Temporal Regulation of Viral and Cellular Gene Expression during Human T-Lymphotropic Virus Type I-Mediated Lymphocyte Immortalization

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An autocrine mechanism of proliferation may play a significant role in the leukemogenesis of adult T-cell leukemia, a mature T-cell malignancy caused by human T-lymphotropic virus type I (HTLV-I). To further delineate the role of HTLV-I and the interleukin 2 (IL2) system in the transformation process, human primary lymphocytes were infected by cocultivation with lethally X-irradiated MT2 cells in the presence or absence of human rIL2; the polymerase chain amplification reaction was used to examine quantitatively the expression of HTLV-I, IL2, and IL2R alpha mRNAs during early and late proliferation phases that displayed polyclonal (days 7 to 49) and oligoclonal (days 100 to 150) proviral integration, respectively. IL2 mRNA and IL2 activity were transiently expressed during the polyclonal phase but were undetectable at later time points. IL2R alpha mRNA expression remained at a constitutively high level throughout the examined time course. Viral transcripts were detectable at each time point. Expression of the *tax-rex* mRNA was inversely related to IL2 mRNA levels; it was low early in the polyclonal phase but increased 30-fold with the development of oligoclonality. In addition, paraformaldehyde-fixed HTLV-I-producing cells activated peripheral blood lymphocytes. These data suggest that HTLV-I activates human T lymphocytes. However, IL2 expression is transient, indicating a limited involvement of an IL2 autocrine growth loop in the transformation process. Lastly, another viral determinant, in addition to the *trans* activator *tax*, may be important in HTLV-I-induced T-cell proliferation.

Human T-lymphotropic virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia (ATL), a malignancy of helper/inducer T cells (reviewed by Ratner and Poiesz in reference 39). Because viremia is not present in asymptomatic HTLV-I-infected individuals or patients with ATL and because viral gene expression appears to be attenuated in infected cells (14, 22, 49), it has been difficult to ascertain the role of HTLV-I in leukemogenesis *in vivo*. Recently, though, an HTLV-I mRNA (*tax-rex*) has been detected in both types of patients by the polymerase chain amplification reaction (PCR) (25). Moreover, *in vitro* studies suggest a direct role of the virus in T-cell transformation. In culture, HTLV-I has been shown to infect and to be expressed in several human and other mammalian cell types. Interestingly, it only immortalizes normal T lymphocytes (7, 23, 32, 34). Infection and transformation of normal peripheral blood lymphocytes (PL) and cord blood lymphocytes (CB) can be accomplished with virions, but it is more efficiently performed by cocultivation with HTLV-I-producing donor cells (8, 10, 30). Generally, the *in vitro* transformed T-cell population is of the helper/inducer phenotype, closely resembling tumor cell lines established from ATL patients. These observations imply that HTLV-I contains genetic determinants capable of immortalizing a particular cell type.

How HTLV-I deregulates T-cell proliferation and thereby causes ATL is of intense interest. HTLV-I has been observed to act at two levels to affect the normal activation and proliferation processes of T lymphocytes. The first mode of action occurs at the cell surface. HTLV-I particles directly

activate resting human T lymphocytes, and viral infection is not a prerequisite for activation (12, 17). This mitogenic activity is neutralized by antisera to HTLV-I as well as by a monoclonal antibody to the surface envelope protein, gp46. Lastly, HTLV-I virions or gp46 alone may contribute to T-cell transformation by inducing a proliferative response of target cells via a T-lymphocyte-specific antigen, CD2 (12).

Trans activation of cellular genes involved in T-cell activation and proliferation is a second mechanism by which the virus appears to affect T lymphocytes. HTLV-I carries no classical oncogene and is not consistently integrated in the host genome at a particular site (43). The 3' end of the viral genome encodes two known novel regulatory proteins, *tax* and *rex*, responsible for transcriptional up-regulation and expression of viral structural proteins, respectively (4, 11, 21, 48). The *tax* protein, in addition to up-regulating viral gene expression, has been shown to *trans* activate the genes for interleukin 2 receptor (IL2R) alpha, IL2, and *c-fos* as well as IL3, IL4, *c-sis*, GM-CSF, and the major histocompatibility complex class I antigen in T cells, fibroblasts, and glial cells (15, 29, 31, 37, 42, 45). The up-regulation of genes associated with T-cell activation and proliferation implies that *tax* may act on the host cell to help maintain the activated state or to induce an autocrine loop mechanism of proliferation. This may then allow a secondary event to occur that leads to a growth factor-independent state. However, *tax* alone has not been shown to transform T lymphocytes. Therefore, it is not clear whether *tax* is necessary or sufficient for T-cell transformation.

Since ATL cells have the helper/inducer T-cell phenotype and display a large number of high-affinity IL2 receptors (20, 35), it has been suggested that HTLV-I immortalizes infected cells by means of a deregulated autocrine loop involv-

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ing the IL2 system (29, 45). However, few of the HTLV-I-infected cell lines examined express IL2, and only in rare instances do they respond to IL2 (2, 3, 18).

In the present study, to further define the role of HTLV-I and the IL2 system in T-cell transformation, the expression of HTLV-I mRNAs and IL2 and IL2R α mRNAs was monitored at intervals after the initiation of cocultivation of lethally X-irradiated MT2 cells with normal primary human lymphocytes. The results suggest that HTLV-I activates primary lymphocytes. However, the levels of IL2 and IL2R α expression do not necessarily correlate with the level of pX expression or the percentage of p19-producing cells. Thus, another viral determinant(s) such as *env*, in addition to *tax*, may be important in HTLV-I-induced lymphocyte proliferation.

