

# Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts

(AIDS/Tat/transcription elongation/U1 cells)

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**ABSTRACT** We have investigated the molecular basis of human immunodeficiency virus type 1 (HIV-1) latency in a tissue culture model and in HIV-infected people. We show that increased levels of Tat, but not Rev, can release the proviruses from latency in U1 cells. The absence of Tat in these cells is manifested by the accumulation of promoter-proximal viral transcripts, whereas the presence of Tat correlates with increased expression of viral proteins and an increase in promoter-distal transcripts. The presence of promoter-proximal transcripts also serves as a marker for latency in humans. We observed the exclusive presence of promoter-proximal viral transcripts in peripheral mononuclear cells from the majority (10/11) of asymptomatic HIV-infected individuals examined. Activation of these cells *in vitro*, and viremia *in vivo*, correlated with a switch from promoter-proximal transcription to promoter-distal transcription. These results suggest that the control between latency and replication of HIV *in vivo* is at the level of transcription elongation.

Although the progression from sero-conversion to the acquired immunodeficiency syndrome (AIDS) frequently takes years, viral replication occurs at all stages of the infection (1–4). In particular, high levels of virus can be detected in lymph nodes during the asymptomatic stages of disease progression (4, 5). Nonetheless, the fact that there is persistent replication of human immunodeficiency virus (HIV) even in stages of clinical latency does not mean that individual cells do not harbor latent proviruses (called “cellular latency”). Indeed, individual infected cells harbor proviruses that are not expressed until further cellular activation (6–8). Moreover, large numbers of latently infected cells can be detected both in lymph nodes (5) and in the blood before the actual onset of AIDS (4).

U1 cells (9) have been used as a convenient tissue culture model of HIV-1 latency because their proviruses are poorly expressed until cellular activation by a number of cytokines/lymphokines or phorbol esters that act through the cellular transcription factor NF- $\kappa$ B (reviewed in ref. 10). U1 cells are derived from U937 cells, which represent immature human CD4-positive monocytes (11), and contain two integrated HIV-1 proviruses.

Here, we find that constitutive expression of the viral protein Tat induces the expression of all major viral transcripts and proteins in U1 cells, whereas the viral protein Rev has no effect. This suggests that U1 cells are held in latency because of a lack of Tat protein. We developed a reverse transcription-polymerase chain reaction (RT-PCR) method

to detect the short, promoter-proximal transcripts that are made from the viral long terminal repeat (LTR) in the absence of Tat and showed that U1 cells synthesize large amounts of promoter-proximal transcripts relative to promoter-distal transcripts. Cellular activation with phorbol esters, or introduction of Tat alone, increased the relative abundance of promoter-distal transcripts.

We used this RT-PCR assay to determine the transcriptional state of proviruses of HIV-infected individuals *in vivo*. In 10 of 11 HIV-infected individuals with high CD4 levels (>400), promoter-proximal transcripts could be readily detected in the absence of promoter-distal transcripts in peripheral blood mononuclear cells (PBMCs). Activation in culture of latently infected cells from an asymptomatic HIV-infected individual correlated with virus production and the induction of promoter-distal transcription. These results indicate that cellular latency by HIV *in vivo* can be detected by the presence of transcriptionally active proviruses that transcribe only promoter-proximal viral RNA.

## MATERIALS AND METHODS

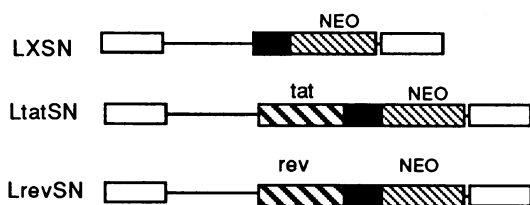
**Cells.** U1 cells were grown in RPMI medium with 10% calf serum and antibiotics. PBMCs were separated from anticoagulated whole blood with Sepacell-MN (Sepratech, Oklahoma City, OK). When cultured,  $4 \times 10^6$  cells were added to 4 ml of RPMI medium with 20% fetal bovine serum, 5% interleukin 2, and 0.12% Polybrene. One microgram of phytohemagglutinin (PHA) per ml and 50 ng of phorbol 12-myristate 13-acetate (PMA) per ml were added to the medium to stimulate PBMCs or U1 cells.

