Molecular Characterization of Human Immunodeficiency Virus Type 1 Cloned Directly from Uncultured Human Brain Tissue: Identification of Replication-Competent and -Defective Viral Genomes

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All presently available replication-competent proviral clones of human immunodeficiency virus type 1 (HIV-1) are derived from cell culture-amplified virus. Since tissue culture is highly selective for viral strains with an *in vitro* growth advantage, such clones may not be representative of the biologically relevant virus present *in vivo*. In this study, we report the molecular cloning and genotypic characterization of 10 HIV-1 genomes directly from uncultured brain tissue of a patient with AIDS dementia complex. Targeting unintegrated circular HIV-1 molecules for recombinant lambda phage cloning, we obtained four full-length genomes with one or two long terminal repeats (LTRs), three defective genomes with internal deletions, two rearranged genomes with inverted LTR sequences, and one integrated proviral half with flanking cellular sequences. Nucleotide sequence analysis of these clones demonstrated chromosomal integration, circle formation, genomic inversion, and LTR-mediated autointegration of HIV-1 genomes *in vivo*. Comparison of a 510-bp hypervariable envelope region among 8 lambda phage-derived and 12 polymerase chain reaction-derived clones from the same brain specimen identified a predominant viral form as well as genetically divergent variants. Variability among 19 of 20 clones ranged between 0.2 and 1.2%. One clone exhibited 8.2% nucleotide sequence differences consisting almost exclusively of G-to-A changes. Transfection of the four full-length HIV-1 genomes identified one clone (YU-2) as replication competent and exhibiting growth characteristics similar to those of tissue culture-derived macrophage tropic strains of HIV-1. These results demonstrate, for the first time, that replication-competent HIV-1 genomes, complex mixtures of defective viral forms, and chromosomally integrated provirus persist *in vivo*. In addition, the brain-derived viral clones are expected to prove valuable for future studies of macrophage and neurotropism as well as for the analysis of other viral properties that are subject to *in vitro* selection pressures.

Infection with human immunodeficiency virus (HIV) causes chronic, progressive destruction of the host's immune system and, in a subset of individuals, neurological dysfunction of variable clinical severity (11, 46). The most common HIV-related central nervous system (CNS) disorder, AIDS dementia complex (ADC), is characterized by motor, behavioral, and cognitive abnormalities. It occurs primarily in late stages of infection and frequently results in severe dementia and physical disability (45, 47, 56). Despite these profound clinical sequelae, the underlying mechanisms responsible for ADC are still unknown. The most consistent laboratory findings associated with ADC have been the presence of HIV-1 in brain tissue of affected individuals and a strong association between HIV-1 expression (RNA and protein) and clinical severity of illness (14, 15, 21, 24, 28, 32, 41, 54, 66, 72). Multinucleated giant cells and microglial nodules have been identified as characteristic histological markers, and their appearance has been interpreted to be the result of HIV-induced cell fusion and cytolytic infection (5, 16, 26, 35, 37, 52, 66, 72). It has also been postulated that alterations of cytokine networks in the brain, the release of viral proteins *per se*, or the production of neurotoxic factors by virus-infected cells may contribute to the pathogenesis of ADC (18).

The development and clinical course of ADC is highly variable, and it is possible that specific viral determinants responsible for cell tropism, replication potential, and cytopathic properties contribute to this heterogeneity (7, 8, 12, 13, 29, 33, 62, 73). Virological analysis has shown that HIV infection of the brain occurs preferentially in macrophages, microglia, and derivative multinucleated cells (15, 16, 28, 29, 66, 72). In addition, only macrophage-tropic HIV-1 isolates establish a productive infection in primary human brain explant cultures and cause a lytic infection of normal human microglia cells (68). T-cell-adapted virus strains generally fail to replicate in these same cell systems (68). These findings thus suggest that certain strains of HIV are neurotropic because of their capacity to productively infect and replicate in brain cells of the monocyte/macrophage lineage. This possibility is supported by the recent finding of independent CNS-derived HIV-1 isolates which share a common envelope region conferring macrophage tropism (39, 55).

Because of the low abundance of proviral DNA in tissues of chronically infected individuals (53, 54), studies of HIV-1 biology, including studies of HIV macrophage and neurotropism, have been performed exclusively with cell culture-adapted virus strains and transfection-competent molecular clones derived from these cultures (7, 8, 29, 39, 55, 70). Little is therefore known about the replicative and biological properties of HIV-1 as it exists in primary infected tissues.

