT 102 cells, total DNA was isolated from the cocultures and applied in twofold dilutions to a nylon membrane through a slot blot manifold. HTLV-1 DNA was detected by using probes derived from a full-length HTLV-1 clone. Densitometric evaluation indicated a mean 35% greater signal in the CD4 row than in the CD8 row. Controls included HuT 102, which is an HTLV-1-infected cell line, and HuT 78, which is a non-HTLV-1-infected T-cell line.

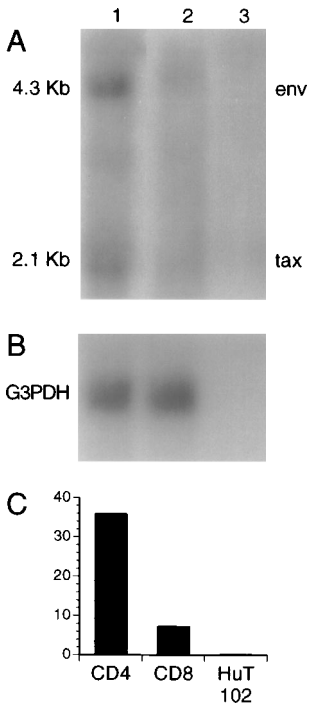

FIG. 4. Short-term $CD4^+$ cocultures produced more viral RNA than did $CD8^+$ cocultures. (A) Irradiated HuT 102 cells were cultured with purified $CD4^+$ (lane 1) or $CD8^+$ (lane 2) T cells or alone as control (lane 3). At day 6, cultures were stimulated with 2 μg of PHAP per ml, and 18 h poststimulation, total RNA was harvested. Northern blot analysis was performed with probes derived from an HTLV-1 LTR-luciferase plasmid. Lane 1 demonstrates greater envelope (env) and *tax* mRNA expression in the $CD4^+$ cultures than in the $CD8^+$ cultures (lane 2) and the control HuT 102 culture (lane 3). The Northern blots are representative of three separate trials. (B) Constitutive expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA indicates equal loading of RNA in the $CD4^+$ (lane 1) and $CD8^+$ (lane 2) cultures, whereas no signal was observed in the HuT 102 culture (lane 3). (C) Densitometric evaluation indicated a fourfold increase of envelope and *tax* mRNA in the $CD4^+$ coculture compared with the $CD8^+$ culture.

$CD4^+$ T cells elicit greater LTR-mediated reporter gene activity following transient transfection. To overcome the inherent problems of cell-associated viral transmission and to determine if $CD4^+$ primary cells could enhance transcription from the HTLV-1 LTR compared with $CD8^+$ primary cells, we transiently transfected the purified T-cell subsets with an HTLV-1 LTR-luciferase construct. Luciferase activity was threefold higher in $CD4^+$ T cells than in $CD8^+$ T cells (Fig. 5A). This result suggests that the promoter and/or enhancer sequences within the HTLV-1 LTR were slightly more responsive to the transcriptional environment within $CD4^+$ primary cells compared with $CD8^+$ primary cells.

The virally encoded Tax protein is a 40-kDa, nuclear localizing protein that acts in *trans* to enhance transcription from the HTLV-1 LTR (14). Recent reports demonstrate that Tax enhances transcription by increasing the dimerization and DNA binding of transcription factors containing leucine zipper dimerization motifs (3, 46). To determine whether Tax differentially influences the rate of transcription from the HTLV-1 LTR in primary T cells, we cotransfected purified $CD4^+$ and $CD8^+$ T-cell subsets with both the HTLV-1 LTR-luciferase construct and an HTLV-1 LTR-*tax* plasmid. We observed a 15- to 20-fold increase in luciferase activity in $CD4^+$ cotransfected cells compared with the $CD8^+$ cotransfected cells (Fig. 5B). Therefore, not only do $CD4^+$ primary cells support a modestly