**Reverse Transcription and PCR.** RNA and DNA were isolated from  $2 \times 10^6$  cultured cells or freshly isolated PBMCs as described (12). RNA and DNA were serially diluted separately in water with 1  $\mu$ g of tRNA per ml. Each serial dilution was separately amplified. cDNA synthesis was performed with random primers in a 20- $\mu$ l reaction volume. A commercially available PCR carry-over prevention kit, and the “hot-start” system Ampli-wax (Perkin-Elmer/Cetus), was also added to each sample and cDNAs were amplified in a final volume of 100  $\mu$ l in an amplification mixture containing 1 unit of *Taq* polymerase, 1 unit of uracil *N*-glycosylase,

Abbreviations: AIDS, acquired immunodeficiency disease syndrome; HIV, human immunodeficiency virus; LTR, long terminal repeat; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription-polymerase chain reaction; MuLV, murine leukemia virus; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate.

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**FIG. 1.** Structure of retroviral vectors. Vectors were based on the MuLV genome as described (15). Open boxes represent the LTRs; black boxes represent the simian virus 40 early promoter, striped boxes are either the *tat* cDNA or the *rev* cDNA, and NEO is the G418-resistance gene. There was a 50-fold increase in chloramphenicol acetyltransferase activity in U937 cells infected with LtatSN compared with U937 cells infected with LXSNS when both were transiently transfected with a plasmid containing an HIV-1 LTR 5' to the *cat* gene. There was a >200-fold increase in p24<sup>gag</sup> in U937 cells infected with LrevSN compared with U937 cells infected with LneoSN when both were transiently transfected with a HIV-1 provirus containing a frame-shift mutation in the second exon of Rev.

0.8–1.0 pmol of each primer, 200  $\mu$ M (each) dATP, dGTP, and dCTP, 100  $\mu$ M dUTP, 10 mM Tris (pH 8.3), 3 mM MgCl<sub>2</sub>, 50 mM KCl, and 200  $\mu$ g of gelatin per ml, for 30 cycles using a thermal profile for 20 sec at 95°C, for 20 sec at 56°C, and for 40 sec at 72°C. Sequences of primers and probes were derived from the HIV-1<sub>Lai</sub> LTR. They were as follows: primer 1, GGGTCTCTGGTTAGA (positions 1–16); primer 2, GGGTCCCTAGTTAGCC (positions 58–42); primer 3, CTGCTAGAGATTTCCACTGAC (positions 181–158), where +1 is the first base of R.

The amplified product of primer pairs 1 and 2 was 59 bp in length and the product of primer pairs 1 and 3 was 182 bp in length. Amplified products were detected by liquid hybridization as described (13). Each sample was separately amplified but electrophoresed in 10% nondenaturing polyacrylamide gels and hybridized together in the same lanes for ease of comparison. The radiolabeled probe was complementary to the TAR loop region of the HIV-1 LTR: TAR loop probe, GCCTGGGAGCTCTCTGG (positions 27–43).

The probe was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and added to a final concentration of 30 pmol to samples that were denatured for 1 min at 95°C and hybridized for 3 min at 56°C.

Some samples were done by an alternative protocol that has equal sensitivity and specificity but is more rapid. Briefly, simultaneous RNA and DNA isolation was done using a Snap-o-sol RNA/DNA isolation kit (Biotech Labo-