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Advances in polymerase chain reaction (PCR) technologies have recently enabled the genetic characterization of HIV-1 directly from uncultured patient material (3, 10, 36, 65), and results from these studies indicate that culture-derived isolates represent a selected subpopulation of viruses with an in vitro growth advantage (36, 65). PCR technology, however, has been limited to the analysis of subgenomic viral fragments and has not yet been established as a method to obtain full-length proviruses from uncultured tissue. As an alternative to the derivation of proviral clones from cell culture-amplified virus, we sought to obtain HIV-1 genomes directly from uncultured human tissues in order to characterize the genetic composition of HIV-1 as it exists in vivo. In this report, we describe the molecular and biological characterization of 10 recombinant HIV-1 lambda phage clones isolated from a genomic library of uncultured brain DNA derived from a patient with ADC. Availability of these clones allowed us, for the first time, to examine the integration properties, genomic organization, genotypic variability, and biological characteristics of HIV-1 viruses not previously subjected to in vitro selection pressures.

MATERIALS AND METHODS

Patient specimen. Brain samples were obtained from a patient with AIDS (patient 1) who died with severe HIV-1-associated encephalopathy (ADC). Autopsy was performed within hours of the patient's death, and brain tissue was dissected into 1- to 2-cm³ pieces, snap frozen in liquid nitrogen, and stored at -70°C. Immunohistochemical studies demonstrating a productive HIV-1 infection in sections of the same brain have been reported previously (37).

DNA extraction and Southern blot analysis. Extraction of high-molecular-weight DNA from uncultured brain tissue has been described previously (53, 54). Briefly, frozen brain specimens were pulverized in liquid nitrogen, lysed in DNA extraction buffer (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]), digested with proteinase K (100 µg/ml) at 50°C for 4 h, and incubated at 37°C overnight. Total genomic DNA was adjusted to 0.3 M sodium acetate (pH 6.0), extracted twice with phenol-chloroform (1:1) and once with chloroform, precipitated with 2 volumes of absolute ethanol, and dissolved in TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA). High-molecular-weight DNA (20 µg) was digested with restriction enzymes, separated by electrophoresis on a 0.6% agarose gel, blotted onto nitrocellulose filters, hybridized to a ³²P-labeled full-length HIV-1 probe (BH10i; 22), and exposed to X-ray film for 3 days. Positive and negative control DNAs (5 µg) were analyzed in parallel. The resulting autoradiogram was used to estimate the relative abundance of HIV-1 sequences in the uncultured brain DNA and to identify restriction enzymes suitable for lambda phage cloning (Fig. 1).

Molecular cloning of unintegrated HIV-1 circles from uncultured brain DNA. A genomic lambda phage library was constructed as described previously (22, 34). Total genomic brain DNA derived from patient 1 was digested with *Eco*RI (which cleaved the provirus once), fractionated by sucrose gradient centrifugation to enrich for linearized HIV-1 circles, and ligated into *Eco*RI arms of λgtWES · λB. The ligation products were then packaged in vitro, titered, and plated on LE392 cells. A total of 8 × 10⁶ recombinant phage clones were screened with a full-length HIV-1 probe (BH10i; 22), and 10 positive signals were identified. All positive recombinants were plaque purified, and their restriction maps were determined by using multiple enzyme digestions as well as

subgenomic HIV-1 fragments (BH8i and BH5i; 22) and sheared normal human DNA as probes.

PCR. For PCR analysis, DNA was extracted from a different frozen aliquot of the same brain 1 year after completion of the lambda phage cloning experiments. This was done in physically separated laboratories, using stringent precautions to avoid PCR carryover as well as sample contamination. Two oligonucleotide primers designed to amplify the V3 loop and adjacent envelope sequences were synthesized on a DuPont DNA synthesizer (model 300) and purified according to the manufacturer's recommendations (primer 1, 5'-TGTCAACTCAACTGCAGTTAAATGGCAGT-3'; primer 2, 5'-TTGTAAACAGGATCCCTGTAATATTTGATG-3'). The primers contained *Pst*I and *Bam*HI restriction enzyme cleavage sites (underlined) to facilitate subsequent M13 cloning and sequence analysis. PCR reactions were carried out in a total volume of 100 µl containing 1 µg of total genomic brain DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 µM deoxynucleotide triphosphates, 10 pmol of each primer, and 2.5 U of *Taq* polymerase. Samples were overlaid with 100 µl of mineral oil to avoid evaporation and then subjected to 40 amplification cycles consisting of a denaturing step (94°C, 1 min), a primer-annealing step (55°C, 1 min), and a primer extension step (72°C, 1.5 min). Negative and positive control DNAs were amplified in parallel with the brain specimen. PCR products were analyzed by agarose gel electrophoresis (1%) for the presence of appropriately sized DNA fragments.