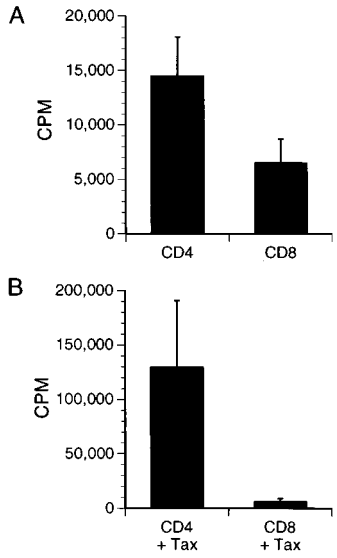

FIG. 5. Enhanced luciferase reporter gene activity in $CD4^+$ T cells compared with $CD8^+$ T cells following transfection with HTLV-1 LTR-luciferase without (A) and with (B) cotransfected *tax*. The figures are representative of three trials, with five replicates per trial. Counts per minute represents the intensity of luciferase activity measured in a scintillation counter. Error bars represent standard deviation values within the depicted trial ($n = 5$). $CD4^+$ cells exhibited a 3-fold increase in luciferase activity without cotransfected *tax* compared with $CD8^+$ cells in the depicted trial (A), whereas the $CD4^+$ cells exhibited a 20-fold increase in luciferase activity with cotransfected *tax* compared with the $CD8^+$ cells (B).

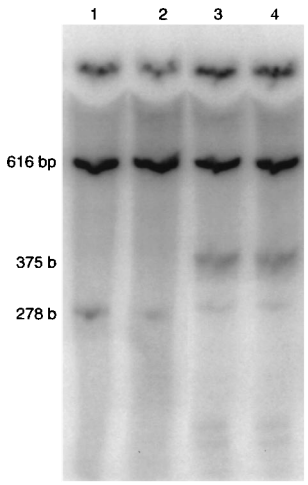

FIG. 6. $CD4^+$ T-cell whole cell extracts direct enhanced basal transcription from the HTLV-1 LTR compared with $CD8^+$ T-cell extracts, using in vitro transcription. Six micrograms of $CD4^+$ whole cell extract (lane 1) directed a modest increase in transcription compared with whole cell extracts derived from $CD8^+$ T cells (lane 2). The $CD4^+$ (lane 3) and $CD8^+$ (lane 4) extracts directed equivocal amounts of transcription from the adenovirus major late promoter. The HTLV-1 promoter directed a transcript of 278 bases (b), whereas the adenovirus promoter directed a transcript of 375 bases. The 616 double-stranded DNA fragment indicates that equivalent amounts of nucleic acid were extracted from the in vitro transcription reactions.

enhanced rate of transcription at the basal LTR level, but these cells also support a strikingly greater rate of transcription in the presence of Tax compared with primary $CD8^+$ cells. This result suggests that the Tax-responsive regions in the HTLV-1 LTR are the primary sequences responsible for the enhanced transcription observed in primary $CD4^+$ T cells and also suggests higher levels or differential phosphorylation states of Tax-responsive transcription factors, particularly of the cyclic AMP (cAMP) element-binding (CREB)/ATF family (13, 55).

In vitro transcription of the HTLV-1 LTR. To further confirm that $CD4^+$ T cells could support basal HTLV-1 transcription at a slightly higher rate than $CD8^+$ T cells, we prepared whole cell extracts from the two subsets of primary T cells and performed in vitro transcription using the HTLV-1 LTR and the adenovirus major late promoter. The extracts derived from $CD4^+$ T cells supported basal transcription from the HTLV-1 LTR at a modestly greater rate than the $CD8^+$ T-cell extracts, whereas both extracts supported equivocal amounts of transcription from the adenovirus major late promoter (Fig. 6). These data support our transient transfection data, which demonstrate a modest increase of basal transcription of the HTLV-1 LTR in purified $CD4^+$ T cells compared with $CD8^+$ T cells.

DISCUSSION

This report demonstrates that the HTLV-1 LTR directs different rates of virus transcription and viral production in primary $CD4^+$ and $CD8^+$ T cells. The data indicate that the observed increased levels of viral mRNA and protein are due to an enhanced rate of transcription in $CD4^+$ T cells; however, other potential differences between the two cell types, such as transcriptional silencers in $CD8^+$ T cells, differential mRNA processing, and virus maturation, may also have contributed to the observed differences. Nevertheless, enhanced HTLV-1 LTR-driven transcription in $CD4^+$ T cells is significant because it furnishes an explanation for the ability of HTLV-1 to infect many cell types yet preferentially replicate in, and transform, $CD4^+$ T cells.

