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# Biological Characterization of Human Immunodeficiency Virus Type 1 Clones Derived from Different Organs of an AIDS Patient by Long-Range PCR

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**In order to characterize the biological properties of human immunodeficiency virus type 1 (HIV-1) variants from different tissues (peripheral blood mononuclear cells [PBMC], lymph node, spleen, brain, and lung) of one patient, we have chosen long-range PCR to amplify virtually full-length HIV proviruses and to construct replication-competent viruses by adding a patient-specific 5' long terminal repeat. To avoid selection during propagation in CD4<sup>+</sup> target cells, we transfected 293 cells and used the supernatants from these cells as challenge viruses for tropism studies after titration on human PBMC. Despite differences in the V3 loop of the major variants found in brain and lung compared to lymphoid tissues all recombinant HIV clones obtained showed identical cell tropism and replicative kinetics. After infection of human PBMC these viruses replicated with similar kinetics, with a slow/low-titer, non-syncytium-inducing phenotype. In contrast to the prediction of macrophage tropism, drawn from the V3 loop sequence, none of these viruses infected monocyte-derived macrophages. The challenge of blood dendritic cells by these recombinant viruses in the presence of tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factor, and interleukin-4 resulted in a productive infection only after adding stimulated CD4<sup>+</sup> T lymphocytes. Therefore, the biological properties of the HIV-1 variants derived from nonlymphoid tissue of this patient did not differ from those of HIV-1 variants from lymphoid tissue with respect to tropism for primary cells such as PBMC, macrophages, and blood dendritic cells.**

The coexistence of natural variants of genetically distinct human immunodeficiency virus type 1 (HIV-1) within individuals has been clearly demonstrated (2, 16, 26, 39, 49). Isolates of HIV-1 derived from peripheral blood mononuclear cells (PBMC) are highly variable over time and show changes in the biological phenotype during the course of infection (15). It has been demonstrated that early in infection HIV-1 isolates show a slow/low-titer, non-syncytium-inducing (NSI) phenotype and preferentially infect monocyte-derived macrophages (MDM) and PBMC. With the onset of AIDS, most but not all patients harbor rapid/high-titer viruses, often with a syncytium-inducing (SI) phenotype, which infect T-cell lines efficiently but may have a reduced ability to infect MDM (1, 52–54).

Studies of HIV-1 variants from tissues other than PBMC suggest they harbor distinct variant populations compared to PBMC. Viruses from the central nervous system are genetically and phenotypically different from viruses in the blood or spleen (10, 23, 45). Some neurological HIV-1 variants infect microglial cells and brain capillary endothelial cells (40, 47), whereas HIV variants recovered from the bowel show differences in induction of cytopathology and sensitivity to neutralization compared to blood isolates (3).

Until recently, there has been no systematic attempt to

quantify and characterize HIV-1 variants throughout the body at different disease stages. Donaldson et al. (20, 21) demonstrated large amounts of HIV provirus in nonlymphoid tissues (brain, lung, colon, and kidney) in AIDS patients, whereas patients who died in the asymptomatic phase did not have consistently detectable provirus in these tissues. The analysis of the V3 loop sequence derived from AIDS patients revealed differences between HIV variants from lymphoid tissues and nonlymphoid tissues (21).

To investigate whether these sequence differences reflected variation in the phenotype of HIV-1 in different tissues, we employed the long-range PCR technique to construct full-length proviruses derived from lymphoid tissue (PBMC, lymph node, and spleen) and nonlymphoid tissue (brain and lung) and compared the biological properties of the molecular clones containing the major V3 loop variants found in these tissues with respect to tropism for primary cells such as PBMC, MDM, and blood dendritic cells (BDC).

To our knowledge, this is the first time that replication-competent viruses have been constructed by long-range PCR amplification of genomic DNA prepared directly from different tissues. Although the sensitivity has to be improved to avoid potential recombination events occurring during the amplification steps (61), the approach described here allows the recovery of full-length infectious molecular clones without passaging on donor PBMC and can provide tissue-specific reference clones with respect to the genotype and phenotype present *in vivo*.

## MATERIALS AND METHODS

**Patient samples.** DNA samples used for PCR amplification were derived from various tissues from an AIDS patient by autopsy. Patient 4 (20) died 5 years after

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infection with HIV-1 and was chosen because of the relative high provirus load in the lymphoid as well as the nonlymphoid tissue, as shown previously (21). Samples of brain (left frontal lobe), lung, mesenteric lymph node, and spleen tissues from patient 4 were dissected into 1- to 2-cm pieces and stored at  $-70^{\circ}\text{C}$ . DNA was extracted from these tissues and whole blood to obtain total DNA as previously described (49).