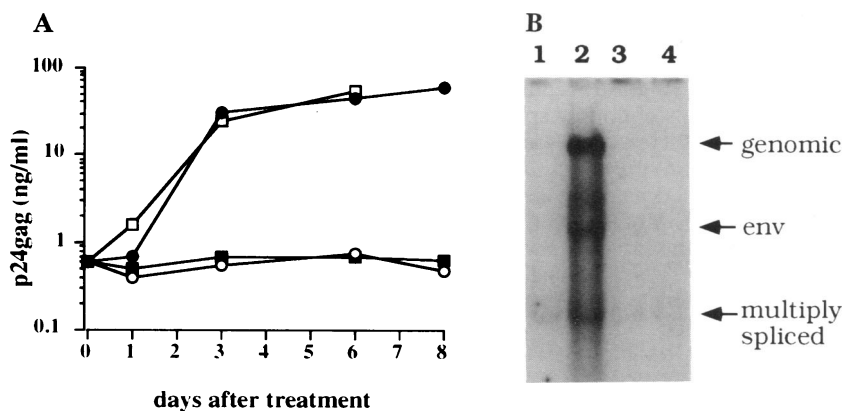
ratories, Houston), and RNA samples were treated with DNase I. Primer 1 (above) was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and gel purified. The PCR Gem-mediated hot-start technique (Perkin-Elmer) was used to increase the specificity of the amplification. PCR reactions were carried out for 25 cycles, and one-fifth of the products were loaded on a 8% denaturing polyacrylamide gel that was run at 25 mA for 2 hr, dried, and exposed to film. Negative controls without RT were performed for all samples in addition to an RNA standard.

## RESULTS

**Tat Expression Induces the HIV-1 Provirus in U1 Cells in the Absence of Cellular Activation.** To gain insight into the molecular basis of latency in HIV-infected people, we first investigated a tissue culture model of cellular latency. The activation of U1 cells by phorbol esters has been shown to increase levels of total viral RNA and especially of larger singly spliced and unspliced viral transcripts (14). To determine if the maintenance of proviral latency in U1 cells is due to insufficient levels of a viral gene product, specifically Tat or Rev, we tested whether or not the increased expression of Tat or Rev from heterologous promoters could induce these proviruses in U1 cells. To this end, retroviral vectors that expressed either Tat or Rev of HIV-1 were constructed using the genome of the murine leukemia virus (MuLV) (Fig. 1). High titer viral stocks were obtained that could infect human epithelial and lymphoid cell lines and transfer functional Tat or Rev into these cells (Fig. 1 and ref. 15).

After infection with retroviral vectors that contained Tat or Rev, viral replication was assessed by measuring levels of secreted p24<sup>gag</sup> in culture supernatants of U1 cells (Fig. 2A). Some cultures were also infected with wild-type amphotropic MuLV to control for possible effects of MuLV proteins (Fig. 2). Infections of U1 cells with the wild-type MuLV and the retroviral vector encoding Rev did not increase levels of p24<sup>gag</sup> (Fig. 2A). On the other hand, infections with the retroviral vector encoding Tat led to rapid and sustained increases in levels of p24<sup>gag</sup>. By day 8 after infection, these levels were 100-fold higher than in the control U1 cells and were nearly equivalent to that observed with PMA (Fig. 2A).

Uninfected U1 cells express small amounts of doubly spliced viral mRNAs (Fig. 2B, lane 1). We found that Tat alone increased the total amounts of viral RNA and increased levels of singly spliced and genomic viral transcripts relative to those of doubly spliced mRNAs (Fig. 2B, lane 2). On the



**FIG. 2.** Effects of Tat on the expression of viral proteins in U1 cells. (A) Tat brings U1 cells out of latency. U1 cells ( $1 \times 10^6$ ) were infected with  $2 \times 10^5$  G418 transforming units of virus (for LtatSN and Lrev SN) or with  $1 \times 10^6$  TCID<sub>50</sub> units of wild-type MuLV. An aliquot of cells was also treated with PMA/PHA. The y axis is a logarithmic scale. ●, LtatSN-infected cells; ○, LrevSN-infected cells; ■, wild-type amphotropic MuLV-infected cells; □, PHA/PMA-treated cells. (B) Seven days after infection, RNA was collected from cultures infected with the same retroviral vectors as in A. Ten micrograms of total RNA was loaded per lane. Lane 1, unstimulated U1 cells; lane 2, U1 cells infected with LtatSN; lane 3, U1 cells infected with LrevSN; lane 4, U1 cells infected with wild-type MuLV. Sizes of the major RNA species, which represent genomic, env, and multiply spliced viral transcripts are marked.

other hand, the quantity and splicing patterns of these mRNAs did not change when *rev* or *neo* genes were introduced into these cells (Fig. 2A, lanes 3 and 4). These data indicate that constitutive expression of Tat induces the expression of integrated proviruses and can substitute for cellular activation of U1 cells. Thus, the previously observed phenotype of increased expression of singly spliced and genomic viral transcripts that followed cellular activation of U1 cells (14) probably reflected the increased synthesis of Rev that occurred after sufficiently high levels of Tat were achieved.