Coculture of $CD4^+$ and $CD8^+$ T-cell subsets with HuT 102 cells allowed us to compare viral production during an early period of in vitro infection in the two types of T cells. An inherent problem with cell-associated viral infection when two types of cells are compared is the potential for different levels of initial and subsequent reinfection, particularly if one type of cell produces more virus than the other. In the present work, both the flow cytometry results and proviral DNA levels in the two cocultures indicated that the levels of infection were similar between the two cultures. Therefore, the large differences in soluble capsid antigen between the two cocultures cannot be explained by different levels of infection, suggesting differences in virus transcriptional or posttranscriptional events. It is interesting that there was not more of a difference in proviral levels between the two cocultures on the basis of the levels of p24 capsid antigen. It is possible that a 6-week coculture does not afford enough time to observe differences at the provirus level, or perhaps many of the particles were defective. The latter scenario would yield high levels of viral antigen but relatively low levels of infectious virus particles.

There appears to be a discrepancy between the amounts of viral protein in the two cocultures as measured by flow cytometry and antigen capture in that there was a much larger difference between soluble virus (fivefold) and gp46 expressing cells (15%) at 6 weeks. However, the tests temporally measure viral protein levels in very different fashions. The antigen capture test measures capsid antigen that has accumulated over time and thus can be considered a dynamic measurement of viral production. Flow cytometric analysis measures cell surface antigen at one point in time and thus can be considered a static measurement. Therefore, the scenario in which both cocultures contain relatively equal numbers of infected cells

with the $CD4^+$ coculture supporting increased virus production is consistent with the observed data.

The cells used in the 1-week coculture, transient transfections, and in vitro transcription experiments were stimulated with mitogen to increase cell numbers and viral transcription as previously described for assays with HTLV-1 (28, 31, 40, 50), human immunodeficiency virus type 1 (30, 42), and simian immunodeficiency virus (38). $CD4^+$ and $CD8^+$ T cells used in the 6-week coculture experiment were not mitogen stimulated after T-cell separation because we felt that this best modeled an early in vivo infection event, and these cells would proliferate and be activated in response to the presence of the irradiated HuT 102 cells (20). Although mitogen-stimulated and nonstimulated cells were not directly compared in this work because of the technical difficulty of obtaining adequate numbers of nonstimulated primary cells, it is probable that mitogen stimulation played a role in the higher levels of viral transcription observed in the $CD4^+$ T cells. It has been shown that different tyrosine kinase activity levels exist in $CD4^+$ and $CD8^+$ T cells poststimulation (19). Mitogen stimulation in T cells increases intracellular levels of cAMP (29, 45, 47). Increases in cAMP are associated with elevated levels of active protein kinase A, which phosphorylates CREB proteins (18, 24). Additionally, mitogen activation of PBMCs has been shown to cause increased binding of CREB proteins to their DNA response element (cAMP response element) (51). Because imperfect cAMP response element sites are present in the three 21-bp repeats within the HTLV-1 LTR (35, 41), it is possible that differential levels of either cAMP or other molecules in the mitogen-activated secondary signalling pathways in $CD4^+$ and $CD8^+$ cells mediated the basal transcription differences observed in the transient transfection and in vitro transcription trials.

The transient transfection and in vitro transcription data clearly showed that whereas there is a modest difference in HTLV-1 basal transcription, the presence of cotransfected *tax* mediated a striking difference in LTR-driven gene expression in $CD4^+$ and $CD8^+$ T cells. The cotransfected *tax* in our transient transfection experiments was under the control of the HTLV-1 LTR, as was the reporter gene, because we felt that this best represented an in vivo infection. However, having *tax* under the control of the LTR may have affected our results. Our data demonstrated that basal transcription is slightly higher in $CD4^+$ T-cells than in $CD8^+$ T cells. This would likely lead to more *tax* being produced in the $CD4^+$ cells, thereby amplifying the differences in the amount of reporter gene being produced in the two T-cell subsets. Nevertheless, our data clearly indicate that the major transcriptional difference of the HTLV-1 LTR in $CD4^+$ and $CD8^+$ T cells is mediated by Tax. The amount of Tax in the $CD4^+$ cells is critical because Tax has recently been shown to cause increased dimerization and DNA binding of transcription factors containing a leucine zipper dimerization motif, notably of the CREB/ATF family (3, 46). The Tax-mediated increase of transcription in $CD4^+$ cells is likely due to either one or both of the following two mechanisms: an increased expression or activation of Tax-responsive transcription factors, or an increased activity of Tax within the $CD4^+$ T-cell population.