**Long-range PCR and construction of a full-length provirus clone.** In order to construct a full-length provirus, we used a primer pair designed to amplify 9.0 kb covering the primer binding site up to the 3' end of the 3' long terminal repeat (LTR) (U5 region). The nucleotide sequences of the primers and the position of the 5' base in the HXB2 provirus (41) were 626(+), 5' AGGGGGCCAAGTCCGGCTCTCTAGCAGTGGCGCCCGAACAGGG 3', and 9690(-), 5' AGTCCGGCGCCGGTCTGAGGGATCTCTAGTTACCAGAGTC 3'. The underlined sequences mark the recognition sites for the restriction enzymes *SfiI*, *NarI*, and *NotI*, respectively, to facilitate the cloning of the 9.0-kb fragment into pGEM13-LTR (see below). The long-range PCRs were performed with a mixture of AmpliTaq (Perkin-Elmer Cetus) and *Pfu* polymerase (Stratagene) in an 8:1 ratio. Between 100 and 500 ng of genomic DNA derived from whole blood from patient 4 were sufficient to obtain a 9.0-kb amplification product in a 100- $\mu\text{l}$  reaction mixture containing 200 mM Tris-HCl (pH 8.7), 100 mM KCl, 20 mM  $\text{MgSO}_4$ , 100 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1 mg of bovine serum albumin/ml, 250  $\mu\text{M}$  (each) deoxynucleoside triphosphate, and 2 pmol of each primer. All reactions were run on an Omnigene Thermal Cycler (40 cycles of  $96^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s, and  $68^{\circ}\text{C}$  for 10 min).

To construct a full-length provirus, we amplified in a separate PCR a 5' LTR with the primer pair 56(+), 5' GCAGGACGTCAAGCTTCACACACAAGGG TACTTGGCTCATTGGCAG 3' and 651(-), 5' GAAGGGCCGACTTGGC CCCTGTTCCGGCGCCACTGCTAGAGAAG 3'. The underlined sequences mark the recognition sites for the restriction enzymes *HindIII*, *SfiI*, and *NarI*, respectively. The resulting 0.6-kb product was digested with *HindIII* and *SfiI* and cloned into pGEM13 to obtain pGEM13-LTR. All 9.0-kb PCR products were cloned into this vector by using the restriction enzymes *NarI* (primer binding site) and *NotI* (3' LTR).

**Construction of recombinant proviruses.** A nested primer pair to amplify the 3' half of the proviral genome was also designed. Modifying primers described by Kusumi et al. (32) in length and restriction enzyme recognition sites, we amplified 4.5 kb with genomic DNA from all tissues obtained (brain, lung, lymph node, and spleen). The outer primer pair was 4955(+), 5' TAGTAGACGCTGGGA AAGGTGAAGGGGCGAGTAGTA 3'; *AatII* recognition site underlined) and 9690(-). The second, nested PCR was performed with the primer pair 5048(+), 5' TGTGTGACGTCACAGATGGCAGGTGATGATTGTGT 3' (*AatII* recognition site underlined) and 9624(-), 5' TAAGGCGGCGCGGCAAGCTT TATTGAGGCTTAG 3'; *NotI* recognition site underlined) (40 cycles of  $96^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s, and  $68^{\circ}\text{C}$  for 6 min were run for each primer pair). The resulting products were cloned into pGEM5 with the restriction enzymes indicated, and the V3 sequence (in parentheses) was determined by using the ABI sequencing kit and primer V3f+ (5' TGGCAGTCTAGCAGAAGAAG 3'). The sequencing of the central region of the 3' halves obtained revealed a single *EcoRI* site at position 5745, 85 nucleotides 5' of the *tat* open reading frame. This *EcoRI* site was used together with the *NotI* site introduced by the inner antisense primer 9624(-) to exchange the 3' half of a PBMC-derived full-length clone with the 3' halves obtained from brain, lung, lymph node, and spleen (see Fig. 2).

**Sensitivity and fidelity of the long-range PCR approach.** To estimate the sensitivity of the 9.0-kb long-range PCR and the 4.5-kb nested long-range PCR we extracted genomic DNA from human PBMC spiked with different amounts of ACH2 cells (these cells carry a single copy of HIV<sub>11B</sub> per genome). The final concentration of proviruses per 200 ng of genomic DNA for each mixture was calculated and used in the PCR as a target. The fidelity of this prolonged PCR approach was checked by digesting the 9.0- and 4.5-kb fragments which were PCR products obtained by using HXB2-containing plasmid as a target. Digestion with 17 selected restriction enzymes, divided throughout the proviral genome, revealed no mutation at least in the restriction enzyme recognition sequences (data not shown).

**Transfection of recombinant HIV clones and titration on PBMC.** The human kidney cell line 293 was transfected with Lipofectamine (GIBCO) according to the manufacturer's protocol, and virus in the cell culture supernatant was harvested 4 days later. After DNase I (Promega) treatment to digest remaining plasmid DNA and chromosomal proviral DNA from dead cells, the amount of p24<sup>gag</sup> was assessed by using an in-house p24<sup>gag</sup> enzyme-linked immunosorbent assay (ELISA) as described earlier (13). The 50% tissue culture infectious dose (TCID<sub>50</sub>) was determined by using stimulated human PBMC as target cells and serial diluted virus in quadruplicate (37).

**Primary cells.** PBMC from HIV-seronegative individuals were purified by the Ficoll-Hypaque method, stimulated with 0.5  $\mu\text{g}$  of phytohemagglutinin A (PHA; Sigma)/ml for 48 h, and maintained in RPMI 1640 with 20% fetal calf serum (FCS), 20 U of recombinant interleukin-2 (IL-2; Boehringer Mannheim)/ml, and 100 U of both penicillin and streptomycin/ml.

