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Envelope Is a Major Viral Determinant of the Distinct In Vitro Cellular Transformation Tropism of Human T-Cell Leukemia Virus Type 1 (HTLV-1) and HTLV-2

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Human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2 are related deltaretroviruses but are distinct in their disease-inducing capacity. These viruses can infect a variety of cell types, but only T lymphocytes become transformed, which is defined in vitro as showing indefinite interleukin-2-independent growth. Studies have indicated that HTLV-1 has a preferential tropism for $CD4^+$ T cells in vivo and is associated with the development of leukemia and neurological disease. Conversely, the in vivo T-cell tropism of HTLV-2 is less clear, although it appears that $CD8^+$ T cells preferentially harbor the provirus, with only a few cases of disease association. The difference in T-cell transformation tropism has been confirmed in vitro as shown by the preferential transformation of $CD4^+$ T cells by HTLV-1 versus the transformation of $CD8^+$ T cells by HTLV-2. Our previous studies showed that Tax and overlapping Rex do not confer the distinct T-cell transformation tropisms between HTLV-1 and HTLV-2. Therefore, for this study HTLV-1 and HTLV-2 recombinants were generated to assess the contribution of LTR and *env* sequences in T-cell transformation tropism. Both sets of proviral recombinants expressed p19 Gag following transfection into cells. Furthermore, recombinant viruses were replication competent and had the capacity to transform T lymphocytes. Our data showed that exchange of the *env* gene resulted in altered T-cell transformation tropism compared to wild-type virus, while exchange of long terminal repeat sequences had no significant effect. HTLV-2/Env1 preferentially transformed $CD4^+$ T cells similarly to wild-type HTLV-1 (wtHTLV-1), whereas HTLV-1/Env2 had a transformation tropism similar to that of wtHTLV-2 ($CD8^+$ T cells). These results indicate that *env* is a major viral determinant for HTLV T-cell transformation tropism in vitro and provides strong evidence implicating its contribution to the distinct pathogenesis resulting from HTLV-1 versus HTLV-2 infections.

Human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2 are related but distinct pathogenic complex retroviruses. HTLV-1 has been identified as the etiologic agent of adult T-cell leukemia/lymphoma, a malignancy of $CD4^+$ T lymphocytes, as well as of a chronic progressive neurological disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (22, 26, 74, 75). In contrast, HTLV-2 has not been conclusively associated with disease; to date only a few cases of variant hairy cell leukemia ($CD8^+$ T-cell origin) and several cases of neurological disease have been reported (28, 34, 60).

It has been shown that HTLV-1 and HTLV-2 exhibit distinct in vivo T-cell tropisms. HTLV-1 has a preferential tropism for $CD4^+$ T lymphocytes in asymptomatic patients and those with leukemia and neurological disease (57, 58, 75). However, $CD8^+$ T cells from patients with HTLV-1-associated myelopathy/tropical spastic paraparesis were identified as an additional reservoir for HTLV-1 (49). In contrast, HTLV-2 in vivo tropism is less clear but seems to favor $CD8^+$ T lymphocytes. Proviral sequences were detected predominantly in $CD8^+$ T lymphocytes from HTLV-2-infected individuals, whereas others have detected HTLV-2 in both $CD4^+$ and $CD8^+$ T-cell subsets, with a greater proviral burden in $CD8^+$ T cells (32, 41).

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The distinct in vivo T-cell tropism of HTLV-1 and HTLV-2 has been recapitulated in vitro using transformation/immortalization assays in which irradiated 729 producer cells were cocultured with freshly isolated human peripheral blood mononuclear cells (PBMCs). Results from these studies showed that the majority of cells transformed by HTLV-1 in vitro were $CD4^+$ T lymphocytes (55, 59, 76). In addition, Tax-mediated HTLV-1 transcription was increased significantly in purified $CD4^+$ versus $CD8^+$ T-cell subsets, suggesting that an enhanced rate of viral transcription may be responsible for the preferential transformation of $CD4^+$ T cells by HTLV-1 (51). Conversely, purified $CD4^+$ and $CD8^+$ T cells were shown to be equally susceptible to HTLV-2 infection and subsequent viral gene expression. However, coculture of HTLV-2 producer cells with freshly isolated, nonstimulated PBMCs or purified T-cell subsets resulted in preferential transformation of $CD8^+$ T cells (62, 71, 76). Since Tax has been shown to be critical for cellular transformation in vitro and interacts with numerous cellular processes involving cell growth and differentiation, cell cycle regulation, and DNA repair (17, 19, 35, 73, 77), it has been hypothesized that the Tax would encode the viral determinant for transformation tropism. However, recent studies using recombinant HTLVs indicated that Tax and overlapping Rex did not confer the distinct HTLV-1 and HTLV-2 transformation tropism in vitro (76). This suggests that other viral genes or sequences are responsible for the differential ability to transform $CD4^+$ or $CD8^+$ T cells.

Here we generated and evaluated recombinant viruses in which the long terminal repeat (LTR) and *env* sequences were exchanged between HTLV-1 and HTLV-2. Our results indicated that the exchange of LTR sequences did not alter HTLV transformation tropisms. Interestingly, we identified Env as a major viral factor that confers the in vitro HTLV transformation tropism; wild-type HTLV-2 (wtHTLV-2) and HTLV-1 with Env-2 (HTLV-1/Env2) preferentially transformed $CD8^+$ T cells, whereas wtHTLV-1 and HTLV-2/Env1 displayed a preferred transformation tropism for $CD4^+$ T cells. This study provides the first biological evidence that the envelope gene may play an important role in the distinct pathogenesis resulting from HTLV-1 and HTLV-2 infections.

MATERIALS AND METHODS

Cells. 293T cells and human osteogenic sarcoma (HOS) cells were maintained in Dulbecco's modified Eagle's medium. The 729 and BJAB human B-cell lines were maintained in Iscove's medium. Jurkat T cells were maintained in RPMI 1640 medium. All media were supplemented to contain 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Human PBMCs were isolated from the blood of normal donors by centrifugation over Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) and were cultured in RPMI 1640 medium supplemented with 20% FBS, 2 mM glutamine, and antibiotics. In selected experiments, transformed T lymphocytes (10 weeks postcoculture) were treated with 10 U/ml of human interleukin-2 (IL-2) (Roche, Indianapolis, IN) to enhance the short-term growth of cells required for molecular analysis.