The CREB/ATF family of transcription factors is the principal family influenced by Tax (13). These transcription factors increase transcription only after phosphorylation (reviewed in references 18 and 24). There is controversy, however, about the effects of phosphorylation of CREB/ATF transcription factors to various cAMP response element sites (10, 33, 52). Anderson and Dynan (3) showed that phosphorylation did not alter CREB binding to the most proximal HTLV-1 Tax-responsive 21-bp repeat region. These authors also concluded that because of a low association constant value, the majority of CREB protein would be present as dimers. Therefore, an increased amount of the CREB/ATF family of transcription factors in $CD4^+$ T cells would account for the observed enhanced transcriptional rate at both the basal and Tax-mediated levels. Additionally, differences in $CD4^+$ and $CD8^+$ T-cell secondary signalling after mitogen stimulation could further exacerbate HTLV-1 transcriptional differences in these primary cells.

This is the first report of differential effects of Tax in primary cells. Interestingly, Fontes et al. (11) have reported that phorbol esters enhance the phosphorylation state of Tax, although functional activity of Tax has not been correlated to phosphorylation state. Ongoing work in our laboratory is addressing the possibility that Tax has more transactivating activity in $CD4^+$ T cells potentially because of differential kinase and/or phosphatase activity levels in $CD4^+$ T cells compared with $CD8^+$ T cells.

We report that transcription from the HTLV-1 LTR is enhanced in $CD4^+$ T cells. Additionally, we have shown that the enhanced transcription is much greater in the presence of Tax. Because $CD4^+$ T cells support transcription of the virus and the viral regulatory proteins, we believe that this accounts for the observation that HTLV-1 is able to infect various types of hemopoietic cells but is preferentially detected in, and transforms, $CD4^+$ T cells.

ACKNOWLEDGMENTS

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REFERENCES

1. **Abken, H., and B. Reinrath.** 1992. A procedure to standardize CAT reporter gene assay. Nucleic Acids Res. **20:**3527.
2. **Akagi, T., Y. Hoshida, T. Yoshino, N. Teramoto, E. Kondo, K. Hayashi, and K. Takahashi.** 1992. Infectivity of human T-lymphotropic virus type I to human nervous tissue cells in vitro. Acta Neuropathol. **84:**147–152.
3. **Anderson, M. G., and W. S. Dynan.** 1994. Quantitative studies of the effect of HTLV-1 tax protein on CREB protein-DNA binding. Nucleic Acids Res. **22:**3194–3201.
4. **Baum, C., P. Forster, S. Hegewisch-Becker, and K. Harbers.** 1994. An optimized electroporation protocol applicable to a wide range of cell lines. BioTechniques **17:**1058–1062.
5. **Boral, A. L., S. A. Okenquist, and J. Lenz.** 1989. Identification of the SL3-3 virus enhancer core as a T-lymphoma cell-specific element. J. Virol. **63:**76–84.
6. **Brown, T.** 1994. Preparation and analysis of DNA, p. 2.9.18–2.9.20. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., Somerset, N.J.

6a. **Brown, T.** 1994. Preparation and analysis of RNA, p. 4.9.1–4.9.14. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., Somerset, N.J.

7. **Chatis, P. A., C. A. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1983. Role for the 3′ end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses. Proc. Natl. Acad. Sci. USA **80:**4408–4411.
8. **Chatis, P. A., C. A. Holland, J. E. Silver, T. N. Frederickson, N. Hopkins, and J. W. Hartley.** 1984. A 3′ end fragment encompassing the transcriptional enhancers of nondefective Friend virus confers erythroleukemogenicity on Moloney leukemia virus. J. Virol. **52:**248–254.
9. **Dalgleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312:**763–767.
10. **de Groot, R. P., J. den Hertog, J. R. Vandenheede, J. Goris, and P. Sassone-Corsi.** 1993. Multiple and cooperative phosphorylation events regulate the CREM activator function. EMBO J. **12:**3903–3911.
11. **Fontes, J. D., J. M. Strawhecker, N. D. Bills, R. E. Lewis, and S. H. Hinrichs.**