DNA sequence analysis. PCR amplification products were visualized by ethidium bromide staining, cleaved with *Pst*I and *Bam*HI, and subcloned into M13mp19. Recombinant M13 clones were identified by hybridization to an HIV-1-specific probe, and 12 individual M13 clones were sequenced in their entirety by the dideoxynucleotide chain termination method (51). Nucleotide and amino acid sequence alignments were performed by using the program MICROGENIE (Beckman).

Selected regions of the recombinant lambda phage clones, including an envelope fragment corresponding to the region amplified by PCR, were also subjected to nucleotide sequence analysis (see shaded regions in Fig. 2). The latter was done to compare HIV-1 viral sequences derived by two independent cloning techniques. In addition, nucleotide sequence analysis was performed to map internal deletions in YU-1, YU-5, and YU-27, genomic rearrangements in YU-3 and YU-4, the circle junction in YU-32, and the flanking cellular sequences in YU-6. Phage clones were analyzed by direct double-stranded sequence analysis of lambda phage DNA (hypervariable envelope region) and by single-stranded sequence analysis after subcloning of appropriate fragments into M13mp18/19 (deletion boundaries, circle junction, long terminal repeat [LTR] regions, and cellular DNA).

DNA transfection and viral infectivity studies. All immortalized T-cell lines, including Molt-4 (clone 8) (27), CEMx174 (50), and SupT1 (60), were maintained in RPMI 1640 supplemented with 15% fetal bovine serum, 2 mM glutamine, and 100 µg of gentamicin per ml (complete medium). Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult volunteers by Ficoll-Hypaque density gradient centrifugation as previously described (9). PBMCs were stimulated with phytohemagglutinin (PHA; 4 µg/ml) for 24 h, washed free of lectin, and maintained in complete medium supplemented with 30 U of recombinant interleukin-2 (Amgen Biologicals) per ml. Cos-1 cells were main-

tained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

To determine the replication competence of all clones representing full-length viruses (YU-2, YU-10, YU-21, and YU-32), lambda phage inserts were isolated by agarose gel electrophoresis following digestion with *EcoRI* and self-ligated to generate viral genomes in nonpermuted form. In addition, full-length nonpermuted YU-2 and YU-10 viral inserts were constructed by ligating a *Sall-EcoRI* fragment (5' half of the genome) and an *EcoRI-SphI* fragment (3' half of the genome) into a *Sall*- and *SphI*-digested plasmid vector (pTZ19R; United States Biochemical). Ten micrograms of self-ligated phage inserts or reconstructed provirus in plasmid was transfected into Cos-1 cells along with control DNAs, using the calcium phosphate precipitation method (2). Twenty-four hours after transfection, PHA-stimulated normal donor lymphocytes or immortalized T-cell lines were added to the Cos-1 monolayer and cocultivated for 48 h. Nonadherent cells were subsequently transferred to a new flask and monitored for retroviral activity by reverse transcriptase and p24 antigen capture assays (9).

Reverse transcriptase assays were performed by pelleting virus particles from culture supernatants ($250,000 \times g$; 35 min) and resuspending the pellet in 100 μ l of lysing buffer (25 mM Tris-HCl [pH 7.9], 0.4 M NaCl, 0.5 mM dithiothreitol, 0.25% Triton X-100, 0.25 mM phenylmethylsulfonyl fluoride, 10% glycerol). Then 20 μ l of sample was mixed with 85 μ l of reverse transcriptase reaction cocktail containing 75 mM Tris-HCl (pH 7.5), 1.5 mM dithiothreitol, 1.2 mM ATP, 15 mM MgCl₂, 5 μ l of poly(rA) · poly(dT)₁₂₋₁₈ (10 U/ml), 0.1 mM TTP, and 0.325 μ l of [³H]dTTP (2.5 mCi/ml) and incubated at 37°C for 1 h. Reactions were stopped by adding 3 ml of cold 10% trichloroacetic acid containing 0.1 M pyrophosphate and 5 mg of tRNA per ml. Samples were filtered, washed extensively with 5% trichloroacetic acid, dried with 70% ethanol, and processed for scintillation counting. HIV-1 p24 antigen capture assays were performed according to the manufacturer's recommendations (9).