MDM were purified by adherence and cultured as described by Gregory et al. (28). Briefly,  $2 \times 10^7$  freshly isolated PBMC were added to 140-mm-diameter bacterial dishes and incubated at  $37^{\circ}\text{C}$  for 2 h. Nonadherent cells were washed off by vigorous pipetting, and the remaining cells were cultured for 4 days in RPMI 1640-5% FCS-10% heat-inactivated human serum. The macrophages were

scraped off, counted, and set up in 24-well plates ( $5 \times 10^5$  cells per well) or 96-well plates ( $10^4$  cells per well), and the next day they were challenged with the recombinant viruses.

BDC were obtained from freshly isolated PBMC after overnight culture in RPMI 1640-10% FCS. The next day, the nonadherent cells were separated from T lymphocytes by centrifugation over 13.7% (wt/wt) metrizamide. These preparations were either negatively selected for dendritic cells by depletion of CD3<sup>+</sup> T lymphocytes, CD14<sup>+</sup> monocytes/macrophages, and CD19<sup>+</sup> B lymphocytes by using the Mini-Macs system (Milteny-Biotec) or positively selected after incubation with the monoclonal antibody HB15a (62), a specific marker for dendritic cells, followed by a second incubation with a bead-conjugated rat anti-mouse immunoglobulin G2a antibody (Milteny-Biotec). Both protocols resulted routinely in a cell population containing less than 1% T and B lymphocytes and monocytes as determined by fluorescence-activated cell sorter analysis. The remaining cells (>98%) were BDC as determined by appearance. BDC ( $10^5$ ) were challenged with recombinant virus and cultured in RPMI 1640-10% FCS containing 100 U of tumor necrosis factor alpha, 800 U of granulocyte-macrophage colony-stimulating factor, and 500 U of IL-4 per ml. After overnight incubation, the cells were washed four times, and stimulated T cells ( $10^5$  cells per well) were added to half of the wells.

**Immunostaining of HIV-infected cells.** The immunostaining method was similar to that described by Chesebro and Wehrly (11), adapted for a  $\beta$ -galactosidase conjugate as described earlier (14).

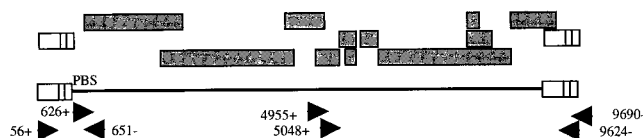
**Detection of late reverse transcriptase products after infection.** Forty-eight hours after challenge, cells were lysed and DNA was extracted by using the Nucleon DNA preparation kit (Scotlabs) according to the manufacturer's protocol. The entry PCRs were performed with 0.2  $\mu\text{g}$  of genomic DNA per 50- $\mu\text{l}$  reaction mixture containing 66 mM Tris-HCl (pH 8.8), 4 mM  $\text{MgCl}_2$ , 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 200  $\mu\text{M}$  (each) deoxynucleoside triphosphate, and 5 pmol of each primer. The expected 175-bp fragment was obtained only after two strand switches had occurred during the reverse transcription (positive primer R region, 5' CTAAGTGGGAACCCACTG 3' [nucleotides 498 to 516], and the minus strand primer *gag* region, 5' TCCTGCGTCGAGAGAGCTC 3' [nucleotides 693 to 675] [35 cycles of  $96^{\circ}\text{C}$  for 30 s,  $65^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 45 s were used] (33). The sensitivity of the entry PCR was determined by using HIV-1<sub>GUN-1w</sub> (titrated on MDM) to infect MDM with multiplicities of infection of 1.0, 0.1, 0.01, and 0.001. The lowest level of input virus detected was approximately 40 infectious units. To verify the standardization of the input DNA, control reactions with primers amplifying a product from the glyceraldehyde-3-phosphate dehydrogenase gene were used. The reactions were performed under the same conditions as for the entry PCR by using the 5' primer 5' TGGATATTGTTGCCATCAA TGACC 3' and the 3' primer 5' GATGGCATGGACTGTGGTCATG 3' for 20 cycles of  $96^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s (33).

**Single-round virus infectivity assay.** *env* genes from selected tissue-derived proviral 3' halves (E4, LN15, S8, B8, and L5) were cloned into plasmid pSVIII-*env* (30) under the control of an HIV-1 LTR promoter. This was done by digesting the tissue-derived 3'-half proviral genomes with *KpnI* and *XhoI* and replacing the HXB2 *env* coding region in pSVIII after (partial) digestion with *KpnI* and *XhoI*.

Cos-1 cells ( $2 \times 10^5$  cells per well) were cotransfected with 5  $\mu\text{g}$  of either pSVIII-E4, pSVIII-LN15, pSVIII-S8, pSVIII-B8, or pSVIII-L5 and 2.5  $\mu\text{g}$  of HXB $\Delta$ envCAT, an *env*-deficient HIV-1 provirus which contains a chloramphenicol acetyltransferase (CAT) gene in place of the *nef* gene, by using Lipofectamine. Three days after transfection supernatant was collected, and equivalent amounts of produced virions (10 ng of p24<sup>gag</sup>) were used to infect CCC/CD4 cells transiently expressing the chemokine receptors CXCR4, CCR5, and CCR3 (50). After two days the cells were lysed and the amount of CAT in the lysate was determined by using a CAT-ELISA (Boehringer Mannheim) according to the manufacturer's protocol. Two different virus stocks were used in at least three different challenge experiments.