1993. Phorbol esters modulate the phosphorylation of human T-cell leukemia virus type I Tax. J. Virol. **67:**4436–4441.
12. **Gazdar, A. F., D. N. Carney, P. A. Bunn, E. K. Russell, E. S. Jaffe, G. P. Schechter, and J. G. Guccion.** 1980. Mitogen requirements for the in vitro propagation of cutaneous T-cell lymphomas. Blood **55:**409–417.
13. **Giam, C.-Z., and Y.-L. Xu.** 1989. HTLV-I *tax* gene product activates transcription via pre-existing cellular factors and cAMP responsive elements. J. Biol. Chem. **264:**15236–15241.
14. **Green, P. L., and I. S. Chen.** 1990. Regulation of human T cell leukemia virus expression. FASEB J. **4:**169–175.
15. **Ho, D. D., T. R. Rota, and M. S. Hirsch.** 1984. Infection of human endothelial cells by human T-lymphotropic virus type I. Proc. Natl. Acad. Sci. USA **81:**7588–7590.
16. **Hoffman, P. M., S. Dhib-Jalbut, J. A. Mikovits, D. S. Robbins, A. L. Wolf, G. K. Bergey, N. C. Lohrey, O. S. Weislow, and F. W. Ruscetti.** 1992. Human T-cell leukemia virus type I infection of monocytes and microglial cells in primary human cultures. Proc. Natl. Acad. Sci. USA **89:**11784–11788.
17. **Hollsberg, P., and D. A. Hafler.** 1993. Pathogenesis of diseases induced by human lymphotropic virus type I infection. N. Engl. J. Med. **328:**1173–1182.
18. **Hunter, T.** 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell **80:**225–236.
19. **Julius, M., C. R. Maroun, and L. Haughn.** 1993. Distinct roles for CD4 and CD8 co-receptors in antigen receptor signalling. Immunol. Today **14:**177–183.
20. **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.
21. **Koyanagi, Y., Y. Itoyama, N. Nakamura, K. Takamatsu, J. Kira, T. Iwamasa, I. Goto, and N. Yamamoto.** 1993. In vivo infection of human T-cell leukemia virus type I in non-T cells. Virology **196:**25–33.
22. **Krichbaum-Stenger, K., B. J. Poiesz, P. Keller, G. Ehrlich, J. Gavalchin, B. H. Davis, and J. L. Moore.** 1987. Specific adsorption of HTLV-I to various target human and animal cells. Blood **70:**1303–1311.
23. **Lairmore, M. D., S. Jacobson, F. Gracia, L. Castillo, M. Larreategui, B. D. Roberts, P. H. Levine, W. A. Blattner, and J. E. Kaplan.** 1990. Isolation of human T-cell lymphotropic virus type 2 from Guaymi Indians in Panama. Proc. Natl. Acad. Sci. USA **87:**8840–8844.
24. **Lalli, E., and P. Sassone-Corsi.** 1994. Signal transduction and gene regulation: the nuclear response to cAMP. J. Biol. Chem. **269:**17359–17362.
25. **Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins.** 1987. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. J. Virol. **61:**693–700.
26. **Macchi, B., M. Popovic, P. Allavena, J. Ortaldo, P. Rossi, R. C. Gallo, and E. Bonmassar.** 1987. In vitro susceptibility of different human T-cell subpopulations and resistance of large granular lymphocytes to HTLV-I infection. Int. J. Cancer **40:**1–6.
27. **Manley, J. L., A. Fire, M. Samuels, and P. A. Sharp.** 1983. In vitro transcription: whole-cell extract. Methods Enzymol. **101:**568–582.
28. **Matsuda, S., Y. Nakao, H. Ohigashi, K. Koshimizu, and Y. Ito.** 1986. Plant-derived diterpene esters enhance HTLV-I-induced colony formation of lymphocytes in co-culture. Int. J. Cancer **38:**859–865.
29. **Munoz, E., U. Beutner, A. Zubiaga, and B. T. Huber.** 1990. IL-1 operates two separate signal transduction pathways in T helper type II cells. J. Immunol. **144:**964–969.
30. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326:**711–713.
31. **Nakao, Y., S. Matsuda, T. Matsui, T. Koizumi, Y. Katakami, T. Fujita, and Y. Ito.** 1986. Inhibitors of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced multinucleated cell formation and HTLV-I p19 antigen expression in HTLV-I-infected T-cell line KH-2Lo. Int. J. Cancer **37:**911–917.
32. **Nerenberg, M., S. H. Hinrichs, R. K. Reynolds, G. Khoury, and G. Jay.** 1987. The tat gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice. Science **237:**1324–1329.
33. **Nichols, M., F. Weih, W. Schmid, C. DeVack, E. Kowenz-Leutz, B. Lucklow, M. Boshart, and G. Schutz.** 1992. Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. EMBO J. **11:**3337–3346.
