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The Long Terminal Repeat Is Not a Major Determinant of the Cellular Tropism of Human Immunodeficiency Virus Type 1

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The long terminal repeats (LTRs) of human immunodeficiency virus type 1 (HIV-1) strains from the central nervous systems of four patients with AIDS and of an HIV-1 isolate which is highly macrophage-tropic were isolated by using the polymerase chain reaction. In transient transfection assays, these LTRs demonstrated no significant difference in basal or stimulated levels of transcription in any of a variety of cell lines tested, compared with expression directed from the LTR of a T-lymphocyte-tropic strain of HIV-1. Chimeric viruses were created with the LTRs of the macrophage-tropic and brain-derived viruses ligated to the viral backbone from a T-lymphocyte-tropic strain. No change in cellular tropism was demonstrated with these chimeric viruses. Thus, unlike the LTRs of some murine retroviruses, the LTR of HIV-1 does not appear to play a major role in determining cellular tropism.

Human immunodeficiency virus type 1 (HIV-1) infects a variety of cell types both in cell culture and in vivo (7, 14, 22, 29, 35). The CD4-positive T lymphocyte and the monocyte/macrophage appear to be the major targets for HIV-1 in vivo (7, 29). Although the CD4-positive T lymphocyte may be the major reservoir for HIV-1 in the bloodstream (29), the monocyte/macrophage is thought to be the primary tissue reservoir (7, 22). The monocyte/macrophage is also the predominant cell type infected in the central nervous system (7, 35).

It has recently been demonstrated that strains of HIV-1 exist which exhibit a specific tropism for either T lymphocytes or monocyte/macrophages (5–7, 14). Many of the macrophage-tropic strains have been isolated from the central nervous system (5, 7, 14). Understanding the molecular mechanisms which determine the cellular tropism of HIV-1 strains would be helpful in elucidating the pathogenesis of HIV-1 infection (11). Because a variety of murine retroviruses appear to exhibit cellular tropism on the basis of specific alterations in their long terminal repeats (LTRs) (4, 12, 26), we investigated whether the LTR of HIV-1 was involved in determining cellular tropism.

To examine the role of the LTR in transcriptional control, plasmids were constructed with the LTRs of different HIV-1 isolates placed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. For this purpose, a macrophage-tropic HIV-1 isolate (Ba-L) (7) (kindly provided by Mikulas Popovic) was grown on primary human macrophages, and genomic DNA was isolated by using standard methods (2). The 3′ LTR of Ba-L was amplified, from nucleotides 9009 to 9686, by using a polymerase chain reaction as previously described (21). After amplification and gel purification, this segment of the Ba-L genome was digested with *Kpn*I and *Hin*dIII. This DNA fragment, containing Ba-L LTRs, was then introduced into a plasmid (SP65; Promega, Madison, Wis.) upstream of the CAT gene, which was cut from a previously described HIV CAT plasmid (19) and introduced into SP65 from the *Hin*dIII to the *Bam*HI sites.

Because the vast majority of cells infected in the central nervous system by HIV-1 are monocyte/macrophages (7, 35), brain tissue from patients who have died from HIV-1-related disease should be an excellent source of macrophage-tropic strains in vivo. We therefore prepared genomic DNA from the brain tissue obtained at autopsy from four HIV-1-infected individuals. By using the above-cited polymerase chain reaction technique, we were able to amplify and isolate LTRs from all four brain samples. They were cloned (*Kpn*I to *Hin*dIII), as well, into the CAT-containing plasmid, upstream of the CAT gene. Because there may be multiple strains of HIV-1 in the tissues of infected individuals and in macrophage cultures of the uncloned Ba-L isolate (6), at least two independent clones from each sample were characterized in detail.

These plasmids were then sequenced by using a chain termination method (Sequenase; United States Biochemical Corp., Cleveland, Ohio) (28). The LTRs differed from each other and from the sequence of the R7 derivative of the HXB2 LTR (15), which was also sequenced in the above plasmid construct, by 2 to 4%. This is in accordance with the findings for other LTRs from various HIV-1 isolates (17). No mutations in the defined regulatory motifs of the HIV-1 LTR were demonstrated (27). Most nucleotide differences clustered in the region of the LTR upstream of the last AP-1 binding site (nucleotides −445 to −365) and between the postulated NFAT-1 binding site and the nuclear factor-κB enhancer motifs (nucleotides −201 to −106) in the U3 region. No mutations were noted in the putative negative regulatory element or downstream of the TATA box (data not shown). The individual clones from brain samples or the Ba-L-infected macrophages differed from one another by only a few nucleotides, which were located within nonregulatory domains (data not shown).