The presence of functional Tat in activated and nonactivated U1 cells was indirectly assayed by fusing U1 cells with polyethylene glycol to an indicator cell line that contained a single integrated copy of the HIV-1 LTR linked to the  $\beta$ -galactosidase reporter gene that is sensitive to levels of Tat (16). The results of these experiments (not shown) demonstrated that nonactivated U1 cells express little Tat but that cellular activation increases the synthesis of Tat. This suggests that activation of U1 cells by phorbol esters acts, at least in part, through increasing Tat levels.

**Tat Affects the Ratio of Promoter-Proximal to Promoter-Distal Viral Transcripts in U1 Cells.** Nuclear run-on experiments demonstrated a steep polarity of HIV-1 transcription from the LTR that was reversed by Tat (17–20). These transcriptional states were reflected in the accumulation of short, prematurely terminated TAR transcripts in the absence of Tat and of long polyadenylated viral transcripts in the presence of Tat (18, 21). Given our results that insufficient levels of Tat were indicative of proviral latency in U1 cells (Fig. 2), we tested whether these different viral RNAs could be used as markers for proviral latency.

To determine levels of short and long viral transcripts, we used quantitative RT-PCR (12, 13). First, pairs of primers that corresponded to 5' and 3' ends of TAR (primers 1 and 2) and to the 3' end of the U5 region (primer 3) were synthesized (Fig. 3A). Primers 1 and 2 would amplify both short and long transcripts, while primers 1 and 3 would amplify only RNA that was longer than the TAR region (Fig. 3A). Given the steep transcriptional polarity in the absence of Tat, and the fact that only prematurely terminated transcripts that contain the TAR RNA stem-loop are stable in cells (17, 19, 22), the second set of primers (1 and 3) should detect very few, if any, prematurely terminated transcripts. Both sets of primers amplified with equal efficiencies *in vitro* transcribed RNA and plasmid DNA and were sensitive to <100 copies of nucleic acid (Fig. 3B and data not shown).

Ratios of short to long transcripts were assessed by comparing autoradiographs of amplified DNA obtained with both sets of primers (Fig. 3B, left). Nonactivated U1 cells transcribed predominantly short transcripts (Fig. 3B, righthand panel). This correlated with the low levels of doubly spliced transcripts in U1 cells (Fig. 2B, lane 1). However, 4 and 8 days after infection with amphotropic retroviruses coding for Tat (Fig. 1), ratios of short to long transcripts decreased by 10- to 100-fold (Fig. 3B, central panels). Moreover, 4 days after the administration of PHA/PMA, the ratios of short to long transcripts decreased similarly (Fig. 3B, righthand panel). Activation of U1 cells with PMA led to a more rapid qualitative change in HIV-1 transcription, which suggests that activated U1 cells expressed Tat earlier than those infected with amphotropic retroviral vectors. These experiments suggest that escape from viral latency is accompanied by Tat-mediated increase in elongation efficiency of RNA polymerase II.

**Detection of Promoter-Proximal Transcripts in PBMCs from HIV-Infected Individuals with High CD4 Levels.** Because of the concordance between assays of Tat function and RT-PCR in U1 cells, we next asked whether RT-PCR could be used to detect this form of proviral latency in PBMCs from HIV-1-