Plasmids. The wtHTLV-1 proviral clone Ach (38) and wtHTLV-2 proviral clone pH6neo (7) were used to generate recombinant proviral clones for this study. To assist in the generation of the recombinant HTLV proviral clones, several restriction enzyme sites were introduced by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specifically, an EcoRI site was generated between the end of the 5′ LTR and the start codon of Gag in the HTLV-1 provirus (^{786}TTATTC^{791} to GAATTC); an EcoRI site already is present at the identical location in the HTLV-2 provirus. An EcoRV site was generated 3′ to the stop codon of *tax-1* in the HTLV-1 provirus (^{8356}TGAAAAG^{8362} to TGATATC) and 3′ to *tax-2* in the HTLV-2 provirus (^{8203}TAGCCTCC^{8210} to TAGATATC) (76). The HTLV-1/LTR2 and HTLV-2/LTR1 recombinants, where both the 5′ LTR and 3′ LTR were exchanged between HTLV-1 and HTLV-2, were generated by exchanging the EcoRI-EcoRV fragments. In order to exchange the *env* genes, an NheI site was generated downstream of the stop codon of *env-1* in the HTLV-1 provirus (^{6651}GCACAC^{6656} to GCTAGC), which corresponds to the location of an identical site already present in the HTLV-2 provirus. HTLV-2/Env1 and HTLV-1/Env2 were generated by exchanging the HTLV-1 and HTLV-2 NcoI (HTLV-1 nucleotide 5177 and HTLV-2 nucleotide 5178)-NheI fragment containing the entire *env* gene.

Transfection and p19 Gag ELISA. In order to measure virion production from the recombinant HTLV proviral clones, 2×10^5 293T cells were transfected using Lipofectamine PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. The total amount of DNA was kept constant and contained 2 μg of HTLV proviral plasmid DNA or a negative control and 0.1 μg of cytomegalovirus-luciferase. After 48 h of growth, culture supernatants were collected and assayed for p19 matrix antigen by using a commercially available enzyme-linked immunosorbent assay (ELISA) (Zeptometrix, Buffalo, NY). The cell lysates also were harvested and assayed for luciferase activity to monitor the transfection efficiency. p19 Gag calibration curves were generated using HTLV-1 p19 antigen standards as described by the manufacturer, with a detection sensitivity of 25 pg/ml. All experiments were performed in triplicate. To generate stable transfectants, proviral plasmid clones containing the Neo^r gene were introduced into cells by electroporation as described previously (4, 24). Stable transfectants containing the desired proviral clones were isolated following incubation in 24-well culture plates in medium containing 1 mg/ml of Geneticin. After 4 to 5 weeks of selection, viable cells were single-cell cloned, expanded, and maintained in culture for further analysis. The clones were screened for p19 Gag expression in the cell supernatants by ELISA.

DNA preparation and PCR. Genomic DNA was isolated from permanently transfected cell clones or from transformed PBMCs by using the PUREGENE DNA purification system (Gentra, Minneapolis, Minn.). One microgram of genomic DNA was subjected to 30-cycle PCR analysis. The PCR-amplified product was separated on an agarose gel and visualized by ethidium bromide staining. The primer pair TRE-1(S) [$^{74(8351)}$AAGTCTGAAAAGGTCAGG G$^{92(8369)}$] and TRE-1(AS) [$^{335(8612)}$CACGCTTTTATAGACTCCTG$^{316(8593)}$] was used to amplify a 262-bp fragment specific for the HTLV-1 LTR, and the primer pair $^{(8197)}$GGCGACTAGCCTCCCAAGCCAG$^{29(8218)}$ and $^{750(8938)}$CGGGAA GACAATGCTCCTAGGGCG$^{727(8916)}$ was used to amplify a 743-bp fragment specific for the HTLV-2 LTR (numbers and numbers in parentheses represent proviral nucleotide positions in the 5′ LTR and 3′LTR, respectively). The primer pair ^{8182}GAGGCGGATGACAATGGCGAC^{8202} (Tax-2/LTR-2 specific) and TRE-1 (AS) was used to amplify a 279-bp fragment and specifically detect a hybrid LTR-2/1 that results from the *tax-2* gene being part of the LTR. The degenerative primer pair $^{5276(5265)}$TCTCCTCMTACCACTCYA$^{5293(5282)}$ and $^{6346(6335)}$CTTGYTCCCAGAAYAGGAG$^{6328(6317)}$ was designed to amplify a 1,071-bp product from both *env-1* and *env-2* sequences (numbers and numbers in parentheses represent proviral nucleotide positions in HTLV-1 and HTLV-2, respectively).

Syncytium and transformation assays. Syncytium and transformation assays were performed as described previously (24, 61). Briefly, 729 producer cells were irradiated with 10,000 rads and then cocultured with BJAB or HOS cells. Syncytia in BJAB cocultures were enumerated microscopically 2 to 5 days postplating. For HOS cocultures, plates were washed after 24 h incubation, Wright's stained, and scored for syncytia containing at least four nuclei per cell as described previously (10).

For the in vitro transformation assays, 10^6 irradiated 729 producer cells were cocultured with 2×10^6 freshly isolated PBMCs in the absence of IL-2 in 24-well culture plates. Transformed cells were defined as cells with continuous proliferation 8 weeks postcoculture in the absence of IL-2. HTLV expression was confirmed by detection of p19 Gag protein in culture supernatants by using an ELISA at weekly intervals. Viable cells were counted weekly by trypan blue exclusion. Wells containing transformed T cells were enumerated and phenotyped by fluorescence-activated cell sorter analysis. Cells were stained with anti-human CD3 antibody–fluorescein isothiocyanate (FITC), anti-human CD4 antibody–phycoerythrin (PE), and anti-human CD8 antibody-PE-Cy5 (BD PharMingen, San Diego, CA) and analyzed using a Coulter Epics Elite flow cytometer.