## RESULTS

**Sensitivity of the long-range PCR approach and construction of PBMC-derived full-length HIV-1 clones.** The long-range PCR performed with different amounts of proviruses per given DNA concentration revealed successful amplification of the 9.0-kb fragment when at least  $4 \times 10^3$  proviruses were present in the reaction. The number of proviruses necessary could not be reduced by employing a second round (Fig. 1). By using the primer pairs to amplify only the 3' half of the proviral genome, a much higher sensitivity was obtained. The nested PCR approach yielded a 4.5-kb fragment when only 20 proviruses were present in the reaction. This high sensitivity allowed the amplification of 3'-half viral genomes from genomic DNA prepared from lymph node, spleen, brain, and lung tissue collected postmortem from patient 4 (21). Because of the high proviral concentration in PBMC from this patient, we were



	primer sets	kb	number of targets / 200ng DNA				
			$2 \times 10^4$	$4 \times 10^3$	$8 \times 10^2$	20	1
1st round	626+ / 9690-	9.1	+	+	-	-	-
2nd round	626+ / 9624-	9.0	+	+	-	-	-
1st round	4955+ / 9690-	4.7	+	+	+	-	-
2nd round	5048+ / 9624-	4.5	+	+	+	+	-

FIG. 1. (Top) Location of primers used in this study. The primers amplified either the 5' LTR, the 9.0-kb amplicon, or the 4.5-kb amplicon. Arrowheads indicate the direction of transcription. (Bottom) Titration of sensitivity of PCR primers. Genomic DNA was prepared from ACH2 cells mixed with human PBMC. The PCR products were resolved by electrophoresis through 0.6% agarose gels and visualized by using ethidium bromide.

able to amplify a 9.0-kb fragment by using 200 ng of genomic DNA. This fragment was cloned into pGEM13-LTR. The sequencing of the V3 loop revealed exactly the same V3 loop amino acid sequences as published previously (21) (Fig. 2). Two of 12 full-length HIV clones derived from PBMC DNA replicated after infection of donor PBMC (data not shown).

It is possible that recombinant genomes are generated during PCR amplification. This could occur if an unfinished strand annealed to a different parental sequence. The strand would then be completed with the second sequence as template. To assess such recombination during the PCR process for the 4.5-kb fragments, we performed long-range PCR using two different provirus-containing plasmids (pHXB2 and pGUN-1wt) at equal target numbers ( $10^2$  targets). After cloning the PCR products, a restriction analysis and partial sequencing revealed that four of 11 (36%) 4.5-kb clones contained recombinants between pHXB2 and pGUN-1wt. (Cloned fragments with 3' sequences characteristic of one infectious clone and 5' sequences characteristic of the other were considered to be recombinants.)

**Construction of recombinant HIV clones with tissue-specific 3' halves.** In contrast to PBMC (see above) the proviral load in other tissues was relatively low. Because of the low sensitivity of the 9.0-kb long-range PCR, we decided to amplify only proviral 3' halves and to construct recombinant viruses with the PBMC-derived full-length infectious clone as a backbone. Restriction enzyme analysis revealed a single *EcoRI* site in all amplified 4.5-kb fragments 85 nucleotides upstream of the *tat* open reading frame. We constructed 20 full-length clones containing 3' halves derived from different tissues as follows: 7 from lymph node, 5 from spleen, 5 from brain, and 3 from lung. Each of these clones showed exactly the same V3 loop sequence described by Donaldson et al. (21) for the major variants found in these different tissues. The major V3 loop sequence from lymphoid tissue (PBMC, lymph node, and spleen) differs in six amino acids from the major sequence found in nonlymphoid tissue (brain and lung). Figure 2 shows only those virus clones that were replication competent in PBMC. These recombinant HIV clones were transfected in 293 cells, and the harvested virus was used for titration on donor PBMC and for all challenge experiments. To investigate the possibility that

inhibitory sequences in the 5' half of the PBMC-derived HIV clone HIV<sub>E4</sub> might interfere with tropism for macrophages (4, 29, 57), we constructed two recombinant viruses carrying the 3' halves of HIV<sub>GUNwt</sub>, a dual-tropic HIV clone infecting T-cell lines and macrophages (38), and the 3' half of HIV<sub>SF162</sub>, a monocyctotropic HIV clone (10), by using long-range PCR and the same cloning strategy as described for the patient 4 tissue-specific recombinant viruses. These recombinant viruses were transfected into 293 cells and titrated the same way.

**Replication kinetics of recombinant viruses after infection of PBMC.** After titration of all recombinant HIV clones,  $10^6$  PBMC were infected with  $10^4$  TCID<sub>50</sub>, and the replication kinetics were monitored by using a p24<sup>gag</sup> ELISA (HIV-1 p24 core profile ELISA; Du Pont) over the following 28 days. As shown in Fig. 3, all viruses showed a peak in p24<sup>gag</sup> production in the culture supernatants after approximately 10 days. No virus-induced syncytia in these cultures or in CD4<sup>+</sup> T-cell lines (C8166, Molt4, and MT-2) were observed. Therefore, all viruses showed a NSI phenotype. The amount of p24<sup>gag</sup> in the culture supernatants after infection with the recombinant clones was about 10 times less than in the culture supernatants infected with the laboratory-adapted, SI HIV-1 strains HIV<sub>HXB2</sub> and HIV<sub>GUNwt</sub> (data not shown). The replication kinetics did not reveal a clear difference between recombinant viruses derived from lymphoid tissue and those derived from nonlymphoid tissue (Fig. 3).