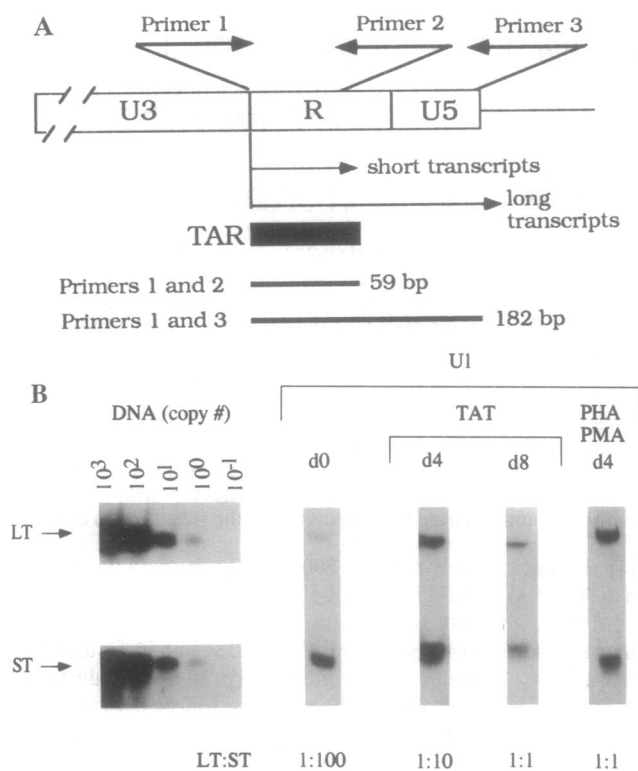


FIG. 3. Detection of transcripts initiated from the HIV-1 LTR in U1 cells by RT-PCR. (A) Schematic representation of the HIV-1 LTR, oligonucleotide primers used to amplify viral transcripts, and expected sizes of PCR products. The three primers are shown above the boxes representing U3, R, and U5 regions of the HIV-1 LTR (TAR is contained within R). Short, prematurely terminated, nonpolyadenylated transcripts (ST) of 59 nucleotides and long transcripts (LT) of 182 nucleotides, which are amplified by primer pairs 1 and 2 and 1 and 3, respectively, are diagrammed below the HIV-1 LTR. The filled box represents the TAR region. (B) Ratios of short to long transcripts in U1 cells infected with retroviruses encoding Tat and treated with PHA/PMA. Ten-fold dilutions of plasmid DNA amplified with both primer pairs are shown on the left. On the right, RNA from U1 cells was amplified with the primer pairs shown in A. d0, Nonactivated U1 cells; TAT d4, U1 cells 4 days after infection with LtatSN (Fig. 1); Tat d8, U1 cells 8 days after infection with LtatSN; PHA/PMA d4, U1 cells 4 days after stimulation with PHA and PMA. Ratios of the long transcripts (LT) to short transcripts (ST) are given below each lane.

infected individuals. To this end, RNA and DNA were extracted from PBMCs of 9 HIV-1-infected individuals with high CD4 levels (CD4 cells per  $\text{mm}^3$  ranged from 1051 to 502, with a median level of 620). None of these individuals was viremic, and all were asymptomatic except for oral candida in two individuals.

In all nine cases, viral DNA could be amplified from cells of these individuals with both primer pair 1 and 3 (Fig. 4C) and primer pair 1 and 2 (Fig. 4D). This indicates that the PBMCs of each individual harbored HIV-1-infected cells. Moreover, in all nine cases the presence of promoter-proximal transcripts (short transcripts) could be readily detected (Fig. 4B) in the absence of promoter-distal transcription (Fig. 4A). Controls of the reactions without reverse transcriptase verified that the promoter-proximal signal was due to RNA rather than DNA (Fig. 4B, lanes marked with a minus sign). This result indicates all (9/9) of these non-AIDS individuals with high CD4 counts harbored latent proviruses that were transcriptionally active, but deficient in transcription elongation.