MATERIALS AND METHODS

Cell lines and culture. The human HTLV-I-producing cell line MT2, uninfected T-lymphoid cell lines H9 and Jurkat E6-1 (AIDS Research and Reference Reagent Program), and promonocytic line U937 were maintained in RPMI 1640 medium containing 10% heat-inactivated (56°C for 30 min) fetal calf serum, 4 mM glutamine, 50 U of penicillin per ml, and 50 μ g of streptomycin per ml (complete medium). All cell lines were either obtained as mycoplasma-negative cultures or were treated for mycoplasma contamination with BM cyclin (Boehringer Mannheim) prior to use. Subsequent tests for mycoplasma in all cultures were negative throughout the study.

HTLV-I infection of lymphocytes by cocultivation. Mononuclear cells were isolated from heparinized umbilical cord blood of healthy newborns or peripheral blood of healthy adult donors by Ficoll-Hypaque (Sigma Chemical Co.) centrifugation. The cells were washed twice with phosphate-buffered saline (PBS) and resuspended in complete medium. Subsequently, the cells were seeded into plastic tissue culture flasks and incubated at 37°C for 1 h to remove adherent cells. Nonadherent cells were used for cocultivation with the MT2 cell line according to the method of Popovic et al. (35).

Prior to cocultivation, the MT2 cells were washed once with complete medium and then lethally X-irradiated (6,000 R). The cells were then mixed either with CB treated for 5 days with 10 μ g of phytohemagglutinin P (PHA-P) (Difco Laboratories) per ml or with unstimulated PL in a ratio of 2:5. Cell mixtures were cultured in complete medium in the presence or absence of human rIL2 (20 U/ml [Boehringer Mannheim] or 50 U/ml [Cetus]) at 37°C in a humidified atmosphere containing 5% carbon dioxide in air.

Immunofluorescence assay. The expression of the HTLV-I structural protein p19 in infected cells was detected by indirect immunofluorescence with mouse monoclonal antibody 12/1-2 (40). Cells were added to wells of toxoplasmosis slides (Bellco Glass Co.) and fixed in 1:2 methanol-acetone. Antibodies were added to the appropriate wells and allowed to incubate for 30 min at 25°C in a humidified chamber. The slides were washed with PBS-0.25% Triton X-100 for 1 h and then stained with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma Chemical Co.) for 30 min. The staining was followed by an overnight wash. Positive cells were scored the next day.

Antigen assay. Antigen assays for the detection of soluble HTLV-I structural protein p19 were performed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's specifications (Cellular Laboratories, Inc.).

Southern blot analysis. High-molecular-weight genomic DNA was prepared according to the method of Sambrook et al. (41). Ten micrograms of DNA were digested with *Bam*HI. Electrophoresis was performed in 0.8% agarose gels, and the DNA was transferred to a nitrocellulose membrane (Nitroplus 2000; Micron Separations, Inc.) The filter was hybridized to ³²P-radiolabeled full-length HTLV-I genome prepared by hexamer priming. Hybridization was for 12 to 16 h at 37°C in 3 $\times$ SSC (0.45 M NaCl, 45 mM sodium citrate), 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution is 0.2 mg of Ficoll per ml, 0.2 mg of polyvinylpyrrolidone per ml, and 0.2 mg of bovine serum albumin per ml), 5 mM Tris HCl (pH 7.5), 0.5% sodium dodecyl sulfate (SDS), 1 mM EDTA, 62 μ g of *Candida utilis* tRNA per ml, 100 μ g of salmon sperm DNA per ml, 40% formamide, and 10% dextran sulfate (molecular weight, 500,000). Blots were washed once at 25°C and four times at 68°C in 0.5 $\times$ SSC-0.1% SDS and then exposed to XAR film (Kodak) with intensifying screens at -80°C.

RNA-PCR. Total RNA was isolated from cells by the method of Chomczynski and Sacchi (6). Reverse transcription was performed in 20- μ l reaction mixtures. Total RNA (250 ng) was added to each reverse transcription mixture containing 200 pmol of the 3' complementary PCR primer, 20 nmol of each deoxynucleoside triphosphate, 1 $\times$ murine leukemia virus reverse transcriptase buffer (50 mM Tris HCl [pH 8.3], 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂), and 20 U of recombinant RNasin (Promega). The mixture was heated at 90°C for 1 min and incubated for an additional 15 min at 37°C to anneal the primer to its template. One hundred units of murine leukemia virus reverse transcriptase (United States Biochemical Co.) was added to each reaction and incubated at 37°C for an additional 40 min.

After reverse transcription, PCR was performed on the reaction products in a 100- μ l volume. Each PCR contained a 20- μ l reverse transcription mixture, plus 200 pmol of the 5' forward PCR primer, 1 $\times$ *Taq* polymerase buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3], 1.5 mM MgCl₂, 0.01% gelatin), and 1 U of *Taq* polymerase (Perkin Elmer Cetus). The amplification profiles involved denaturation at 94°C for 2 min, annealing at 56°C for 2 min, and extension at 72°C for 3 to 5 min. The number of cycles ranged from 20 to 40, depending on the number of cycles found to generate products in the linear range of amplification with respect to the initial cDNA concentration.