For cell-free infectivity studies, supernatants of primary transfectants or PBMC cocultures were clarified by low-speed centrifugation ($600 \times g$; 10 min), filtered (1.2- μ m-pore-size filter), incubated for 18 h at 37°C with the appropriate target cells, and washed. The appearance of multinucleated giant cells was monitored on a daily basis, and supernatants were harvested every 4 to 7 days and tested for reverse transcriptase activity and HIV-1 p24 antigen as described above.

Western immunoblot analysis. The protein profile of transfection-derived virions was determined by Western blot analysis, using a procedure previously described (4). Briefly, viral antigen was prepared from culture supernatants by ultracentrifugation, solubilized in electrophoresis buffer, boiled, and separated on a 12% polyacrylamide slab gel containing SDS. Following electrophoresis, proteins were transferred to nitrocellulose by electroblotting and incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk–0.1% Tween 20 in phosphate-buffered saline) and then overnight with HIV-1-positive patient serum. Viral proteins were visualized by incubation of the blots for 2 h at room temperature with ¹²⁵I-labeled protein A, followed by autoradiography.

Electron microscopy. Thin-section electron microscopy was done essentially as described previously (17). Virus-infected cells were pelleted, fixed in 1% glutaraldehyde for 18 h at 4°C, and washed in phosphate-buffered saline. Cells were treated with 1% osmium tetroxide, dehydrated in

FIG. 1. Molecular cloning of full-length HIV-1 genomes from uncultured human brain. (A) Southern blot analysis of high-molecular-weight brain DNA derived from a patient with ADC. The restriction enzyme cleavage patterns and relative intensities of HIV-1 viral sequences in uncultured brain DNA (AS) are compared with those of positive (+; H9/HTLV-IIIb) and negative (–; uninfected PBMCs) control DNAs. Undig, undigested. (B) Schematic representation of the cloning strategy. Unintegrated circular viral DNA was linearized with *EcoRI* and subsequently cloned in permuted form into *EcoRI*-cleaved phage arms of λ gtWES · λ B.

graded alcohols, and embedded in an epoxy resin mixture. Thin sections were cut, stained with uranyl acetate and lead nitrate, and examined with a Philips 301 electron microscope.

RESULTS

Lambda phage cloning of full-length HIV-1 from uncultured human brain. Patients with ADC are known to harbor considerable amounts of HIV-1 sequences within their brains, both in chromosomally integrated form and in unintegrated form (41, 54). While both linear and circular viral molecules contain a complete viral genome, viral circles can be cloned in their entirety after linearization with an appropriate restriction enzyme. The abundance of circular viral DNA in the CNS of certain patients with ADC therefore prompted us to attempt molecular cloning of replication-competent HIV-1 genomes directly from this viral population.

Screening several brain DNAs from patients with ADC, we identified one sample to be particularly suitable for lambda phage cloning. This DNA preparation was available in sufficient quantities, exhibited no signs of degradation, and contained enough HIV-1 viral sequences to be visualized by Southern blot analysis. Restriction enzyme mapping with *EcoRI* and *SstI* (alone and in combination) confirmed that *EcoRI* cleaved the provirus only once and indicated that

FIG. 2. Recombinant lambda phage clones obtained from a genomic library of uncultured brain DNA. Restriction enzyme analysis identified nine clones as containing unintegrated HIV-1 circles in permuted orientation and one clone as representing an integrated proviral half with flanking cellular sequences (wavy lines). Hatched boxes represent LTR sequences; arrows indicate the transcriptional direction. Triangles depict the positions of internal deletions. Locations of restriction enzymes sites (E, *EcoRI*; S, *SstI*) and relative positions of major HIV-1 open reading frames are shown. Genomic regions confirmed by nucleotide sequence analysis are shaded.

viral circles comprised the majority of HIV-1 sequences within this particular sample (Fig. 1A). We therefore constructed a genomic lambda phage library in λ gtWES $\cdot\lambda$ B, using *EcoRI*-digested brain DNA fractions 9 to 12 kb in length (Fig. 1B). Screening approximately 30 genomic equivalents (8×10^6 lambda phage plaques), we identified 10 recombinant clones that hybridized to a probe specific for HIV-1.