**Tropism studies with MDM and BDC.** Because of the close resemblance of the V3 loop sequences of all recombinant HIV clones with the consensus sequence for primary lymphocyte and macrophage-tropic viruses (34), we expected productive infection of MDM and BDC by these viruses. The infection experiments were performed by using  $5 \times 10^5$  infectious units of each recombinant virus (assessed on PBMC) plated onto  $5 \times 10^5$  MDM and BDC (multiplicity of infection = 1), respectively. The MDM cultures were maintained over 21 days, and supernatants collected on days 7, 14, and 21 did not contain detectable levels of p24<sup>gag</sup> (HIV-1 p24 core profile ELISA; detection limit, 12.5 pg/ml). After 21 days PHA-stimulated PBMC were added to the cultured MDM in order to amplify a low-level HIV-1 replication. But this virus rescue assay as well as an immunostaining for p24<sup>gag</sup> on MDM 21 days after infection did not reveal any replication of HIV-1 in these cultures (Table 1). The control viruses (E4/GUNwt and E4/SF162) were successfully rescued even after an infection at a multiplicity of infection of 0.01. This result was confirmed by the failure to amplify late reverse transcription products in lysates of macrophages prepared 48 h after challenge (data not shown). The detection of p24<sup>gag</sup> in culture supernatants after challenge with the recombinant viruses E4/GUNwt and E4/SF162 (Fig. 2) clearly indicates the absence of inhibitory sequences in the 5' half of the PBMC-derived provirus, such as mutations in the basic domain of p17<sup>gag</sup> (4). The sequences of the chimeric *vpr* genes obtained due to the recombination at the *EcoRI* site do not differ from those of the wild-type *vpr* and do not encode premature stop codons (29, 57). Although the recombinant viruses derived from patient 4 contain V3 loop amino acid sequences that predict a macrophage-tropic virus, none of them could infect macrophages.

After challenge of  $5 \times 10^5$  BDC with the recombinant viruses ( $5 \times 10^5$  TCID<sub>50</sub>), we did not detect p24<sup>gag</sup> in the cell supernatants up to 20 days, nor could we see immunostained cells after fixation (Table 1). However, if stimulated autologous T cells were added one day after challenge, p24<sup>gag</sup> was detected as early as 5 days after infection. Using CD19<sup>+</sup> primary B cells instead of BDC, we did not detect p24<sup>gag</sup> after the coculture with autologous T cells (data not shown). Therefore,