RESULTS

Construction of recombinant HTLV-1 and HTLV-2 proviral clones. We constructed recombinant proviruses in which the LTRs or the *env* genes of HTLV-1 and HTLV-2 were exchanged to determine if these sequences might be responsible for the different biological properties of these two related viruses, with specific emphasis on transformation tropism in vitro. Figure 1 shows the genome structure of HTLV and schematics of the wild-type and recombinant proviruses. The wtHTLV-1 molecular clone Ach and wtHTLV-2 molecular clone pH6neo were the parental clones used in these studies. Upon transfection into cells, both clones were capable of virion synthesis, resulting in infection and transformation of human PBMCs as determined by coculture assay. Specific restriction enzyme sites were generated by site-directed mutagenesis and were used to exchange the LTRs or *env* genes between HTLV-1 and HTLV-2 (Fig. 1B) (see Materials and Methods). There is approximately 65% homology between HTLV-1 and HTLV-2 at the nucleotide sequence level, with the lowest homology in the LTR region (31%). The amino acid homologies between HTLV-1 Env (Env-1) and HTLV-2 Env (Env-2) are 61% for the surface glycoprotein (SU) and 84% for the transmembrane protein (TM), respectively (23).

p19 Gag production by recombinant proviruses. Although the HTLV-1 and HTLV-2 genome structures are relatively similar and highly homologous, the ability of regions or genes, excluding *tax*/*rex*, to substitute for each other in a proviral context that results in virion production has not been assessed

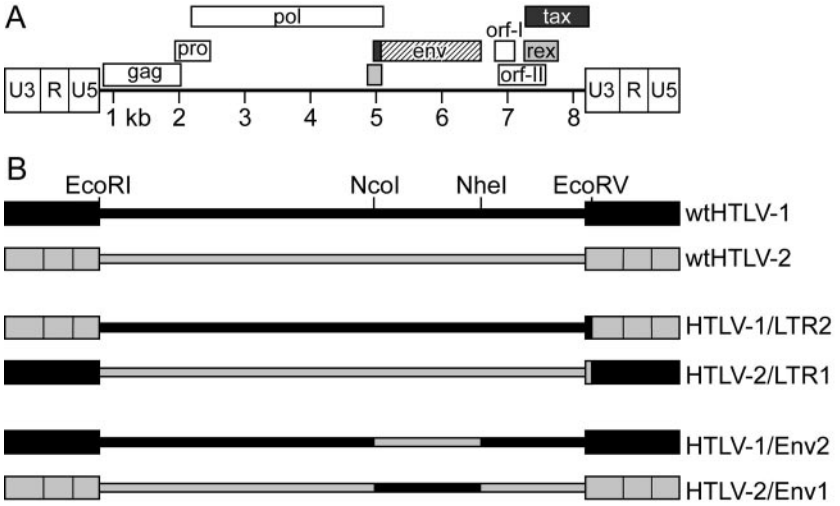

FIG. 1. Organization of the HTLV genome and the exchanged regions in recombinant proviral clones. (A) The complete HTLV proviral genome is shown schematically. LTRs are depicted with U3, R, and U5 regions. The locations of *gag*, *pro*, *pol*, *env*, *tax*, and *rex* genes and their corresponding reading frames are indicated, along with *orf-I* and *orf-II* of HTLV-1. (B) Genomic organization of the parental and recombinant proviral clones used in this study. Black boxes indicate HTLV-1 origin. Gray boxes indicate HTLV-2 origin. Specific restriction endonucleases used in the construction of recombinant HTLV are shown on the wtHTLV-1 genome.

directly. Efficient p19 Gag production from proviral clones requires a functional viral promoter as well as viral transregulatory proteins Tax and Rex; the concentration of p19 Gag in the supernatant of transfected cells has been used as a measure of virion production. The parental and LTR or *env* recombinant proviral clones were transfected into 293T cells, and p19 Gag production in the culture supernatant was quantified by ELISA. As shown in Fig. 2A, all recombinant proviral clones produced p19 Gag. Consistent with our previous studies, p19 Gag production from wtHTLV-1-transfected cells was approximately fourfold higher than that from wtHTLV-2-transfected cells (76). Cells transfected with HTLV-1/LTR2 produced slightly lower levels of p19 Gag than those transfected with wtHTLV-1, and p19 Gag production from HTLV-2/LTR1 was approximately fourfold less than that from wtHTLV-2. These differences suggest that Tax-1 can activate both LTR-1 and LTR-2 at similar levels, whereas Tax-2 displays a greater transactivation capacity on LTR-2 than on LTR-1. As would be predicted, exchange of the *env* gene did not significantly alter p19 Gag production compared to wild-type proviruses. Taken together, these results indicate that HTLV-1 and HTLV-2 LTR sequences or *env* genes are functionally interchangeable. However, levels of gene expression from the LTR recombinants were altered, likely due to differences in promoter/enhancer sequences and/or intrinsic Tax-1 and Tax-2 transactivation activities.

Establishment of 729 stable transfectants for recombinant HTLV production. To determine the capacity of recombinant proviral clones to synthesize viral proteins, direct viral replication, and induce cellular transformation, permanent 729 B-cell transfectants expressing wild-type and recombinant proviral clones were generated and further characterized. Each of the stable transfectants contained complete copies of the provirus, and the presence of the expected LTR or *env* sequences was confirmed by diagnostic DNA PCR (data not shown). To monitor the production of viral protein in these stable transfectants, the concentrations of p19 Gag in the culture supernatants were quantified by ELISA. As shown in Fig. 2B, representative stable cell clones selected for this study had p19 Gag expression similar to levels observed in transiently transfected cells (compare Fig. 2A and B).