To determine whether the various cloned LTRs exhibited differing levels of function, the HIV-1 LTR CAT constructs were transfected into a variety of cell lines by using a DEAE-dextran method previously described (23). The levels

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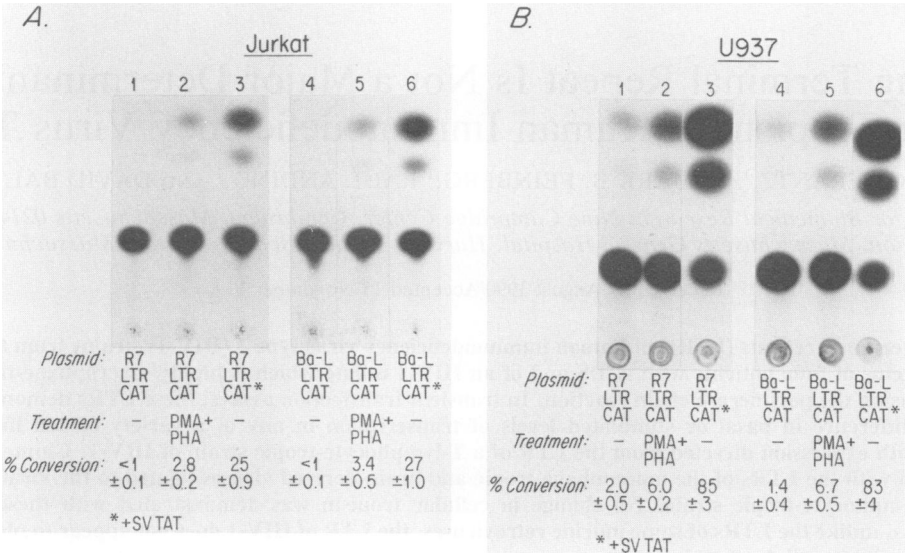

FIG. 1. Transient transfections of HIV-1 LTR CAT constructs. (A) Jurkat cells; (B) U937 cells. Lanes 1, R7 LTR CAT, no treatment; lanes 2, R7 LTR CAT treated with phorbol 12-myristate 13-acetate (50 ng/ml) and phytohemagglutinin (2 μg/ml); lanes 3, R7 LTR CAT cotransfected with SVTAT; lanes 4, Ba-L LTR CAT, no treatment; lanes 5, Ba-L LTR CAT treated with phorbol 12-myristate 13-acetate and phytohemagglutinin; lanes 6, Ba-L LTR CAT cotransfected with SVTAT. The LTRs used in these constructs were amplified by using an oligonucleotide encompassing nucleotides 9009 to 9030 (5′-CCTCAGGTACCTTTAAGACCA-3′) as a sense primer and an antisense primer including nucleotides 9665 to 9686 (5′-GAGGGATCTCTAGTTACCAG-3′) on the HXB2 genome (17, 25). These represent extremely conserved regions of the HIV-1 genome (17). Ten micrograms of the LTR CAT plasmids was transfected into 10^7 cells. Two micrograms of the SVTAT plasmid was cotransfected in appropriate experiments. The quantity of protein used in the CAT assays was normalized to 70 μg for U937 and 100 μg for Jurkat cells. The percent conversion of [^{14}C]chloramphenicol was measured, and the data are represented as the arithmetic mean of at least three transfections ± the standard error of the mean from two plasmid preparations. The autoradiographs are representative examples of these experiments.

of LTR-directed transcription were compared with those of a CAT construct containing the LTR of the R7 derivative of HXB2 (R7 LTR CAT). The HXB2 virus is a T-lymphocyte-tropic HIV-1 clone which replicates poorly in macrophages (6, 7). The R7 derivative of HXB2 has a deleted restriction site, *Eco*RI, in the 3′ sequences flanking the viral genome but otherwise is indistinguishable from the original HXB2 clone (15, 25).