Ten microliters of each PCR was used for analysis. Electrophoresis of the samples was performed in 1.5% agarose gels; the samples were then transferred to nitrocellulose membranes and hybridized to hexamer-primed radiolabeled probes or end-labeled oligonucleotides. The hybridization conditions with proviral or cDNA probes were as described in the previous section. Oligonucleotide probes were hybridized to blots in 5 $\times$ SSC, 1% Sarkosyl, 5 $\times$ Denhardt's solution, 100 μ g of salmon sperm DNA per ml, and 10% dextran sulfate for 12 to 16 h at 37°C. Filters were washed once in 1 $\times$ SSC-0.01% Sarkosyl for 15 min at 25°C followed by four washes in 0.5 $\times$ SSC-0.1% Sarkosyl for 15 min each at 56°C. Filters were then exposed to XAR film (Kodak) with intensifying screens at -80°C.

PCR primers. The primers used for amplification of specific mRNA were as follows. For detection of HTLV-I mRNA, five primers were used; the numbers indicating their positions in the genome correspond to the sequence of HTLV-I-ATK (44) (see Fig. 3A). To detect the full-length *gag-pol* (8.5-kb) HTLV-I mRNA, a primer upstream of the first splice donor, B16 (5' GGCTCGCATCTCTCCTTC

ACGCGC 3' [378 to 402]) was used with complementary primer C2 (5' CGCTACGGGAAAAGATTTGGCCCAT 3' [824 to 849]) located downstream of the first splice donor site but before the first splice acceptor site. For detection of the 4.2-kb spliced *env* mRNA, B16 served as the 5' primer and ENV2D (5' TTTTGAAAAATCGAGATTAATATT 3' [5647 to 5671]) was the 3' complementary primer; ENV2D is located between the second splice donor and second splice acceptor site. ENV6A (5' AGCTGCAGCCCAAGACCCGT CGGA 3' [5141 to 5166]) was used as a 5' primer and C1 (5' CCATTTCCGAAGGGGGAGTATTTGC 3' [7648 to 7673]) was used as the 3' complementary primer for the detection of the 2.1-kb pX (*tax-rex*) mRNA. ENV6A is located between the first splice acceptor site and second splice donor; C1 is downstream from the second splice junction.

For the detection of the IL2 mRNA, a primer set that spans exon 1 to exon 4 of the IL2 gene (24) was used for PCR amplification (see Fig. 4C). The 5' primer was IL2/1 (5' GCTACAAGTGGAGCATTACTGCTG 3' [exon 1]), and the 3' complementary primer was IL2/4 (5' CTACAATGGT GCTGTCTCATCAGC 3' [exon 4]).

Primers used for the amplification of the IL2R alpha chain mRNA were located in exons 1 and 3 as defined by the sequence of Leonard et al. (26) (see Fig. 3A). The 5' forward primer R1 (5' GATGGATTCATACCTGCTGATGTGG 3') is in exon 1, and the complementary primer R4 (5' TCCAC TGGCTGCATTGGACTTTGCA 3') is in exon 3.

Primers for the constitutively expressed aldolase A mRNA (H20, H23, and A25) were as previously described by Chelly et al. (5) (see Fig. 3A). Amplification of this mRNA was used as a control.

In vitro transcription. For *in vitro* transcription of the IL2 gene, a 1.0-kb cDNA clone of the human IL2 gene was excised from clone Pst-5 (kindly provided by S. Arya and R. C. Gallo, National Institutes of Health) and inserted into the *Pst*I site of SP64. Likewise, regions of HTLV-I mRNAs containing the primer sites used for PCR amplification of *gag-pol*, *env*, and pX were cloned into Bluescript KS. The resultant clones were transcribed *in vitro* by the Riboprobe transcription method (Promega).

Proliferative response assay. Peripheral blood mononuclear cells (PBMC) were isolated as previously indicated. After the removal of adherent cells, the remaining cells were treated with PHA-P (10 µg/ml) for 7 days. The cells were washed twice with RPMI complete medium. Cells (4×10^4) in 100 µl of RPMI complete medium were seeded into wells of a 96-well plate that contained 100 µl of serially diluted supernatant from cocultivated cultures or human rIL2 (Cetus). During the last 6 h of a 48-h incubation at 37°C, 0.5 µCi of [³H]thymidine (20 Ci/mmol, NEN) was added to each well. Incorporated [³H]thymidine was harvested with a Skatron cell harvester and measured by standard liquid scintillation counting.

Neutralization of IL2 activity. Polyclonal anti-IL2 antibody EP-100 (Genzyme) was utilized to inhibit IL2 activity (47). Prior to adding PHA-stimulated cells to test wells containing supernatants with proliferation induction activity, EP-100 or control serum was added to the supernatants at a dilution of 1:20 and incubated at 37°C for 2 h. The proliferation assay was then carried out as indicated in the previous section.