To evaluate the capacity of the stable transfectants to produce infectious progeny virions, the transfectants were irradiated and cocultured with HOS or BJAB cells. Productive HTLV infection of these cells results in the rapid induction of syncytia (24, 40, 56). Interestingly, infection of HOS cells by either HTLV-1 or HTLV-2 results in induction of syncytia, whereas efficient syncytium induction following infection of BJAB cells generally is restricted to HTLV-2 infection. Syncytium formation is an indirect measure of viral Env expression, such that the presence of infectious virus capable of spreading throughout the culture dramatically reduces the time required for syncytium induction. As summarized in Table 1, coculture of 10^6 729-wtHTLV-2, 729-HTLV-2/LTR1, or 729-HTLV-1/Env2 cells with 5×10^5 BJAB cells resulted in syncytium formation as early as 2 days postplating. To address the efficiency with which the viruses could 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 1), and there was no apparent difference in the time course of syncytium induction between wtHTLV-2 and recombinant viruses containing the HTLV-2 Env. Stable transfectants expressing the HTLV-1 Env failed to produce appreciable syncytia in BJAB cells up to 5 days postplating. Consistent with previous studies, stable cell clones expressing either wtHTLV-1, wtHTLV-2, or recombinant viruses, irrespective of LTR or Env, were competent to induce syncytia in HOS cells (Table 1) (24, 40). These results demonstrated that the wild-type and recombinant viruses could replicate and spread efficiently throughout the culture.

Recombinant viruses can transform PBMCs. We determined whether the recombinant viruses had the capacity to

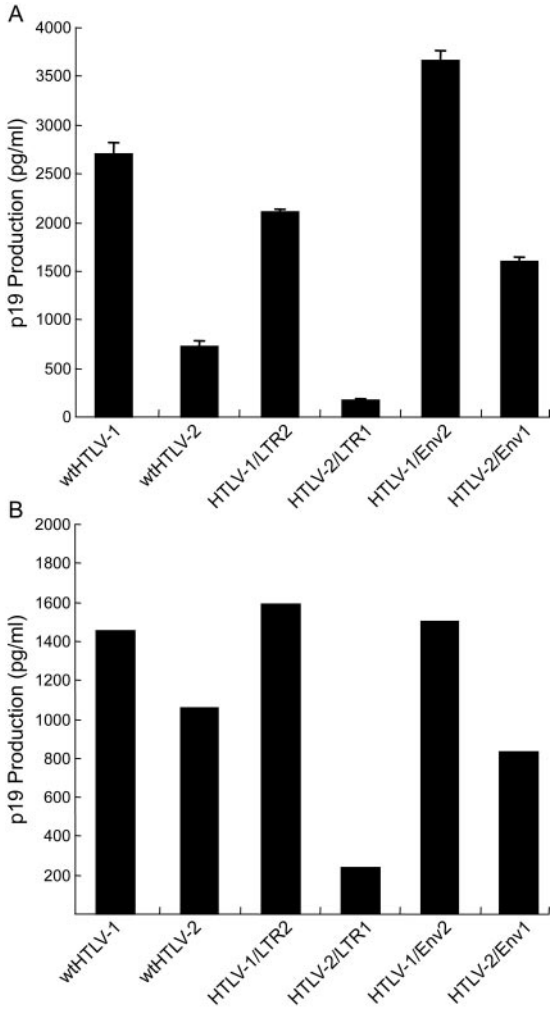

FIG. 2. Viral protein production from recombinant proviral clones. (A) p19 Gag expression in transiently transfected 293T cells. 293T cells (2×10^5) were transfected with 2 μg of proviral DNA by using Lipofectamine PLUS reagent. At 72 h posttransfection, p19 Gag expression in the culture medium was measured by ELISA. The values, which represent p19 Gag levels for three independent experiments, are normalized for transfection efficiency. Error bars indicate standard deviations. (B) 729 stable transfectants containing wtHTLV-1, wtHTLV-2, HTLV-2/LTR1, HTLV-1/LTR2, HTLV-1/Env2, or HTLV-2/Env1 were isolated as described in Materials and Methods. A representative of each cell clone used in syncytium and transformation assays is shown. Culture supernatants were harvested after 48 h of growth and tested for p19 Gag production by ELISA. In general, p19 Gag production from these stable producer cell lines showed a level similar to that from transiently transfected 293T cells.

transform human PBMCs. These experiments used a stringent transformation assay that closely mimics the in vivo infection. Irradiated 729 stable producer cells were cocultured with freshly isolated, nonstimulated PBMCs in the absence of exogenous lectins or IL-2. However, the cell-to-cell contact between irradiated HTLV producer cells and PBMCs likely results in a mixed lymphocyte reaction which activates PBMCs and HTLV receptor expression, facilitating efficient HTLV infection (36). Cell number and viability were examined at weekly intervals to monitor the transformation process and the

TABLE 1. HTLV induction of syncytia in BJAB and HOS cells

Stable transfectants	Syncytium induction in:	
	BJAB cells[a]	HOS cells[b]
HTLV-1	−	+
HTLV-2	+	+
HTLV-1/LTR2	−	+
HTLV-2/LTR1	+	+
HTLV-1/Env2	+	+
HTLV-2/Env1	−	+
Untransfected 729 cells	−	−

[a] BJAB cells (5×10^5) were cocultivated with serial 10-fold dilutions of irradiated 729 stable producer cells (10^6, 10^5, 10^4, 10^3, 10^2, and 10^1). Syncytia were counted at 2 to 5 days postplating. At least 100 irradiated 729 producer cells and 2 days of cocultivation were required for significant microscopic syncytium formation.

[b] Confluent HOS cells were cocultured with 10^6 irradiated 729 stable producer cells. Syncytia were counted after 24 hours postplating.

characteristic expansion of cells from the PBMC mixed cell population. A growth curve from a representative experiment is depicted in Fig. 3A. When cocultured with irradiated uninfected 729 cells, PBMCs showed a progressive loss of viable cells over time and eventually died off at approximately 5 to 6 weeks postplating. In contrast, the transformation process of PBMCs was apparent in coculture wells containing producer cells of wtHTLV-1, wtHTLV-2, and both LTR and Env recombinant viruses. Cell number and viability were similar for all virus-producing cells throughout the experiment. It is noteworthy that although HTLV-2/LTR1 expression, as determined in transfected cells and stable cell clones, was consistently lower than expression of other proviruses, its capacity to transform PBMCs was not significantly different. Viral replication was assessed by quantitation of p19 Gag production in the culture supernatant starting at 3 weeks postcocultivation. This is the time point at which productively HTLV-infected PBMCs typically produce viral particles and the particle production from residual irradiated viral producer cells becomes negligible (Fig. 3B). Our results indicated that the recombinant viruses, like the parental viruses, were capable of productively infecting PBMCs and inducing sustained proliferation or transformation of the cells in the absence of exogenous cytokines.