To determine if, as in U1 cells, activation of latently infected cells could change the ratio of promoter-proximal to

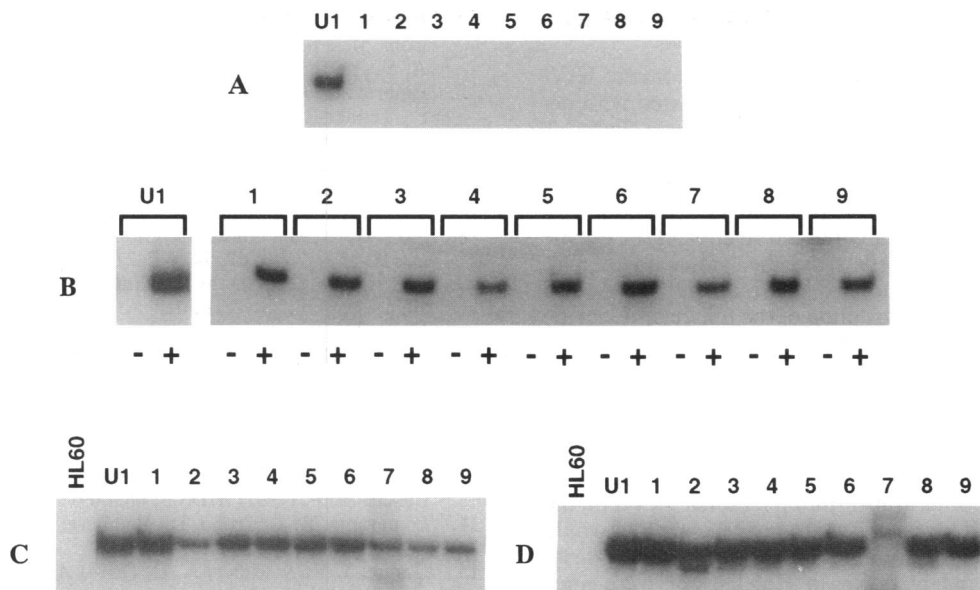


FIG. 4. Analysis of HIV-1 transcripts in PBMCs of infected individuals with high CD4 counts. RNA and DNA were isolated from buffy coats of individuals labeled 1-9 and subjected to PCR in the presence or absence of reverse transcriptase (see text). (A) RT-PCR with primer pair 1 and 3 (long transcripts, Fig. 3A) using an RNA template from the PBMCs of individuals 1-9. The lane marked U1 is RNA from activated U1 cells. (B) RT-PCR with primer pair 1 and 2 (short transcripts, Fig. 3A) using an RNA template from the PBMCs of individuals 1-9. The lanes marked U1 are RNA from activated U1 cells. The minus or plus sign indicates whether or not reverse transcriptase was added to the reaction prior to the PCR. (C) PCR with primer pair 1 and 3 using a DNA template from the PBMCs of individuals 1-9. HL60 is DNA from HL60 cells as a negative control. (D) PCR with primer pair 1 and 2 using a DNA template from the PBMCs of individuals 1 through 9. CD4 counts of individuals 1-9 were 1051, 636, 545, 771, 595, 620, 840, 546, and 505, respectively. All patients had received AZT except no. 9.

promoter-distal transcription, the PBMCs of one individual with a CD4 count of 420 were activated by coculture with autologous cells in the presence of PHA. As in Fig. 4, only promoter-proximal transcripts could be detected in these uncultured cells (Fig. 5, day 0). However, after activation, ratios of short to long transcripts declined to <10-fold (Fig. 5, day 3 and day 14). Increased levels of long transcripts were also correlated with the appearance of p24<sup>agg</sup> in culture supernatants (undetectable at day 0; 9 pg/ml and >100 pg/ml

on days 3 and 14 after cellular activation, respectively). RNA was also extracted from PBMCs of two AIDS patients with CD4 counts of 10 and 36 (Fig. 5). As expected, because of increased levels of viral replication observed late in the disease (23, 24), the ratio of short to long transcripts in these patients approached one (Fig. 5). Promoter-distal transcripts could also be detected in the PBMCs of an asymptomatic and p24<sup>agg</sup> negative individual with a CD4 count of 412 (Fig. 5). These results indicate that cellular latency *in vivo* can be detected by the presence of promoter-proximal transcripts and that activation of proviruses, either in culture or *in vivo* during disease progression, marks a transition to promoter-distal transcription.