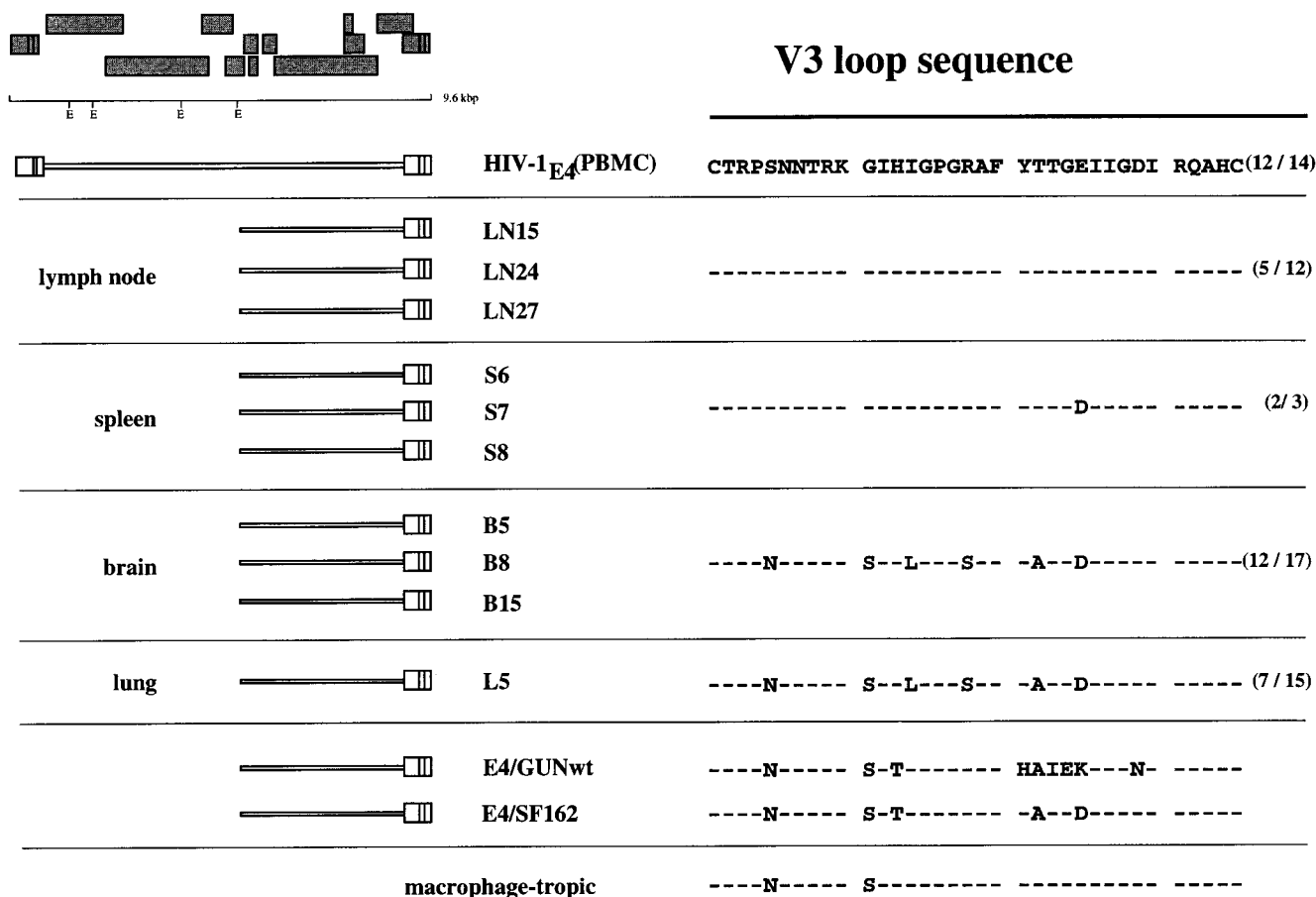


FIG. 2. Genomic structures of PBMC-derived full-length clone HIV<sub>E4</sub> and the subsequently cloned recombinant viruses containing the 3' half from the different organs indicated. V3 loop amino acid sequences were determined for all replication-competent clones. All recombinant viruses contain the tissue-specific V3 loop amino acid sequence representing the major variant in each tissue as determined by Donaldson et al. (22). Ratios in parentheses are the frequencies of the corresponding V3 loop variant in the observed variants (data from reference 22).

BDC were able to pass virus to T cells in the coculture system, but they do not support a productive infection with HIV-1 on their own. The replication kinetics in this BDC-T-cell coculture system did not show any difference between molecular clones derived from lymphoid tissue (PBMC, lymph node, and spleen) and molecular clones derived from nonlymphoid tissues (brain and lung).

**Coreceptor use of tissue-derived envelope glycoproteins.** To assess the efficiency with which the tissue-derived envelope glycoproteins mediate early events of HIV-1 infection, an *env* complementation assay (30) was utilized. Pseudotype HIV-1 viruses were produced by cotransfection of COS-1 cells with two plasmids, pHXB2 $\Delta$ envCAT and pSVIII. Different pSVIII plasmids encoding the envelope glycoproteins derived from selected tissue-derived clones, E4 (PBMC), LN15 (lymph node), S8 (spleen), B8 (brain), and L5 (lung), and two control viruses, GUN-1wt (dual tropic) and HXB2 (T-cell line tropic), were used. An equal amount of p2<sup>Agg</sup>-containing supernatant was incubated with CCC/CD4 cells transiently expressing the chemokine receptors CCR3, CCR5, and CXCR4. A CAT-ELISA performed 3 days after the infection of the target cells revealed that all tissue-derived envelope glycoproteins are able to utilize the chemokine receptors CCR3 and CCR5 but not CXCR4 (Fig. 4). The usage of CCR3 and CCR5 correlates with the finding that all recombinant viruses are of the NSI

phenotype but does not explain the lack of macrophage tropism. The use of U87/CD4 cells stably expressing the chemokine receptors CCR3, CCR5, and CXCR4 (kindly provided by D. Littman) and the recombinant viruses confirmed the findings of the *env* complementation assay (data not shown).

## DISCUSSION

**Construction of replication-competent HIV clones by using long-range PCR.** In order to construct replication-competent HIV clones representing the genotype present at a specific time point in a specific tissue we used long-range PCR. This approach allowed us to construct full-length proviruses containing the major V3 loop sequence found in PBMC of patient 4. The procedure led to successful amplifications provided at least  $4 \times 10^3$  proviruses were present in the reaction. This limited sensitivity could result in the amplification of recombinant proviruses generated during PCR (7, 46, 61). However, the low recovery of replication-competent proviral clones (2 of 12) may reflect the high concentration of defective proviruses in the peripheral blood cells (due to recombination and reverse transcription errors in vivo) described by others or due to recombination during PCR (17, 35). Our validation of recombination events during long-range PCR to obtain 4.5-kb fragments, however, showed a lower recombination frequency than