We assessed the presence of HTLV sequences in PBMCs transformed by both wild-type and recombinant viruses. Diagnostic DNA PCR was performed to determine if transformed cells contained the expected viral sequences. Figure 4 shows that high-molecular-weight DNA from cells transformed by all parental and recombinant viruses contain HTLV sequences. Sets of specific primer pairs were used to confirm the presence of the expected viral sequences. LTR-specific primer pairs distinguished between LTR-1, LTR-2, or an LTR-2/LTR-1 that results from the 3′ portion of the *tax* gene being part of the LTR (Fig. 4A). The appropriate *env* sequences were confirmed using a degenerative set of primers that detects both *env-1* and *env-2*. The amplified fragment then was digested with BamHI and ClaI, which distinguishes *env-1* from *env-2* (Fig. 4B). Together these results indicated that the expected wild-type and recombinant proviral sequences were present in transformed PBMCs.

Envelope is a major viral determinant for HTLV transformation tropism. To determine if the exchange of LTR or *env*

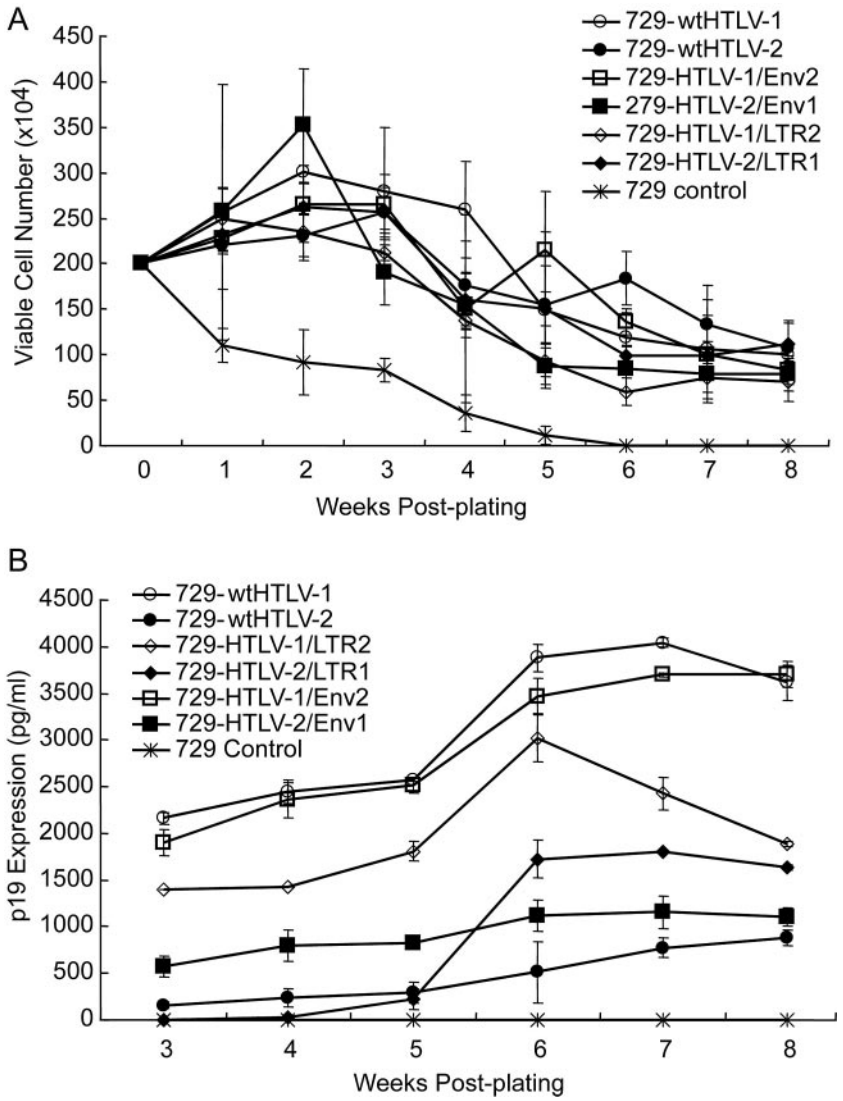

FIG. 3. Growth curve for HTLV T-lymphocyte transformation assay. Human PBMCs were isolated by Ficoll-Paque and cocultivated with irradiated (10,000 rads) 729 producer cells (729-wtHTLV-1, 729-wtHTLV-2, 729-HTLV-2/LTR1, 729-HTLV-1/LTR2, 729-HTLV-1/Env2, or 729-HTLV-2/Env1) or 729 uninfected control cells as indicated. PBMCs (2×10^6) were cultured with donor cells (1×10^6) in 24-well plates. Cells were fed once per week with medium containing 20% FBS. (A) Cell viability was determined weekly by trypan blue exclusion from 0 to 8 weeks postcocultivation. The mean and standard deviation for each time point were determined from three independent samples. (B) The presence of HTLV gene expression was confirmed by detection of structural Gag protein in the culture supernatant by p19 ELISA at 3, 4, 5, 6, 7, and 8 weeks postcocultivation. The mean and standard deviation for each time point were determined from three independent samples.