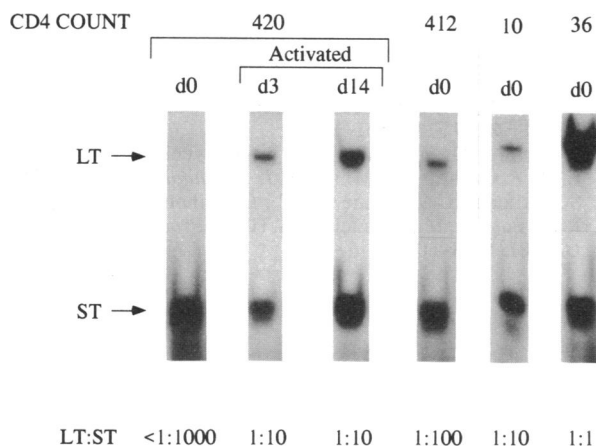


FIG. 5. Activation of latently infected cells results in promoter-distal transcription. RT-PCR products from PBMCs of infected but asymptomatic patients (CD4 counts of 420 and 412) and AIDS patients (CD4 counts of 10 and 36) are shown. The asymptomatic patients were Walter Reed stage I and were not receiving antiviral therapy at the time the blood was taken. The AIDS patients were Walter Reed stage V and stage VI and were receiving 3'-azido-3'-deoxythymidine (AZT) at the time the blood was taken. PBMCs from one asymptomatic patient (CD4 count 420) were activated with PHA/PMA for 3 days and/or by cocultivation with stimulated feeder cells for 14 days. Ratios of long to short transcripts (LT:ST) were estimated as in Fig. 3 and are shown under each lane.

### DISCUSSION

We show that the HIV-1 provirus in U1 cells can be released from latency by an increase in the intracellular level of the viral transactivator, Tat. The absence of Tat is correlated with a predominance of promoter-proximal transcripts over promoter-distal transcripts. The addition of Tat alone, or stimulation of the cells with phorbol esters, changes the ratio of promoter-proximal to promoter-distal transcripts. These results allowed us to develop an assay for the detection of HIV cellular latency in infected people.

The molecular basis of HIV-1 latency in cells can be explained by blocks at several stages of the viral life cycle. There is evidence to support incompletely reverse transcribed RNA (25), unintegrated proviral DNA (7), and integrated proviruses that are either transcriptionally silent or express only doubly spliced viral mRNAs (26). Our study suggests the existence of another state in which promoter-proximal transcription from the HIV-1 LTR predominates. These states of viral latency and replication are distinguished by elongation-deficient and elongation-competent transcription complexes that produce short transcripts and long transcripts, respectively. In all individuals with a CD4 count above 500 studied here, the presence of promoter-proximal

transcripts could be detected in the absence of promoter-distal transcripts.

In U1 cells, and in the PBMCs of an asymptomatic HIV-1-infected individual, cellular activation correlated with decreased ratios of short to long transcripts and new synthesis of viral proteins. We also observe this phenotype in AIDS patients and in one HIV-infected individual with an intermediate CD4 level (Fig. 5). It is possible that detection of promoter-distal transcripts might be a reflection of the escape of the activated infected cells from the lymph nodes and, therefore, detection of promoter-distal transcripts might be a sensitive marker for the destruction of the follicular dendritic cell networks that occurs early in disease progression (reviewed in ref. 27).

Various mechanisms have been proposed to explain the regulation of assembly of elongation-competent polymerase complexes (28). Presumably, in latently infected cells that transcribe only promoter-proximal viral RNA, the basal levels of NF- $\kappa$ B (or other transcription factors that act on the LTR) are so low that levels of Tat are not reached that would affect a change from promoter-proximal to promoter-distal transcription. This might occur in T cells that were once activated such that viral integration occurred (25, 29) but then became quiescent and part of the pool of infected "memory" T cells. Indeed, these T cells as defined by the CD45R0 marker contain abundant proviral DNA (30, 31). Activation of these cells by antigen would increase the basal level of transcription from the LTR, which, in turn, would increase the level of Tat and activate proviral expression. Given that increased levels of viremia are observed late in the disease (23, 24), it is possible that transcriptional activation of these latent proviruses in the periphery plays a role in T-cell depletion and in the progression to AIDS.

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