RESULTS

HTLV-I infection of CB. To study the expression of HTLV-I mRNA and IL2 and IL2R alpha mRNA during long-term growth of infected cells, PHA-stimulated normal

FIG. 1. Growth of HTLV-I-infected CB in the presence of exogenous rIL2 and expression of viral p19 structural protein. (A) Extrapolated total viable cell number of X-irradiated MT2 cells (irr MT2), PHA-stimulated CB (CB), and irradiated MT2 cells cocultivated with CB (MT/CB). The number of viable cells was determined by trypan blue exclusion and corrected for cell losses due to splitting and removal of cells for assays. (B) Percentage of MT/CB cells positive for the expression of the HTLV-I structural protein p19 by indirect immunofluorescence and amount of soluble p19 present in supernatants at time intervals from the initiation of cocultivation. IFA, immunofluorescence assay.

human CB were cocultivated with a lethally X-irradiated HTLV-I-producing cell line, MT2. The cells were cultured in the presence of human rIL2 (Fig. 1A). Both PHA-stimulated CB and the cocultivated cells (MT/CB) proliferated during the first 7 days. By day 10, however, CB entered a growth crisis that resulted in the death of all cells in the culture by day 25. In contrast, MT/CB continued to divide, although at a slower rate; they doubled every 10 to 12 days. The irradiated MT2 cells did not divide and died by day 21.

At intervals from the initiation of cocultivation, cells and culture supernatants were monitored for expression of the HTLV-I core protein p19^{gag}. An indirect immunofluorescence assay monitored intracellular expression of p19, whereas an antigen ELISA was used to monitor p19^{gag} released into the culture medium, most likely in virus particles. As shown in Fig. 1B, the level of soluble p19 and the percentage of p19-positive cells displayed similar kinetics. The percentage of p19-positive and soluble p19 decreased during the first 7 days. The drop in p19 expression was probably a reflection of the death of the X-irradiated MT2 cells and the simultaneous proliferation of T lymphocytes. Between days 24 and 42, the percentage of p19-positive cells rose from 13% to 36% and remained constant through day 100. By day 150, the percentage of positive cells had increased to 87%. The soluble p19 level

FIG. 2. Southern blot of HTLV-I-infected CB at time intervals from the initiation of cocultivation of CB with X-irradiated MT2 cells. (A) DNA was isolated from cells harvested at each time point (indicated in days [d] at top) and digested with *Bam*HI. Electrophoresis was performed on a 0.8% agarose gel. The DNA was transferred to nitrocellulose and hybridized to a radiolabeled full-length HTLV-I probe (38). The right lane containing MT2 is a shorter exposure of lane 3. The first two lanes are DNA from uninfected U937 and H9 cells. The positions of selected marker fragments from an *Eco*RI and *Hind*III digest of lambda DNA are indicated on the left. (B) The location of the *Bam*HI restriction sites in the HTLV-I proviral genome according to the sequence of the proviral clone HTLV-I-ATK (44) is shown. Fragments with viral DNA sequences or viral DNA and flanking cellular sequences that hybridize with a full-length HTLV-I probe are shown at the top. LTR, long terminal repeat; Pr, protease; B, *Bam*HI.

showed similar kinetics, suggesting that virion release was directly related to the number of infected cells expressing intracellular p19.

In order to determine when the HTLV-I-infected cell population displayed a clonal pattern of proviral integration, *Bam*HI digests of DNA prepared from MT2, CB, and MT/CB cultures at selected time intervals were hybridized to a full-length HTLV-I probe (Fig. 2A). *Bam*HI cleaves at two locations in the HTLV-I genome, giving a 1.0-kb internal fragment and 5' and 3' flanking sequences associated with cellular DNA (Fig. 2B). At the early time points (days 7 and

49), DNA digests from the cocultivated cells yielded the 1.0-kb *env* fragment and a smear, indicating that the provirus was integrated at a large number of cellular loci. By day 100, oligoclonal selection had occurred that persisted through day 150. This was manifested by the presence of distinct bands of proviral DNA-cellular flanking sequence fragments. In addition, the banding pattern for proviral DNA-cellular flanking sequences of MT2 cells and MT/CB at both day 100 and 150 was distinct. This implied that the resultant cell line from cocultivation was not the MT2 cell line but was an immortalized CB cell line. Furthermore, fluorescence-activated cell sorter (FACS) analysis of the MT/CB cells indicated that they were 98% CD4+ and 83.5% CD3+, while the MT2 cells were 79.2% CD4+ and 1.7% CD3+. Human leukocyte antigen (HLA) analysis verified that MT2 and MT/CB were distinct. The HLA phenotypes of the MT/CB cells were A26 and A30, B14 and B17, Cw⁻, DR7 and DRw11, DRw52 and DRw53, DQw2, DQw3, and DQw7, and the phenotypes of the MT2 cells were A24, B51 and B60, Cw3, DR2 (DR16) and DR4, DRw52 and DRw53, and DQ1 and DQ3. Lastly, as with U937 and H9 cells which were not HTLV-I infected, DNA from CB did not hybridize to the HTLV-I probe.

Expression of HTLV-I mRNA. While the kinetics of HTLV-I mRNA expression have been examined in transient transfection experiments using a proviral clone (21), steady-state mRNA levels have not been investigated during the course of HTLV-I infection of human lymphocytes in vitro. Previous reports indicated that viral mRNA was undetectable early in HTLV-I-infected cell cultures, prior to day 50 of cultivation (10). Lack of detection may have been due to the insensitivity of techniques utilized in previous studies. Hence, to examine the expression of viral and cellular mRNAs, we adapted the PCR to amplify cDNA generated from viral mRNA transcripts (Fig. 3A). Figure 3B shows PCR-amplified HTLV-I *gag-pol*, *env*, and pX mRNA from the initiation of cocultivation through day 150. For quantitation, their concentrations were compared with known concentrations of in vitro transcribed viral RNA containing the appropriate primer sites (Fig. 3C; see Fig. 8).