The human T-cell line Jurkat (20) and the promonocytic cell line U937 (32) were used as target cells to test the efficiency of these LTR CAT constructs (Fig. 1 and 2). In some cases, transfected cells were stimulated 24 h after transfection with phytohemagglutinin (Sigma Chemical Co., St. Louis, Mo.) and phorbol 12-myristate 13-acetate (Sigma), which stimulate LTR-driven transcription by generation of active nuclear factor-κB (19). Cell lysates were harvested at

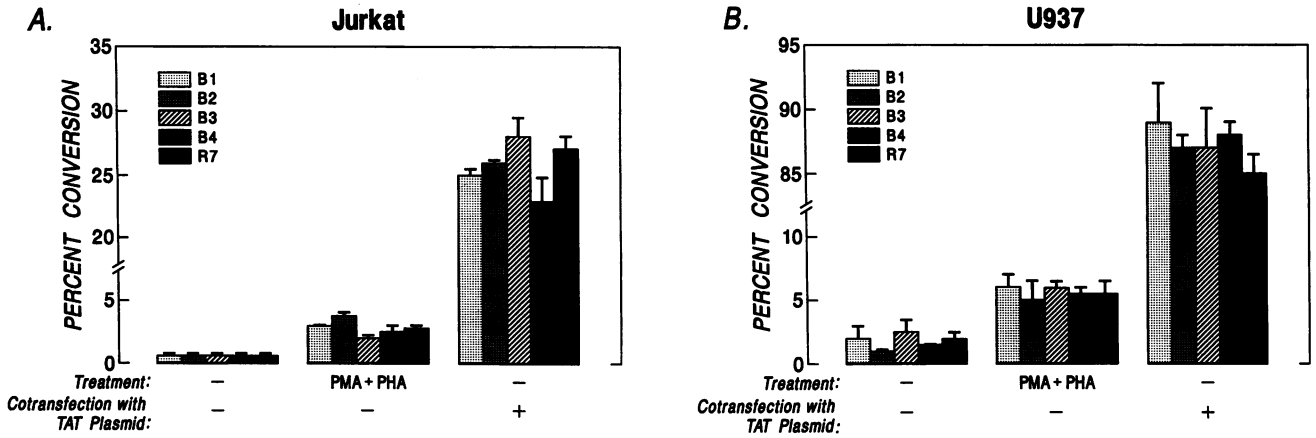

FIG. 2. Transient transfections of brain-derived LTR CAT constructs. (A) Jurkat cells; (B) U937 cells. The bar graphs represent the percent conversion of [^{14}C]chloramphenicol presented as the arithmetic mean of at least three independent transfections plus the standard error of the mean from at least two plasmid preparations. B1, Brain 1 LTR; B2, brain 2 LTR; B3, brain 3 LTR; B4, brain 4 LTR; R7, R7 LTR. PMA, Phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin.