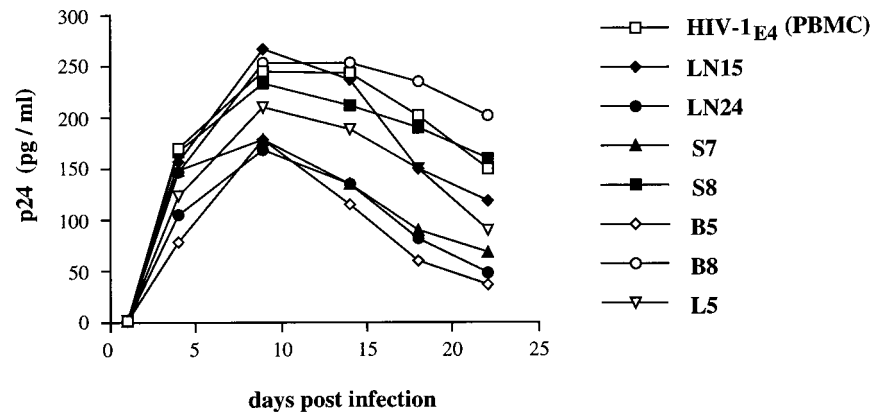


FIG. 3. Replication kinetics on PBMC after infection at a multiplicity of infection of 0.01. Infections were carried out with  $10^4$  TCID<sub>50</sub> of each virus on  $10^6$  PHA-stimulated PBMC. The cells were washed after 1 day, and virus replication was assessed by measuring HIV p24<sup>gag</sup> in the culture supernatants.

did the study of Yang et al. (61). The differences might result from a lower input of targets per PCR and the amplification of only a 4.5-kb fragment, whereas Yang et al. (61) amplified a 8.2-kb fragment. Thus, a minority of the 3'-half proviral clones described here could result from recombination events during the PCR. Since we were unable to find any phenotypic differences in the molecular clones generated, it is likely that any recombinants resemble the bona fide clones generated. Nevertheless, the sensitivity to detect and obtain long-range PCR products needs to be improved to reduce the frequency of such recombinations.

The recently described molecular cloning of full-length HIV genomes directly from plasma viral RNA (24) is likely to yield a larger amount of replication-competent clones, although this has yet to be formally demonstrated. Successful amplification of proviral 3' halves derived from different tissues made it possible to construct recombinant proviruses containing the *tat*, *rev*, *vpu*, and *nef* genes together with the entire *env* gene present in each tissue. The envelope genes in the recombinant viruses reflect copies of the actual envelope genes present in these tissues. Since the envelope contains the major determinants for tropism (31, 42, 48, 51, 59, 60), the characterization of the recombinant viruses constructed as described here might provide a better understanding of the biological properties of HIV variants present in different tissues. Full-length molecular clones of viruses, amplified directly from genomic DNA or from viral RNA obtained from tissues, should produce viruses with the actual phenotype present at a specific time point in a specific tissue. The long-range PCR will result in a remarkable increase of reference sequences from different viral subtypes (46) and aid the analysis of the structure of mosaic subtypes such as A/E recombinations (7). Furthermore, this method allows the characterization of cell-type-specific virus variants by using separated cell populations (BDC, monocytes, and CD4<sup>+</sup> T lymphocytes) to prepare genomic DNA. The availability of cell-type-specific clones could provide definitive evidence for tropism switches to BDC (43) and immature thymocytes (56) late in the infection.

**Biological characterization of recombinant HIV clones derived from different tissues.** Following transfection in 293 cells, the rescued virus was titrated on uninfected PBMC and the replication kinetics were assessed after challenging donor PBMC at a multiplicity of infection of 0.01 (Fig. 3). All recombinant clones replicated like slow/low-titer viruses and did not induce syncytia or infect any of the T-cell lines tested (C8166, Molt4, and MT-2).

It has been shown that a rapid decline in CD4<sup>+</sup> T lymphocytes often correlates with the development of rapidly replicating SI viruses (15, 52–54). However, in 50% of cases disease progression also occurs in patients from whom only NSI viruses can be isolated from the peripheral blood (54). Patient 4 died of AIDS about 5 years after HIV-1 infection without rescue of SI viruses at the time of death. The molecular clones HIV-1<sub>E4</sub>, LN15, S8, B8, and L5 were also tested for their coreceptor usage and in a single-round infectivity assay showed positive results only when the chemokine receptors CCR5 and CCR3 were present (19) (Fig. 4). They were unable to utilize the chemokine receptor CXCR4, which has been described as the main coreceptor for SI isolates adapted to replicate in T-cell lines (25).

NSI virus variants are reportedly able to infect blood MDM, although with variable kinetics (9, 15). The HIV-1 recombinant clones described here encode a V3 loop amino acid sequence resembling the sequence described for primary lymphocyte- and macrophage-tropic viruses (34) and are able to use the coreceptors CCR5 and CCR3 (12, 18, 19, 22). However, they do not infect MDM, suggesting that macrophages might express these chemokine receptors differently than transfected cell lines or PBMC. Thus, some HIV-1 isolates or HIV-1 molecular clones are able to use CCR5 or CCR3 only if it is expressed in a certain way. Our results, however, underline that macrophage tropism can be correctly assessed only by performing an infectivity assay using macrophages as target cells rather than cell lines expressing CD4 and different chemokine receptors. The possibility that mutations in the basic domain of p17<sup>gag</sup> or the *vpr* gene by these recombinant clones resulted in the nonproductive infection of macrophages observed (4, 29, 57) appears unlikely since the recombinant viruses E4/GUNwt and E4/SF162 readily infected macrophages. The *nef* gene is also required for the replication of HIV in macrophages (36). However, a *nef* defect would not explain the restriction for virus entry, as shown by entry PCR.