Detailed restriction enzyme analysis and Southern blot hybridization studies confirmed the presence of HIV-1 sequences in all 10 recombinant clones and determined the lengths and orientations of their viral inserts (Fig. 2). Nine of ten clones represented unintegrated linearized circles which were cloned in permuted form at a single *EcoRI* site located in the central viral region. The restriction enzyme cleavage

pattern of a 10th clone (YU-6) suggested the presence of an integrated provirus (5' half) flanked by normal cellular sequences. Side-by-side comparison with a prototype HIV-1 construct known to possess a complete proviral genome (pHXB2D; 48, 53) identified four brain clones as comprising full-length HIV-1 inserts with either one (YU-2, YU-10, and YU-21) or two (YU-32) tandem copies of the viral LTR. Three clones (YU-1, YU-5, and YU-27) contained internal deletions, while two other clones (YU-3 and YU-4) exhibited genomic rearrangements which were consistent with the presence of an extraneous LTR in reverse orientation.

Molecular characterization of HIV-1-containing lambda phage clones. Nucleotide sequence analysis was performed to further characterize all clones in regions that could not be definitively mapped by restriction enzyme analysis alone.

FIG. 3. Nucleotide sequence analysis of selected genomic regions in brain-derived HIV-1 genomes. (A) Molecular characterization of internal deletions. The extents and locations of internal deletions in clones YU-1, YU-5, and YU-27 are depicted. Sequences are numbered according to the HXB2 reference clone (48). (B) Schematic representation of the circle junction in clone YU-32. The orientation of both LTRs was confirmed by sequence analysis. An additional 4 bp located between the 3' terminus of the 5' LTR and the 5' terminus of the 3' LTR are shaded. (C) Sequence analysis of the junction between HIV-1 proviral DNA and flanking cellular sequences in clone YU-6. The boundaries of the integration site are depicted. aa, amino acids.

Internal deletions of different lengths were identified in three molecular clones (Fig. 2 and 3A). YU-5 contained a 3,044-bp deletion in the 3' half of its genome, which included portions of *tat* and *rev*, the entire *vpu* and *env* genes, and the N-terminal half of *nef*. YU-27 was characterized by a less extensive deletion (549 bp) which included the C terminus of the *env* gene and the N terminus of the *nef* gene. YU-1 contained a 555-bp deletion in its *gag* gene which involved the entire p17 protein and a portion of the p24 protein but did not include sequences 5' of *gag* such as the viral packaging signal. None of the deleted fragments extended to the U3 or U5 region of the viral LTR. The *EcoRI* cloning site was mapped within the *vpr* gene (position 5742 of the HXB2D sequence; 48).

Integration of most retroviruses involves the removal of 2 bp from the termini of the unintegrated linear DNA precursor as well as a duplication of the target sequence at the insertion site (19, 30, 64). Viral circles with two LTRs generally contain those nucleotides which are lost during the integration process within their circle junction, since they result from blunt-end ligation of unintegrated linear molecules (19, 30, 64). To examine whether HIV-1 proviral integration in primary tissue involved similar mechanisms, we sequenced the LTR-LTR circle junction in clone YU-32 (Fig. 3B). The results demonstrated the presence of four additional nucleotides between the conserved CA and TG dinucleotides that generally delineate the boundaries of integrated HIV-1 proviruses (.CA-GTAC-TG.). These data thus indicated that linear HIV-1 molecules contain two dinucleotides at each terminus which are lost before or during recombination with the host genome in vivo.

Nucleotide sequence analysis was also required to further characterize the nature of viral and nonviral sequences in clone YU-6. Restriction enzyme analysis indicated several enzyme site differences 5' of the YU-6 LTR which distinguished this phage clone from all other brain-derived constructs. Subgenomic probes of HIV-1 failed to detect envelope sequences 5' of the LTR, whereas this same region hybridized to nick-translated normal human PBMC DNA, thus indicating the presence of human repetitive sequences (data not shown). Nucleotide sequence analysis of the LTR junction fragment of YU-6 identified the expected boundaries between an integrated HIV-1 provirus and adjoining cellular sequences (Fig. 3C).