The three major viral transcripts were detectable at all the examined time points. The level of mRNA expression for *gag-pol* correlated with the expression of the viral capsid protein, p19 (Fig. 1B). Initially, the level of *gag-pol* mRNA decreased between days 0 and 4 and remained low until day 100, when the oligoclonality of proviral integration became evident. At that point, *gag-pol* expression increased sixfold. *gag-pol* mRNA was always expressed at a lower level than *env* and pX mRNA. *env* mRNA expression initially decreased; it increased 10-fold between days 4 and 25, decreased 2.5-fold between days 25 and 49, and again increased 2.5-fold between days 49 and 100. pX mRNA was expressed at low levels until day 25, at which point it increased 30-fold. Initially, the pX copy number was twofold less than that of *env*, but between days 4 and 25 it increased by four- to sixfold over the level of *env* expression (Fig. 3B and C; see Fig. 8). This dramatic increase in pX mRNA expression was associated with the survival of the crisis phase observed in the uninfected cultures and the outgrowth of a cell population displaying oligoclonal proviral integration. HTLV-I mRNA was not detected in the uninfected CB or Jurkat cell total RNA.

IL2 and IL2R alpha expression. The IL2 system plays a primary role in T-cell proliferation (46). Normally, resting T cells do not produce IL2 and are not capable of responding to it. However, in response to antigen, the IL2 and IL2R alpha genes are transiently transcriptionally activated. This

A

B

C

FIG. 3. Expression of viral mRNA and IL2R alpha mRNA. (A) The positions of the primers used for PCR amplification of HTLV-I, IL2R alpha, and aldolase A mRNA are shown. The diagram for PCR of HTLV-I mRNA is shown at the top of the figure, with nucleotide positions, the positions of splice donor (sd) and splice acceptor (sa) sites, the relative positions of viral genes, and the three classes of viral mRNA indicated. Dotted lines indicate the positions of introns spliced out of the mature mRNA. Arrows represent the positions of the PCR primers. They also indicate whether the primer sequence is 5' to 3' (→) with respect to the viral RNA sequence and noncomplementary or 3' to 5' (←) and complementary to the viral RNA sequence. For the IL2R alpha gene, the positions for each of the eight exons are shown by boxes, and the location of sequences for the noncomplementary (R1) and complementary (R4) PCR primers are indicated. For the aldolase A gene, the six exons that are

results in IL2 production and high levels of high-affinity IL2R expression. Binding of IL2 to the high-affinity receptor initiates a series of events that culminate in cell division. However, the responsiveness to IL2 is transient; the cells become refractory to the proliferative signal of IL2. Because both ATL cells and in vitro HTLV-I-transformed T-cell lines express high levels of IL2 receptors (9, 20, 35) and a few of these lines produce IL2, there may be an autocrine mechanism of proliferation involved in transformation. Hence, we examined the ability of HTLV-I to induce IL2 and IL2R alpha expression (Fig. 3B and 4). During both the polyclonal and oligoclonal stages, the IL2R alpha mRNA was expressed at a constitutively high level in MT/CB cells similar to that seen in MT2 cells (Fig. 3B). In contrast, IL2R alpha expression decreased fourfold in the uninfected lymphocytes from the day of initiation to day 10, the time at which cell growth was arrested.

The expression of IL2 mRNA in MT/CB is shown in Fig. 4A. Cocultivation of irradiated MT2 cells with CB induced IL2 mRNA expression to a level one-fifth that of PL treated with PHA for 4 h. The IL2 mRNA expression was transient; it decreased approximately 14-fold between day 0 and day 4, from which time it remained expressed at a low level through day 49. Neither the irradiated MT2 cells nor the uninfected CB expressed IL2 transcripts at a detectable level. Furthermore, no IL2 mRNA was detected in RNA from unstimulated Jurkat cells.

In order to quantitate the number of IL2 mRNA molecules, we compared the intensity of the hybridizing PCR products obtained from cellular RNA with those obtained from in vitro transcribed IL2 mRNA diluted to measurable concentrations with Jurkat cell RNA (Fig. 4B). The ratios of signals, measured by densitometry, for IL2 mRNA and aldolase A mRNA of the samples from different time points after the initiation of cocultivation (Fig. 4A) were compared with those obtained from the standard values (Fig. 4B). The level of IL2 mRNA expression was found to have decreased from about 1,000 transcripts per cell at day 0, 4 h postinitiation, to a level 10-fold less than 147 transcripts per cell on days 4 to 49. Despite this residual IL2 production, the cell