Patterson et al. (43) reported a higher provirus concentration in BDC from AIDS patients compared to asymptomatic HIV-positive patients. To study the susceptibility of BDC to infection with late HIV-1 variants, we challenged these cells with each of the recombinant infectious clones we had constructed. We did not detect any p24<sup>gag</sup> in the supernatant of purified BDC unless they were cocultured one day after infection with autologous T lymphocytes. Although data for the susceptibility of dendritic cells are inconsistent (reviewed in reference 6), it appears that BDC do not support HIV repli-

TABLE 1. Summary of the challenge experiments

HIV-1 recombinant molecular clones	Amt (pg/ml)/presence of p24 <sup>gag</sup> in <sup>a</sup> :						
	PBMC <sup>a</sup> (7 d.p.i.)	MDM			MDM and PBMC <sup>a</sup> (21 d.p.i.)	BDC <sup>a</sup> (10 d.p.i.)	BDC and T cells <sup>a</sup> (10 d.p.i.)
		ELISA <sup>a</sup> (21 d.p.i.)	Immunostaining assay <sup>b</sup> (21 d.p.i.)	Entry PCR <sup>c</sup> (2 d.p.i.)			
HIV-1 E4 (PBMC)	1,028	<12.5	—	—	<12.5	<12.5	2,426
LN15	812	<12.5	—	—	<12.5	<12.5	1,428
LN24	961	<12.5	—	—	<12.5	<12.5	1,285
LN27	912	<12.5	—	—	<12.5	<12.5	1,616
S6	387	<12.5	—	—	<12.5	<12.5	1,670
S7	520	<12.5	—	—	<12.5	<12.5	935
S8	1,054	<12.5	—	—	<12.5	<12.5	3,180
B5	894	<12.5	—	—	<12.5	<12.5	2,060
B8	1,001	<12.5	—	—	<12.5	<12.5	2,174
B15	793	<12.5	—	—	<12.5	<12.5	1,818
L5	894	<12.5	—	—	<12.5	<12.5	1,816
E4/GUNwt	1,618	2,341	+	+	ND <sup>d</sup>	<12.5	3,180
E4/SF162	1,415	1,876	+	+	ND	<12.5	2,989

<sup>a</sup> All infections were carried out using a multiplicity of infection of 1. The amount of p24<sup>gag</sup> (pg/ml) was assessed by using the DuPont p24 core profile ELISA with a detection limit of 12.5 pg of p24<sup>gag</sup> per ml of cell culture supernatant. Representative results from at least three independent experiments are shown. d.p.i., days postinfection.

<sup>b</sup> +, presence of immunostained MDM.

<sup>c</sup> +, positive results after entry PCR.

<sup>d</sup> ND, not done.

on their own (5, 44) in vitro, although several studies have reported a productive infection with macrophage-tropic viruses (8, 33, 58). Tsunetsugu-Yokota et al. (55) detected a higher provirus concentration in BDC infected with the SI strain HIV<sub>LAI</sub> than in BDC infected with the NSI, macrophage-tropic strain HIV<sub>Ba-L</sub>, but they did not detect p24<sup>gag</sup> in culture supernatants. Recent evidence shows that cultured dendritic cells express the chemokine receptors CXCR4 and CCR5 and are sensitive to HIV-1 entry (27). We have shown that several of the recombinant clones derived from different tissues utilize CCR5 (19). However, it is currently not known whether virus rescued in the BDC-T-cell coculture is derived from virus adsorbed on the surface of BDC or from a reactivation of HIV replication in BDC halted during the reverse transcription stage. Whether cultured dendritic cells express CCR3 and are susceptible to HIV isolates which can use CCR3 as a coreceptor remains to be shown.

Using genomic DNA from different tissues obtained post-

mortem from one AIDS patient, we found that the long-range PCR approach allows the construction and characterization of HIV variants present at a single time point in individual tissues. By avoiding any selection due to isolation of HIV variants capable of replicating in PHA-stimulated PBMC, these molecular clones reflect genotypes and phenotypes actually present in these tissues. Characterization of these recombinant clones revealed no differences in tropism for any primary cell tested.

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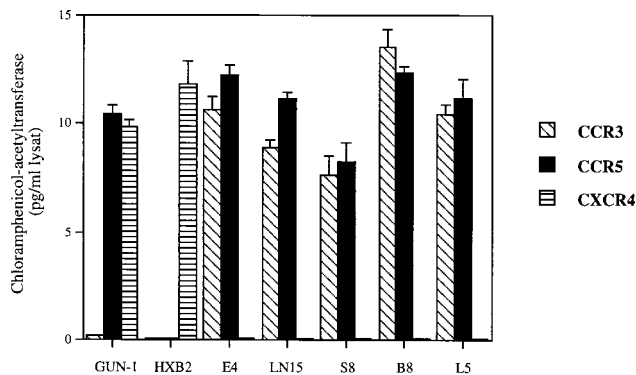


FIG. 4. Single-round infectivity assay using pseudotype viruses with tissue-derived envelope glycoproteins. Amounts of CAT (determined by CAT ELISA) in lysates of CCC/CD4 cells transiently expressing CCR3, CCR5, and CXCR4, respectively, three days after challenge with equal amounts of pseudotype viruses (10 ng of p24<sup>gag</sup>) are shown.

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