Self-integrated HIV-1 circles in primary infected tissue. Detailed restriction enzyme analysis identified two lambda phage clones (YU-3 and YU-4) as exhibiting extensive genomic rearrangements in the 5' halves of their genomes. Further characterization revealed that both clones contained extraneous HIV-1 LTR sequences in reverse orientation with respect to the remainder of the viral genome. To analyze more precisely the nature of these inverted LTR fragments and to elucidate the mechanisms by which they were generated, we determined their nucleotide sequences as well as the sequences of their insertion sites (Fig. 4).

Sequence analysis revealed that both HIV-1 genomes contained inverted LTR sequences within their *pol* genes. YU-4 contained an inverted LTR which was inserted in its entirety without additional deletions or rearrangements of the target sequence except for the presence of a 5-bp direct repeat (AATAC) directly adjacent to the insertion site. The boundaries of the inverted LTR itself were identical to those

FIG. 4. Identification of inverted LTR sequences in the *pol* gene of two unintegrated HIV-1 circles. Nucleotide sequence analysis confirmed the boundaries of the insertion sites. YU-3 contains deletions in both inverted LTR and adjacent *pol* sequences. YU-4 is characterized by an intact inverted LTR flanked by a 5-bp direct repeat (AATAC) immediately adjacent to the insertion site. Sequences are numbered according to the HXB2 reference clone (48).

of an integrated HIV-1 provirus (5'-TG...AC-3'). This fact, as well as the observed duplication of sequences (AATAC) normally present only once in this region of the HIV-1 genome indicated that the inverted LTR in YU-4 was the result of an autointegration event. Inverted LTR sequences were also identified in the *pol* gene of YU-3. However, this inversion was associated with deletions involving both LTR and *pol* sequences. The LTR itself lacked 6 bp on its 3' and 3 bp on its 5' end, while a major portion of the *pol* gene (1,197 bp) was also missing. No direct repeats were identified in the vicinity of the insertion site, and there were no additional deletions or alterations within the inverted LTR. Because of the absence of the characteristic target sequence duplication, the mechanisms responsible for the genomic rearrangements in YU-3 are less clear. Interestingly, genomic inversions in both YU-3 and YU-4 involved solely LTR sequences and did not include adjacent viral sequences as has been observed in other retroviral systems (57, 58, 61, 63). Also, there was no evidence for consensus sequences for target site recognition in regions adjacent to both inverted LTRs.

Genetic variability of HIV-1 in uncultured human brain.

Current data regarding the genetic heterogeneity of HIV-1 have been derived from studies of virus culture (49, 53) and from direct PCR amplification of primary, uncultured tissues (3, 10, 36, 65). Important differences in results from these two approaches include a lesser degree of HIV-1 genetic variation *in vitro* and a surprisingly high proportion of defective viral genomes *in vivo* (36). The availability of both recombinant lambda- and PCR-derived molecular clones of HIV-1 from the same brain specimen allowed us to directly compare HIV-1 variability by these two approaches. Moreover, the PCR analyses served to independently confirm the origin and authenticity of the lambda phage clones. A 510-bp fragment containing the V3 loop and adjacent envelope sequences was amplified by single-round PCR, subcloned into M13, and subjected to nucleotide sequence analysis. Twelve independent M13 clones and eight lambda phage clones were sequenced in the same envelope region. An alignment of their sequences is depicted in Fig. 5.

Comparing a total of 20 independent envelope sequences, we identified 1 predominant viral genotype and 11 minor viral variants. Importantly, the nucleotide sequence of the

predominant genotype was identical for lambda phage- and PCR-derived clones, thus proving unequivocally their common origin. Overall genotypic variability in 19 of 20 clones was 1.2% or less. Only one clone exhibited 8.2% nucleotide sequence differences. Lambda phage clones comprised a homogeneous group of viruses, with five clones having identical nucleotide sequences and the sequences of three others differing by only one or two point mutations (0.2 to 0.4% nucleotide sequence differences). PCR-derived sequences varied more, both with respect to the number of nucleotide sequence substitutions between individual clones (0.2 to 8.2% nucleotide sequence differences) and with respect to the total number of distinguishable variants (8 of 12 versus 3 of 8 for lambda phage clones). In addition, only PCR-derived sequences contained single-base-pair deletions as well as frequent G-to-A transitions. One PCR clone was characterized by a particularly large number of G-to-A changes, which comprised 40 of 42 total nucleotide substitutions.