differentially spliced to form three different mRNAs (27) are shown. The noncomplementary primer (H20) is located in the housekeeping specific exon, H. The complementary primers H23 and A25 are in common exons C1 and C2, respectively. N1 and N2 are nonspecific exons, and M is the muscle-specific exon. LTR, long-terminal repeat. (B) HTLV-I and cellular mRNA were detected by the RNA-PCR method outlined in Materials and Methods. All PCR reactions were for 20 cycles. 5' long terminal repeat *gag*, *env*, and *pX* sequences were excised from pMC1⁵ (38) and used as probes for hybridization to amplified viral transcripts. A cDNA clone of IL2R alpha was used to hybridize with the IL2R alpha product. Oligonucleotide H23 was used to detect the amplified aldolase A product (5). The expected sizes of the PCR products are given to the right. To eliminate possible DNA contamination that could interfere with the quantitation of the *gag-pol* mRNA, the RNA samples used in the amplification of that mRNA were treated with 0.25 U of RQ1 DNase (Promega) for 15 min at 37°C and then incubated at 65°C for 5 min to inactivate the enzyme. This treatment was capable of digesting at least 1 ng of plasmid pMC1⁵, thereby eliminating amplification of the long terminal repeat *gag* sequence contained in DNA. (C) In vitro transcribed HTLV-I RNAs corresponding to the viral mRNAs in panel B. RNAs were diluted at 10-fold serial dilutions with total RNA from Jurkat cells. The number above each lane represents the viral RNA copy number (copies per cell). Samples in panels B and C were processed and hybridized simultaneously.

FIG. 4. IL2 mRNA expression in HTLV-I-infected CB cultured in the presence of exogenous rIL2. (A) Detection of IL2 mRNA in HTLV-I-infected CB. Total RNA was isolated from MT/CB or CB at the indicated time points (in days [d] at top) or from MT2 cells or Jurkat cells. The RNA sample from day 0 was RNA harvested 4 h after the initiation of cocultivation. (B) In vitro transcribed IL2 cDNA diluted at 10-fold serial dilutions with total RNA from Jurkat cells. The number above each column represents the concentration of IL2 transcripts (transcripts per cell). PCRs for the detection of IL2 and aldolase A were for 40 and 20 cycles, respectively. Samples in panels A and B were processed and hybridized simultaneously to a radiolabeled IL2 cDNA that had been excised from clone Pst-5. (C) Diagram of the IL2 gene and the positions of the primers used for PCR of the IL2 mRNA. The positions of exons 1 to 4 are indicated by boxes, and the locations of the noncomplementary (IL2/1) and complementary (IL2/4) primers are indicated.

proliferation remained dependent on exogenously added rIL2 during the late portion of the polyclonal phase (data not shown).

HTLV-I-induced proliferation of PL in the absence of exogenous rIL2. To determine whether biologically active IL2 was produced during HTLV-I-induced proliferation of normal lymphocytes, cocultivation experiments were repeated with PBMC in the absence of exogenously added human rIL2. PBMC, like cord blood mononuclear cells, can be transformed by HTLV-I; the growth curves of both types of primary human lymphocytes infected with HTLV-I are similar (1, 24a). Figure 5 shows growth curves of a representative experiment in which PL were cocultivated with X-irradiated MT2 cells in the presence or absence of PHA. PL stimulated with PHA proliferated for about 7 to 10 days before growth was arrested and the cells began to die. In contrast, both MT/PL and MT/PL plus PHA grew rapidly for 16 days. Between day 17 and day 42, 75% of the MT/PL cells grown in the absence of PHA remained viable; total cell death occurred by day 55. MT/PL cells cultured with PHA continued to proliferate through day 45, albeit at a lower rate than in the first 2 weeks of culture. Between days 45 and 75, the viable cell number remained constant. Neither the X-irradiated MT2 cells nor the unstimulated PL divided, and there was complete cell death in both cases within 14 days. In addition, other T-lymphoid cell lines (H9 and Jurkat) did not cause PL to proliferate. The growth curves were similar

FIG. 5. Growth of HTLV-I-infected PL in the absence of exogenous rIL2. Extrapolated viable cell numbers of PL cultured in the presence or absence of PHA, in the presence of X-irradiated (6,000 R) MT2 cells alone (MT/PL), or in the presence of both X-irradiated MT2 cells and PHA (MT/PL + PHA) are shown. Cell number and viability were determined as stated in the legend to Fig. 1A.

FIG. 6. IL2 and IL2R alpha mRNA expression during cocultivation in the absence of exogenous rIL2. Total RNA was harvested at the indicated time points (in days [d] at top), and the IL2 and IL2R alpha mRNA were detected by RNA-PCR using 40 and 20 cycles, respectively. The samples at day 0 represent RNA isolated at 6 h postcocultivation with or without PHA addition. RNA isolated from Jurkat cells served as a negative control. Ald A, aldolase A.

to those of the unstimulated lymphocytes (data not shown). Lastly, FACS analysis of the cells cocultivated with X-irradiated MT2 cells indicated that the cell population after 14 days was 98% CD2+, 32% CD4+, and 34% CD8+.

The percentage of p19-positive cells in MT/PL decreased to 0% by day 10 and remained negative through day 25. In the MT/PL culture with PHA, the percentage of p19-positive cells decreased from 14.4 to 4.1% between days 4 and 25 but then increased to 6.6% by day 38. Despite a low level or absence of virus expression, cell viability was prolonged and the number of cell divisions was greater than in the noncocultivated lymphocytes.

Expression of other viral proteins has been examined through day 30 by Western blot analysis with antiserum to HTLV-I. All viral proteins present in MT2 cells were detectable and decreased with time after cocultivation in MT/PL cultures, similar to what is observed by immunofluorescence analysis. Furthermore, the level of HTLV-I transcripts decreased to near background level during this time in both cocultivated cultures. HTLV-I mRNA was undetectable in the PL culture (data not shown).