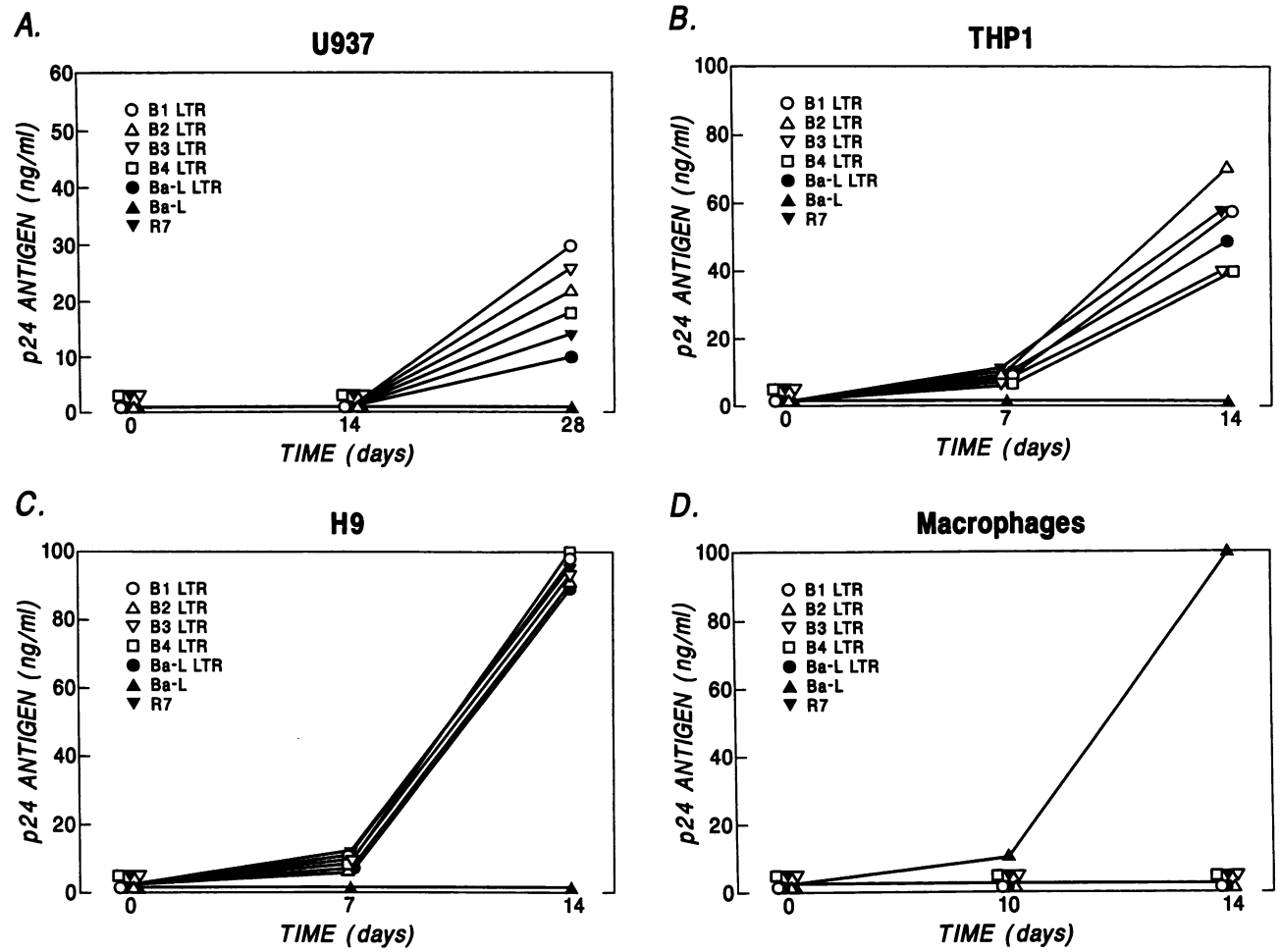

FIG. 3. Viral growth curves of chimeric viruses. (A) U937 cells; (B) THP-1 cells; (C) H9 cells; (D) primary macrophages. Two hundred nanograms of p24 antigen was used to infect cells with a variety of viruses. The p24 antigen level was measured in the supernatant at various times. B1 LTR, B2 LTR, B3 LTR, B4 LTR, and Ba-L LTR are viruses with R7 HXB2 backbones and brain- or Ba-L-derived LTRs. Ba-L and R7 viruses are nonchimeric virus strains.

44 h posttransfection, and the protein levels were normalized by using the method of Bradford (3). CAT activity was determined as previously described (10). Some cultures were cotransfected with a plasmid, SVTAT, which contains the HIV-1 transactivator gene, *tat* (1), under the control of the simian virus 40 promoter (18).

As demonstrated in Fig. 1A (lanes 1 and 4) and B (lanes 1 and 4), the levels of baseline CAT activity did not differ when driven by LTRs from R7 (T-lymphocyte-tropic) or Ba-L (macrophage-tropic) in either the Jurkat or the U937 cell line. Furthermore, stimulation of these transfections by using phytohemagglutinin plus phorbol 12-myristate 13-acetate yielded no significant differences in activity (Fig. 1A and B, lanes 2 and 5). The R7 and Ba-L LTR CAT constructs were strongly transactivated by Tat in both cell lines (Fig. 1A and B, lanes 3 and 6). Further dilutions of the cell extracts from the cultures of U937 cells cotransfected with the Tat-producing plasmid, into the linear range for CAT activity, demonstrated similar levels of transactivation of both plasmids (data not shown). Transfections of the more mature monocytic cell line THP-1 (33) also revealed no significant differences in baseline or stimulated CAT activities between these plasmids (data not shown).

When the constructs containing the LTRs obtained from brain tissues or R7 were transfected into Jurkat and U937 cells, there were no significant differences in the unstimulated or stimulated levels of CAT activity among them (Fig. 2). The brain-derived and R7 LTRs were also equally transactivated by Tat in both cell lines (Fig. 2).