All 12 distinguishable HIV-1 genotypes were also unique in deduced amino acid sequence, indicating that the majority of nucleotide changes were nonsilent in nature. Three of eight lambda phage clones differed from the predominant genotype by only one or two amino acid sequence changes, none of which interrupted the envelope open reading frame. Three of eight PCR-derived clones varied from the predominant strain by a similar number of substitutions, while five other PCR clones contained frameshift mutations or in-frame stop codons. This finding indicated that at least 42% of the PCR-derived clones would correspond to defective viral genomes.

Biological characterization of full-length lambda phage clones derived from uncultured brain. To investigate whether any of the four full-length HIV-1 genomes were replication competent, we isolated viral inserts from YU-2, YU-10, YU-21, and YU-32, self-ligated these inserts to generate viral genomes in nonpermuted orientation, and transfected them into Cos-1 cells. Primary transfectants were subsequently cocultivated with PHA-stimulated normal donor PBMCs or immortalized T-cell lines in an attempt to transmit replication-competent virus. Cultures were inspected daily for the appearance of virus-induced syncytia and monitored for retrovirus activity by p24 antigen capture and reverse tran-

FIG. 5. Genetic variation of HIV-1 in primary human brain. A 510-bp stretch of envelope sequence is aligned between 8 lambda phage-derived and 12 PCR-derived clones. The deduced amino acid sequence, including the position of the major V3 neutralizing epitope, is shown above the nucleotide sequence. Sequence changes are depicted in reference to the predominant viral form, which represents five lambda phage-derived and four PCR-derived clones. Dashes indicate nucleotide sequence identity; asterisks represent single-base-pair deletions. Underlined nucleotides indicate the positions of in-frame translational stop codons. The frequencies of lambda phage- and PCR-derived clones with identical sequences along with the total number of clones analyzed are shown.

To improve the transfection efficiency, we reconstructed two of the four full-length lambda phage clones (YU-2 and

FIG. 6. Biological analysis of a replication-competent HIV-1 genome cloned directly from uncultured human brain. (A) Reconstruction of viral genomes in nonpermuted orientation. Lambda phage inserts were isolated following digestion with *EcoRI* and subsequently self-ligated. Correctly oriented viral inserts were also obtained by ligating a *SalI-EcoRI* fragment and an *EcoRI-SphI* fragment into the plasmid vector pTZ19R. (B) Electron micrograph of transfection derived YU-2 virus in normal donor PBMCs. (C) Virus-induced syncytium formation after cocultivation of YU2-infected PBMCs with Molt-4 (clone 8) cells. (D) Western blot analysis of cell-free YU-2 virus. All major viral gene products are compared with respect to presence and size with HIV-1 from H9/HTLV-IIIb-infected cells.

YU-10) to generate viral genomes in nonpermuted orientation. This was achieved by subcloning a *SalI-EcoRI* fragment (containing the 5' half of the virus) and a *EcoRI-SphI* fragment (containing the 3' half of the virus) into the same plasmid vector (Fig. 6A). Subsequent transfection experiments confirmed the results obtained with use of self-ligated lambda phage inserts. pYU-2-derived virus replicated well in primary lymphocytes and monocytes and induced syncytia when cocultivated with Molt-4 (clone 8) cells (Fig. 6C). Electron microscopy demonstrated normal particle morphology, and Western blot analysis confirmed the presence and appropriate size of all virus-specific proteins (Fig. 6B and D). Importantly, pYU-2-derived virus infected primary macrophages and replicated to high titers, exhibiting growth characteristics similar to those previously identified for macrophage-tropic HIV-1 strains (47a, 69). Transfection of pYU-10 failed to result in a productive viral infection even in the nonpermuted plasmid construct. This result strongly suggested that YU-10 was replication defective, although certain viral genes, including the *gag* and *env* genes, appeared to be functional, since transient p24 production and syncytium formation were observed.

DISCUSSION

In this report, we describe the molecular cloning of 10 HIV-1 viral genomes from uncultured human tissue and describe their genetic and basic biological properties. The

availability of these clones allowed us to address a number of questions relating to the molecular biology of HIV-1 in vivo which could not be studied previously. These included an analysis of the genetic structure and complexity of unintegrated HIV-1 circles in primary infected tissue, characterization of defective and rearranged genomes, evaluation of in vivo genetic variability as determined independently by lambda phage cloning and PCR analysis, and biological characterization of HIV-1 genomes not previously subjected to tissue culture selection pressures.