Figure 6 shows the expression of IL2 and IL2R alpha mRNA from 6 h postinitiation of cocultivation through day 16. IL2 and IL2R alpha mRNA were transiently induced in the PHA-stimulated PL (PL + PHA). The peak level of mRNA expression occurred on day 0 for IL2 and on day 4 for IL2R alpha. PL cocultivated with X-irradiated MT2 in the absence of PHA (MT/PL) attained maximum IL2 mRNA expression at day 10. The level of IL2R alpha transcripts remained high at all time points, two- to threefold higher than the expression in the X-irradiated MT2 cells at day 0. Cocultivation in the presence of PHA resulted in a twofold higher level of IL2 mRNA expression than was attained with PHA-stimulated PL at day 0. IL2 mRNA expression remained high in MT/PL cells grown with PHA through day 10 and then decreased at day 16 to a level similar to that seen in MT/PL grown without PHA or PL grown with PHA. The IL2R alpha mRNA expression in MT/PL cells grown with PHA also remained constitutively high throughout this time.

Supernatants from cocultivated cultures possess IL2 activity. At several time intervals after the initiation of cocultiva-

TABLE 1. Proliferative response of PHA blasts to supernatants from PL Cocultivated with irradiated MT2 cells

Culture	Day ^a	[³ H]thymidine incorporation ^b
Media		303 ± 79
rIL2		14,758 ± 883
Irradiated MT2	4	1,294 ± 273
PL	4	494 ± 57
PL + PHA	4	7,363 ± 620
	10	2,437 ± 240
MT/PL	4	28,032 ± 312
	10	5,296 ± 1,251
MT/PL + PHA	4	40,914 ± 4,300
	10	38,380 ± 192

^a Supernatants were saved from cultures at time intervals from the initiation of cocultivation and/or PHA addition. rIL2 concentration is 25 U/ml (Cetus).

^b Values are mean counts per minute ± standard errors of the mean of triplicate cultures.

tion of PL with irradiated MT2 cells, supernatants were harvested and assayed for the ability to induce [³H]thymidine incorporation in primary human lymphocytes (Table 1). Culture supernatants from X-irradiated MT2 cells alone, PHA-stimulated PL (PL + PHA), and PL cocultivated with MT2 cells (MT/PL) in the presence or absence of PHA contained significant amounts of activity compared with background levels. Furthermore, the MT/PL + PHA supernatants possessed 5- to 15-fold greater activity than the PHA-stimulated PL.

Because the amount of activity in the supernatants correlated with IL2 mRNA expression, we utilized a polyclonal anti-IL2 antiserum to determine whether the proliferation-inducing activity could be neutralized. Table 2 shows that the activity in the supernatants from PL cocultivated with the irradiated MT2 cells in the presence or absence of PHA was inhibited (>95%) by the anti-IL2 antibody at a level similar to that observed in control samples of rIL2-containing medium. A control rabbit serum had no effect on the lymphoproliferative response. This suggests that IL2 was the predominant factor responsible for the proliferation-induction activity found in the culture supernatants.

Paraformaldehyde-fixed HTLV-I-producing cells induce proliferation of PL. Since few of the proliferating cells early in the cocultivated cultures appeared to be infected, as determined by p19^{gag} expression, we cocultivated paraformaldehyde-fixed MT2 cells or non-virus-producing lymphoid

TABLE 2. Neutralization of proliferation by an anti-IL2 antibody

Culture ^a	[³ H]thymidine incorporation ^b with:		
	No antibody	EP-100 ^c	Control
Media	134	147	226
rIL2	13,263	247	13,619
Irradiated MT2	363	373	287
PL + PHA	2,021	1,710	2,960
MT/PL	16,954	413	16,562
MT/PL + PHA	19,060	284	17,566

^a Supernatants tested were the day 4 samples shown in Table 1. The amount of human rIL2, MT/PL, and MT/PL-plus-PHA supernatants used for neutralization were the dilutions that gave 50% of the maximum [³H]thymidine incorporation.

^b Values are the mean counts per minute of duplicate cultures.

^c EP-100 is a rabbit polyclonal anti-IL2 antibody. Normal rabbit serum served as a control.

FIG. 7. Cocultivation of paraformaldehyde-fixed HTLV-I-producing and nonproducing cell lines with PL. PL (10^5) depleted of adherent cells were mixed with 5×10^4 paraformaldehyde-fixed cells (Molt 3, Jurkat, or MT2) in wells of a 96-well plate. Cell lines were fixed with 1% paraformaldehyde in PBS for 1 h. After 5 days, 0.5 μ Ci of [3 H]thymidine was added to each well. Cells were harvested after 12 h. [3 H]thymidine incorporation was monitored by standard scintillation counting. Each value represents the mean counts per minute $\pm$ standard error of the mean of triplicate cultures.

lines with unstimulated normal primary human lymphocytes to determine whether HTLV-I-producing cells would induce lymphocyte proliferation through direct cell contact without virus infection. Whereas the non-virus-producing cells (MOLT3 and Jurkat) did not induce DNA synthesis, the fixed MT2 cells induced a level of [3 H]thymidine incorporation 60% of that of PHA-stimulated PL (Fig. 7). These data suggest that the early activation event in the immortalization process can be mediated by a cell surface-associated viral protein prior to virus infection.