sequences altered transformation tropism, we evaluated phenotypes of cells transformed by wtHTLV-1, wtHTLV-2, HTLV-1/LTR2, HTLV-2/LTR1, HTLV-1/Env2, and HTLV-2/Env1. Since it has been well documented that HTLV transforms only T lymphocytes, individual wells of cells at 10 weeks postcoculture were stained with anti-CD3–FITC, anti-CD4–PE, and anti-CD8–PE-Cy5 and subjected to flow cytometry analysis. The data from multiple wells from at least three independent experiments are summarized in Fig. 5A. Results with wtHTLV-1 and wtHTLV-2 were consistent with previous reports by us and others in that wtHTLV-1 preferentially transforms $CD4^+$ T cells in vitro and wtHTLV-2 has a preferential transformation tropism for $CD8^+$ T cells (59, 62, 71, 76). Importantly, we showed that exchange of the *env* gene significantly alters the transformation tropism ($P < 0.0001$). HTLV $CD4^+$ T-cell transformation tropism correlated with Env-1, and HTLV $CD8^+$ transformation tropism correlated with Env-2. Exchange of the LTRs had no significant effect on transformation tropism (Fig. 5A). In addition, we also observed two minor $CD3^+$ T-cell populations that included CD4/CD8 double-positive and double-negative cells. Although the percentage of these T cells in each well was variable (approximately 0.1 to 12%), there was no significant difference in the number of CD4/CD8 double-positive or double-negative cells transformed by HTLV-1, HTLV-2, or the recombinant viruses (Fig. 5B).

In order to confirm that the cellular tropism defined by HTLV Env at the early transformation stage (8 to 10 weeks) is

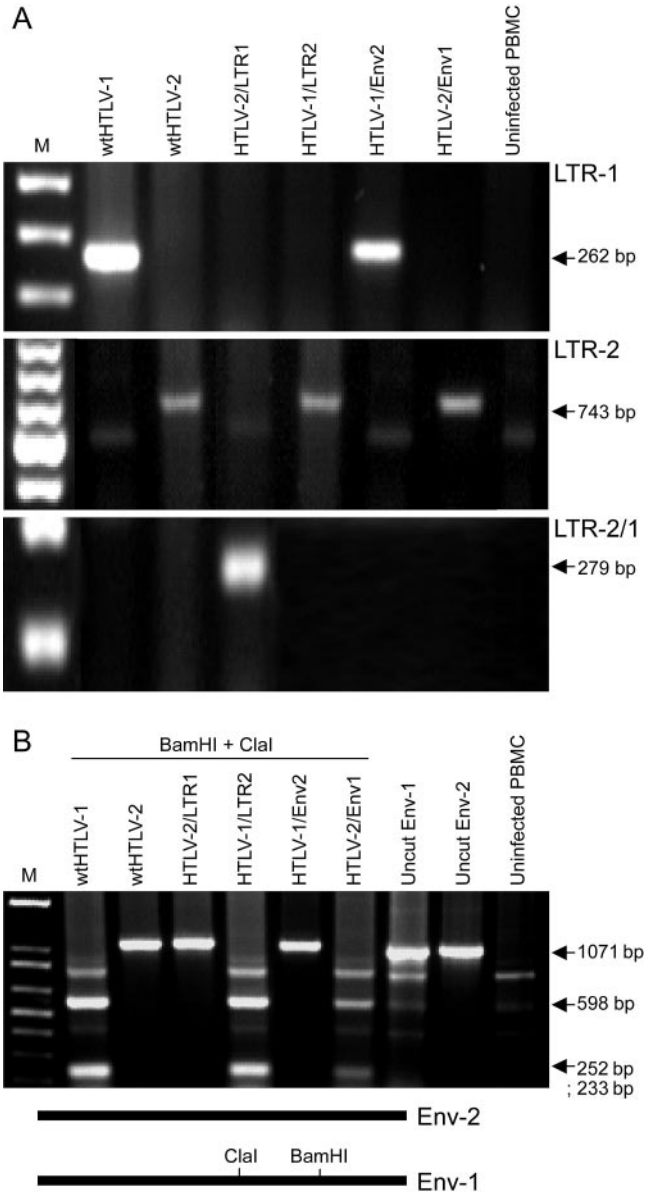

FIG. 4. Transformed PBMCs contain the expected HTLV-1 or HTLV-2 LTR and *env* sequences. (A) Specific HTLV-1 and HTLV-2 LTR sequences were PCR amplified from high-molecular-weight DNA obtained from PBMCs transformed with wtHTLV-1, wtHTLV-2, HTLV-2/LTR1, HTLV-1/LTR2, HTLV-1/Env2, or HTLV-2/Env1. Primers designed to specifically amplify LTR-1 (262 bp), LTR-2 (743 bp), or LTR-2/1 hybrid (279 bp) are as indicated. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. (B) HTLV-1/2 *env* sequences were PCR amplified from transformed PBMC DNA as indicated. The PCR-amplified product (1,071 bp) was incubated in the presence or absence of BamHI and ClaI. Products were separated on a 2% agarose gel and visualized by ethidium bromide staining. The locations of the BamHI and ClaI restriction sites that distinguish Env-1 from Env-2 are shown at the bottom.