34. **Oliff, A., S. Ruscetti, E. C. Douglass, and E. M. Scolnick.** 1981. Isolation of transplantable erythroleukemia cells from mice infected with helper-independent Friend murine leukemia virus. Blood **58:**244–254.
35. **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.
36. **Piras, G., F. Kashanchi, M. F. Radonovich, J. F. Duvall, and J. N. Brady.** 1994. Transcription of the human T-cell lymphotropic virus type I promoter by an alpha-amanitin-resistant polymerase. J. Virol. **68:**6170–6179.
37. **Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA **77:**7415–7419.
38. **Polacino, P. S., H. A. Liang, E. J. Firpo, and E. A. Clark.** 1993. T-cell activation influences initial DNA synthesis of simian immunodeficiency virus in resting T lymphocytes from macaques. J. Virol. **67:**7008–7016.
39. **Pomerantz, R. J., M. B. Feinberg, R. Andino, and D. Baltimore.** 1991. The long terminal repeat is not a major determinant of the cellular tropism of human immunodeficiency virus type 1. J. Virol. **65:**1041–1045.
40. **Radonovich, M., and K. Jeang.** 1989. Activation of the human T-cell leukemia virus type I long terminal repeat by 12-*O*-tetradecanoylphorbol-13-acetate and by Tax (p40) occurs through similar but functionally distinct target sequences. J. Virol. **63:**2987–2994.
41. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. Location of *cis*-acting regulatory sequences in the human T-cell leukemia virus type I long terminal repeat. Proc. Natl. Acad. Sci. USA **82:**6502–6506.
42. **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.
43. **Silver, J. E., and J. M. Fredrickson.** 1983. Susceptibility to Friend helper virus leukemias in CXB recombinant inbred mice. J. Exp. Med. **158:**1693–1702.
44. **Sodroski, J. G., C. A. Rosen, and W. A. Haseltine.** 1984. *Trans*-acting transcriptional activation of the long terminal repeat of human T-lymphotropic viruses in infected cells. Science **225:**381–385.
45. **Takigawa, M., and B. H. Waksman.** 1981. Mechanisms of lymphocyte "deletion" by high concentrations of ligand. I. Cyclic AMP levels and cell death in T-lymphocytes exposed to high concentrations of concanavalin A. Cell. Immunol. **58:**29–38.
46. **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.
47. **Wang, T., J. R. Sheppard, and J. E. Foker.** 1978. Rise and fall of cyclic AMP required for onset of lymphocyte DNA synthesis. Science **201:**155–157.
48. **Weiss, R. A.** 1992. Human immunodeficiency virus receptors. Semin. Virol. **3:**79–84.
49. **Weiss, R. A., P. Clapham, K. Nagy, and H. Hoshino.** 1985. Envelope properties of human T-cell leukemia viruses. Curr. Top. Microbiol. Immunol. **115:**235–246.
50. **Wolfson, M., M. Lev, I. Avinoah, Z. Malik, M. Lochelt, R. M. Flugel, A. Dombrovski, and M. Aboud.** 1994. Stimulation of virus production and induction of self-syncytium formation in human T-cell leukemia virus type I and type II-infected cells by 12-*O*-tetradecanoylphorbol-13-acetate. J. Virol. **68:**4695–4699.
51. **Wollberg, P., H. Soderqvist, and B. D. Nelson.** 1994. Mitogen activation of human peripheral T lymphocytes induces the formation of new cyclic AMP response element-binding protein nuclear complexes. J. Biol. Chem. **269:**19719–19724.
52. **Yamamoto, K. K., G. A. Gonzales, W. H. Briggs III, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334:**494–498.
53. **Yamamoto, N., T. Chosa, Y. Koyanagi, T. Tochikura, J. Schneider, and Y. Hinuma.** 1984. Binding of adult T-cell leukemia virus to various hematopoietic cells. Cancer Lett. **21:**261–268.
54. **Yoshida, M., I. Miyoshi, and Y. Hinuma.** 1981. Isolation and characterization of a retrovirus from cell lines of adult T-cell leukemia and its implication in the disease. Proc. Natl. Acad. Sci. USA **79:**2031–2035.
55. **Zhao, L.-J., and C.-Z. Giam.** 1992. Human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator, Tax, enhances CREB binding to HTLV-I 21-base-pair repeats by protein-protein interaction. Proc. Natl. Acad. Sci. USA **89:**7070–7074.