DISCUSSION

We have analyzed HTLV-I, IL2, and IL2R alpha mRNA expression levels during virus-mediated immortalization of human T lymphocytes to further characterize their role in the transformation process. The temporal relationship of the expression of the viral and IL2 mRNAs to each other is summarized in Fig. 8 in the context of the polyclonal and oligoclonal proviral integration phases. It seems that HTLV-I induces early proliferation of lymphocytes mainly through the activation of the IL2 system via a preexisting viral factor on the surface of HTLV-I-producing cells, while high-level pX expression is associated with the outgrowth of immortalized infected cells.

tax has been shown to activate the IL2 and IL2R alpha genes (29, 45, 52) in transient assays and stable transfectants. While *tax* significantly up-regulates IL2R alpha, its effect on IL2 induction is weak, except in the presence of mitogens, lectins, or ionomycin. In this study, we found that during the polyclonal phase, IL2 was induced to a high level within 4 h of the initiation of cocultivation but decreased rapidly. IL2 transcripts became undetectable with oligoclonality. Moreover, the expression of IL2 was dissociated temporally from the level of *tax* expression and the percent-

FIG. 8. Expression of viral and IL2 mRNA during HTLV-I-mediated immortalization. Phases in which virally infected cells display polyclonal and oligoclonal proviral integration are indicated, as well as the points at which cells in the infected cultures grow beyond the stage at which cells of the uninfected cultures stop proliferating. mRNA expression levels are represented as copies per cell.

age of p19-expressing cells (Fig. 1B and 8). Also, while the IL2R alpha mRNA was up-regulated throughout the examined time course, its consistently high level of expression was independent of both the level of pX transcripts and the percentage of viral p19-expressing cells. Thus, one or more viral determinants other than *tax* may be important in the activation of T lymphocytes by HTLV-I.

There are at least two possible viral mechanisms that could explain how a few virally infected cells may induce T-cell proliferation and thus account for why a large proportion of the dividing cells in the polyclonal phase are p19^{gag} negative. First, envelope could be involved in T-cell activation; it has previously been shown to have mitogenic activity (17). We show here that paraformaldehyde-fixed HTLV-I-producing cells activate normal resting lymphocytes. Duc Dodon et al. (13) also reported similar data and, in addition, have shown that paraformaldehyde-fixed cells infected with vaccinia virus expressing the HTLV-I *env* gene are able to deliver a mitogenic signal. Second, it has been shown that soluble *tax* can induce the proliferation of PL (16, 28). In both studies, however, prior activation of the lymphocytes was required for soluble *tax* to have an effect. Attempts to block proliferation during the early stages of proliferation (first 3 weeks) with an anti-*tax* monoclonal antibody (kindly provided by R. Gartenhaus) at concentrations known to block soluble *tax* activity were negative (24a). Hence, it seems likely that envelope alone or in cooperation with a cellular surface protein can mediate virally induced lymphocyte proliferation.

Recently, Tendler et al. provided data showing the spontaneous proliferation of PBMC isolated from patients with HTLV-I-associated myelopathy/tropical spastic paraparesis

and from asymptomatic HTLV-I-seropositive individuals (51). PBMC from uninfected individuals and patients with ATL did not demonstrate this characteristic. The spontaneous proliferation correlated with expression of pX, IL2, and IL2R alpha. The results reported in the current study show that high-level pX expression does not necessarily correlate with IL2 expression.

Although the IL2 system may be important early in the activation of T lymphocytes by HTLV-I, it may not be sufficient for transformation. Polyclonal growth of infected cells would have persisted if an IL2 autocrine loop was the only factor important in HTLV-I-mediated transformation. However, a subset of the HTLV-I-infected cells grew which did not express IL2 mRNA. High-level pX expression was associated with this event. pX mRNA increased 30-fold with oligoclonality (Fig. 8). This steady-state level was 10-fold greater than that in MT2 cells. Thus, while *tax* may confer upon infected T cells prolonged competence, it may be that high-level pX expression induces additional cellular alterations which establish a cell state that undergoes a secondary event resulting in IL2-independent growth. Alternatively, HTLV-I may be infecting a T-cell population already predisposed for such a secondary event. This population may also be permissive for a high level of virus replication.

The transforming potential of *tax* has been demonstrated in transgenic mice and in rat fibroblasts (33, 36, 50). While these studies indicate the oncogenic potential of *tax*, they do not explain the T-cell-specific nature of HTLV-I-mediated transformation. Thus, it may be that *tax* is insufficient for T-lymphocyte transformation. A novel approach to this question was reported by Grassmann and colleagues. They found that the pX region of HTLV-I possessed immortalizing function when expressed in a recombinant herpesvirus saimiri vector (19). However, it is unclear which pX-encoded activity is responsible for the immortalizing potential and whether products derived from the vector cooperate with a pX product(s).

Further characterization of the HTLV-I *env* protein and identification of the cellular receptor for HTLV-I is necessary to determine its potential involvement in T-cell activation and transformation. It will also be important to determine whether *tax* induces permanent cellular alterations or genetic damage, since it does not appear that *trans* activation by *tax* induces continuous autocrine growth via the IL2 system. An understanding of the role of both proteins in activation and transformation would be greatly aided with the availability of an infectious clone of HTLV-I.

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