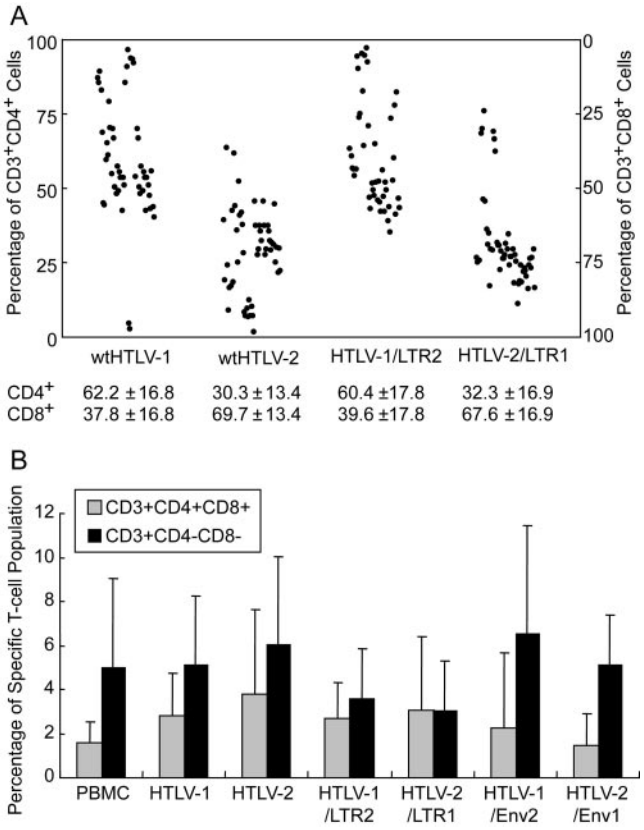

FIG. 5. Cell surface phenotype of HTLV-transformed cells. (A) Exchange of viral envelope altered the major cell types transformed by HTLV-1 and HTLV-2. Transformation assays were performed as described in the legend to Fig. 3. Wells containing transformed T cells, defined as cells with continuous growth for at least 8 weeks postplating in the absence of exogenous IL-2, were stained with anti-CD3 antibody–FITC, anti-CD4 antibody–PE, and anti-CD8–PE-Cy5 and analyzed on a Coulter Epics Elite flow cytometer. The percentages of transformed $CD4^+$ and $CD8^+$ cells in individual wells from three independent experiments for wtHTLV-1 ($n = 50$), wtHTLV-2 ($n = 50$), HTLV-1/LTR2 ($n = 44$), HTLV-2/LTR1 ($n = 50$), HTLV-1/Env2 ($n = 42$), and HTLV-2/Env1 ($n = 42$) are plotted. Mean values and standard deviations for $CD4^+$ and $CD8^+$ viral transformants are indicated. Statistics analyses using analysis of variance and Tukey's honestly significant difference tests indicate that there is significant difference between the wtHTLV-1 mean and each of the means for wtHTLV-2, HTLV-2/LTR1, and HTLV-1/Env2 ($P < 0.0001$). In addition, there is significant difference between the wtHTLV-2 mean and each of the means for wtHTLV-1, HTLV-1/LTR2, and HTLV-2/Env1 ($P < 0.0001$). The data indicate that *env* is the genetic determinant responsible for the distinct transformation tropism between HTLV-1 and HTLV-2. (B) Percentages of transformed $CD4^+/CD8^+$ double-positive and $CD4^-/CD8^-$ double-negative T cells. The mean values and standard deviations indicated are from individual wells in three independent experiments as shown in panel A. The results indicate that there is no significant difference in the percentage of these cell populations in cultures transformed by HTLV-1, HTLV-2, or recombinant viruses.

reflective of long-term cultures, we further analyzed the cell surface phenotypes of PBMC lines that have been in culture for at least 18 weeks. It should be noted that at 12 to 14 weeks postcoculture, a low concentration of IL-2 (5 U/ml) was added to the medium to facilitate the expansion of cells required for analysis. It has been previously shown that the cell population at this stage of the transformation process display mono-/oligoclonal HTLV integration patterns (37). As shown in Table 2, PBMC lines transformed by wtHTLV-1, HTLV-1/LTR2, and HTLV-2/Env1 are primarily $CD3^+/CD4^+/CD8^-$, whereas

TABLE 2. Cell surface phenotypes of long-term HTLV transformed T-cell lines[a]

PBMC culture[b]	% of specific T-cell type			
	$CD4^+$ $CD8^-$	$CD4^-$ $CD8^+$	$CD4^-$ $CD8^-$	$CD4^+$ $CD8^+$
wtHTLV-1				
1	97.1	0	1.8	0.3
2	99.6	0	0.1	0.1
wtHTLV-2				
1	22.5	72.4	1.4	3.3
2	7.1	77.6	0.3	6.7
HTLV-1/LTR2				
1	92.9	1.7	4.8	0.1
2	97.4	0.1	2.0	0.2
HTLV-2/LTR1				
1	15.8	78.1	3.5	1.7
2	9.9	75.2	4.8	7.7
HTLV-1/Env2 (1)	30.1	66.5	0	3.4
HTLV-2/Env1				
1	87.9	0	0	0.3
2	98.2	0	0	1.5

[a] PBMC cultures (18 weeks) were derived from 24-well coculture transformation assay. Exogenous IL-2 (5 U/ml) was provided after 14 weeks postplating to facilitate cell growth and expansion.
[b] 1 and 2, PBMCs from different donors.

PBMC lines transformed by wtHTLV-2, HTLV-2/LTR1, and HTLV-1/Env2 are $CD3^+/CD4^-/CD8^+$. Overall, our results indicate that the viral envelope, but not the viral LTR, is a major viral determinant of the distinct transformation tropism of HTLV-1 and HTLV-2 in vitro.

DISCUSSION

Consistent with their disease association, HTLV-1 and HTLV-2 display distinct in vivo and in vitro cellular transformation tropisms. Therefore, identification of the viral determinant(s) of cellular transformation tropism may provide the basis for understanding HTLV pathogenesis. In a recent study, we showed that the viral oncoprotein Tax and overlapping Rex did not confer the distinct difference in transformation tropism between HTLV-1 and HTLV-2. In this study, we assessed the role of the viral LTR and *env* gene in HTLV-1 and HTLV-2 cellular transformation tropism in vitro. Our results revealed that LTR or *env* gene recombinant viruses were replication competent and could transform primary human T lymphocytes. Flow cytometry analysis indicated that wtHTLV-1, HTLV-1/LTR2, and HTLV-2/Env1 had a preferential transformation tropism for $CD4^+$ T cells and that wtHTLV-2, HTLV-2/LTR1, and HTLV-1/Env2 had a preferential tropism for $CD8^+$ T cells. We conclude from our study that the *env* gene is a major viral determinant of the distinct differences in transformation tropism between HTLV-1 and HTLV-2.

The precedent for LTR-mediated cellular tropism and disease induction has been clearly demonstrated for the murine leukemia viruses (MuLV). Moloney MuLV induces T-cell lymphomas, whereas Friend MuLV induces erythroleukemia when injected into newborn mice (54, 65). Using recombinant viruses, disease tropism was mapped to the viral LTR (5, 6). Further studies demonstrated that specific enhancer sequences within the Moloney MuLV LTR in cooperation with unique T-cell transcription factors were the primary determinants for the distinct cell tropism (3, 42). Sequence comparison of the HTLV-1 and HTLV-2 LTRs revealed that they share approximately 31% homology at the nucleotide sequence level, indicating that the LTRs are the least homologous viral region between HTLV-1 and HTLV-2. The HTLV LTR contains viral transcriptional enhancer and promoter elements with binding sites for numerous cellular transcriptional factors (23), and studies suggest that there are differences in cellular transcription factor loading between LTR-1 and LTR-2 (13; Hung Fan, personal communication). Interestingly, an HTLV-2 mutant in which the three imperfect 21-nucleotide repeats in the U3 region of LTR were replaced with the cytomegalovirus immediate-early enhancer preferentially transformed $CD8^+$ T cells, similarly to wtHTLV-2 (62). This finding is consistent with the results of the present study, where we showed that exchange of the LTR does not confer the differential cellular transformation tropism of HTLV-1 and HTLV-2.