In summary, when tested by transient transfection, the LTRs from a macrophage-tropic virus and HIV-1-infected brain tissue did not differ in transcription efficiency from each other or from the LTR of a T-lymphocyte-tropic strain in various cell types.

To further examine whether HIV-1 tropism might be based on differences in the LTR, chimeric viruses were constructed. A plasmid (pGEM3; Promega) containing the 3′ region of HXB2, from the *Bam*HI site at position 8,474 to an *Xba*I site in the 3′ cellular flanking sequences, was partially digested with *Hin*dIII, and the linearized plasmid was digested with *Kpn*I. This removed the U3 and R regions of the 3′ LTR. The various LTRs from Ba-L and the brain tissues were then ligated to the above plasmid at the *Kpn*I and *Hin*dIII sites. This plasmid, with a substituted LTR, was then digested with *Bam*HI and *Xba*I, which cut at position 8,474 and outside the HXB2 genome, respectively. This

*Bam*HI-*Xba*I fragment was then ligated to a plasmid containing the HXB2 genome from which the native *Bam*HI-*Xba*I fragment was removed. This procedure produced a plasmid with an HXB2 backbone and a variant LTR (U3 and R regions) in the 3′ position. The replacements of the LTRs were confirmed by sequence analyses. After transfection into COS-1 cells (8) and one round of reverse transcription, the U3 region of the viral RNA would be derived solely from the 3′ LTR of the provirus (34). This procedure, therefore, yielded viruses with a U3 region containing all of the recognized regulatory regions of the variant LTRs (27). This was verified on one occasion by using PCR amplification, cloning, and sequencing (data not shown).

Supernatants from these transfected COS-1 cells were harvested, and p24 antigen levels were measured by using a sensitive enzyme-linked immunosorbent assay (DuPont Inc., Wilmington, Del.). Two hundred nanograms of p24 antigen of each chimeric virus was added to a variety of cell lines (2×10^6 cells in 10 ml of RPMI 1640 plus 10% fetal calf serum [Gibco Laboratories, Grand Island, N.Y.]). After an overnight incubation, the cells were washed twice with phosphate-buffered saline and resuspended in 10 ml of complete RPMI 1640. p24 antigen production was measured at various times after infection. The Ba-L isolate was also used, at the same level of p24 antigen input, to infect these cell lines (Fig. 3A, B, and C). The kinetics and levels of viral growth in the T-cell line H9 (24) and in two monocytic lines, U937 and THP-1, were virtually identical for each of the chimeric viruses and for the R7 version of HXB2. Ba-L, by contrast, did not grow in any of these three cell lines, as monitored by p24 antigen production for up to 6 weeks (Fig. 3A, B, and C; data not shown). A lower input (20 ng of p24 antigen) of each viral chimera also revealed no differences in growth kinetics in these cell lines, although U937 cells were not infectable by using this viral input (data not shown).

Primary macrophages, isolated by adherence from the blood of HIV-1 seronegative donors and maintained as previously described (6), were also infected with the above viruses (Fig. 3D). Neither the chimeric viruses nor the R7 virus grew in these cells. As has been previously demonstrated (7), the Ba-L isolate grew well in these primary human macrophages (Fig. 3D).

Chimeric viruses with a T-lymphocyte-tropic HIV-1 strain backbone and various LTRs do not demonstrate significant differences in cellular tropism compared with that of the parent virus. Thus, the HIV-1 LTR does not appear to be a major determinant of cellular tropism. This is in contradistinction to the murine leukemia viruses, in which the LTR may be responsible for differing tissue tropisms and pathogenicities of various strains (4, 12, 26, 30). Recent data have demonstrated that cellular tropism of HIV-1 is at least partially determined at cell entry and involves variations in the envelope glycoproteins (6, 13, 36).

Although we show here that the cellular tropism of HIV-1 is not determined by the LTR, others have indicated that differences in the growth kinetics of various strains of HIV-1 may be mediated by sequence variations in control regions of the LTR (9). Our approach, in which LTRs were directly amplified and cloned from tissue infected in vivo, suggests that the variations in the regulatory elements found when comparing viral strains replicating in cell cultures (9) may be rare in HIV-1-infected tissues. Use of portions of HIV-1 obtained without propagation in cell culture should be a reliable methodology, because mutations or overgrowth by minor viral populations has been noted in HIV-1 strains propagated over time in vitro (16, 31).

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