The interactions between viral Env and cellular receptors mediate viral entry into specific cell types. Evidence suggests that HTLV-1 and HTLV-2 have the same primary receptor, which is expressed ubiquitously on the surface of numerous cell types (11, 45, 68, 69). In contrast to their restricted in vivo and in vitro transformation tropism, in vitro infection with both HTLV-1 and HTLV-2 can be established in many vertebrate cell lines, including T cells, B cells, endothelial cells, glial cells, and monocytes (1, 29–31). Therefore, it would seem unlikely that the HTLV Env would be responsible for the distinct cellular transformation tropism between HTLV-1 and HTLV-2. Studies of other retroviruses have suggested that Env may affect cellular tropism by utilization of specific coreceptors and/or modulation of the intracellular environment through postentry events. In fact, different human immunodeficiency virus type 1 stains utilize different coreceptors to preferentially infect specific cell types, CXCR4 for T cells and CCR5 for macrophages (16, 48). Moreover, it was found that multiple sequences in the Env surface unit (SU), including those outside of the receptor-binding domain, dictate the T-cell tropism and cytopathic properties of feline leukemia virus (25).

In spite of the fact that both HTLV-1 and HTLV-2 can productively infect numerous cell types of different species and likely employ the same primary receptor, this study indicates that Env plays an important role in determining the major T-cell population transformed by HTLV-1 versus HTLV-2. Further investigation of the underlying mechanisms utilized by HTLV Env will be a most worthy pursuit. To date, many cellular factors have been implicated in Env-mediated HTLV infection and syncytium formation, including heat shock protein (HSP-70), various adhesion molecules (VCAM-1 and ICAM-1), membrane glycoprotein C33, HLA A2 receptor, IL-2 receptor, lipid rafts, and the glucose transporter GLUT-1 (9, 12, 20, 27, 39, 45, 47, 64, 72). The failure to identify a conclusive receptor for HTLV suggests that more than one cell surface molecule may play a critical role in HTLV entry. This raises the possibility that these molecules may have different expression levels or subcellular distributions in certain cell types or possess different affinities for HTLV-1 Env or

HTLV-2 Env. Therefore, the susceptibility of particular cells to HTLV-1 or HTLV-2 infection may be modulated by different receptor density and/or affinity, contributing to distinct transformation tropism phenotypes. The important role of receptor density in efficient viral infection of different cell types has been demonstrated for human immunodeficiency virus (14, 70). In support of this theory is the observation by us (in this study) and others of the differences in syncytium induction between Env-1 and Env-2 (10, 20, 56, 67). HTLV-2 Env expression induces efficient syncytium formation in both BJAB cells and HOS cells, whereas Env-1 mediates syncytium formation upon coculture with HOS cells only.

Another plausible explanation is that Env-1 and Env-2 may trigger specific postentry signaling pathway(s) that promote HTLV-mediated cellular transformation in different T-cell types. Env-1 gp46, in conjunction with the CD2/LFA-3 activation pathway, is mitogenic to resting T lymphocytes (15, 21, 36). Furthermore, two recent reports indicated that HTLV receptor expression is induced by T-cell activation and possibly plays a role in the immunobiology of activated T cells (46, 50). Interestingly, both Env-1-mediated syncytium formation and T-cell antigen-receptor signaling require the presence of lipid rafts (33, 53, 72). Lipid rafts are distinct cell membrane structures formed by dynamic clustering of sphingolipids and cholesterol, which are enriched in many glycosyl-phosphatidylinositol-anchored proteins, as well as Src family kinases, protein kinase C, heterotrimeric G proteins, actin, and actin binding proteins (2, 8, 66). Therefore, it is possible that differences in the interactions between Env-1 and Env-2 and a cellular receptor(s) in certain cell membrane microenvironments may induce unique downstream signaling events, leading to distinct transformation tropism. In fact, previous studies have shown that the envelope proteins of some other retroviruses can contribute to the viral transforming activities via activation of certain cellular signaling pathways. For instance, both the Akt/protein kinase B pathway and the mitogen-activated protein kinase pathway have been implicated in Jaagsiekte sheep retrovirus envelope protein-induced cellular transformation (43, 44). The envelope gp55 encoded by Friend spleen focus-forming virus can interact with and constitutively activate a truncated form of the receptor tyrosine kinase Stk, inducing erythropoietin-independent proliferation and differentiation of erythroid cells (52, 63).

Cellular transformation induced by HTLV infection is prerequisite to but is not sufficient for the eventual leukemogenesis. An additional posttransformation event(s) is ultimately required for disease development. It is clear that the viral oncoprotein Tax plays a key role in HTLV-induced cellular transformation and pathogenesis. Furthermore, comparative studies of Tax from HTLV-1 and HTLV-2 revealed that these proteins display many similarities but also some major differences (18). These differences could lead to certain unique physiological and molecular changes of transformed cells that account for the different pathogeneses of HTLV-1 and HTLV-2. In addition, both HTLV-1 and HTLV-2 encode several accessory proteins, which may also contribute to HTLV pathogenesis at the posttransformation stage. Our work indicates that the *env* gene is a major viral genetic determinant of distinct T-cell transformation tropism between HTLV-1 and HTLV-2, implying a more important role for Env in HTLV pathogenesis in addition to cellular entry. With this knowledge, we can further search for its cellular partner(s) that plays a critical role in HTLV cellular tropism to ultimately gain important insights into the mechanisms of HTLV pathogenesis.

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