Complexity of HIV-1 circular DNA in primary infected brain tissue. HIV-1 exists in primary tissues of infected individuals in both chromosomally integrated and unintegrated forms, although the ratio of unintegrated versus integrated molecules differs considerably according to the organ system analyzed (41). Brain tissue contains the highest proportion of extrachromosomal DNA, and patients who suffer from ADC harbor more unintegrated DNA within their CNS than do individuals without neurological symptoms (41, 54). Unintegrated DNA intermediates are generally synthesized immediately following viral entry (19, 64), and an accumulation of such molecules would be expected to represent acute infection or reinfection of susceptible target cells. However, the failure to find evidence of cell-free virus in brain by p24 antigen capture assay (41) that could account for ongoing viral infection and DNA synthesis suggests that viral DNA may be produced or accumulate by other mechanisms. Since the presence of unintegrated HIV-1 DNA

within the brain may be linked to the pathogenesis of ADC (41), a better understanding of the structure and function of these molecules is needed to evaluate their biological significance.

Our study allowed, for the first time, a determination of the genomic organization of unintegrated circular HIV-1 molecules as they exist *in vivo*. Similar to what is found for other retroviruses, HIV-1 circles were found to represent intact as well as defective molecules, contain either one or two copies of the viral LTR, and comprise rearranged genomes with internal inversions and deletions (19, 64). There were, however, structural differences that distinguished the brain-derived HIV-1 circles from all previously studied retroviruses. For example, three of nine clones (30%) exhibited internal deletions that involved various parts of the HIV-1 genome. Olsen and colleagues reported a similar fraction of deleted molecules among 193 circles of Rous sarcoma virus (40). However, 75% of these deletions terminated either within or in close proximity of the viral LTR and included *cis*-acting sequences essential for RNA transcription and packaging (40). Surprisingly, none of the deletions in our clones extended into the viral LTR or involved adjacent regulatory regions such as the packaging signal. These HIV-1 molecules could therefore, at least theoretically, replicate via a helper virus genome. The circular forms of oncogenic retroviruses are also known to contain genomic inversions which involve LTR sequences and adjacent genomic regions (57, 58, 61, 63). In our study of nine unintegrated HIV-1 circles, two molecules with similar internal inversions were identified, although both clones contained exclusively LTR sequences. It is conceivable that these structural differences result from processes specific for HIV-1 replication and persistence in brain-resident target cells.

Retroviral integration results in a loss of 2 bp from the termini of the unintegrated linear DNA precursor as well as a duplication of host cell sequences at the site of proviral insertion (19, 30, 64). The length of the target sequence duplication is specific for individual retroviruses and, in the case of HIV-1, comprises a direct repeat of 5 bp (6, 38, 67). Two of the nine HIV-1 brain clones (YU-3 and YU-4) contained inverted LTR sequences within the viral *pol* gene. A 5-bp duplication immediately adjacent to the recombination site in one of these clones (YU-4), as well as the boundaries of the inverted LTR itself, strongly suggested that LTR-mediated autointegration was responsible for this genomic rearrangement. While similar alterations have been shown to occur in the genomes of oncogenic retroviruses (57, 58, 61, 63), the existence of self-integrated HIV-1 circles in primary human tissue has not yet been described. Moreover, none of the previously reported autointegration models provided an adequate explanation for the generation of the rearranged HIV-1 molecules. To better accommodate the structural characteristics of YU-3 and YU-4, we propose an alternative model (Fig. 7). This model proposes that a circular and a linear HIV-1 molecule integrate into each other. As a result of this autointegration event, a 5-bp direct repeat is created at the insertion site (viral *pol* gene), while 2 bp are lost from the end of the unintegrated linear molecule. As a result of subsequent homologous recombination between the two LTRs of the linear molecule, a whole virus with one LTR is looped out, creating a second viral genome with two LTRs in opposite orientation. Instability of the autointegration site followed by deletions in that region would explain the genomic organization of YU-3.

A careful analysis of self-integrated HIV-1 circles derived

FIG. 7. Hypothetical model explaining the generation of HIV-1 circles with self-integrated LTR sequences. A circular and a linear viral molecule are proposed to integrate into each other, resulting in the loss of 2 bp from the termini of the linear molecule and the duplication of target sequences (5 bp) flanking the insertion site. As a result of subsequent homologous recombination, an entire genome is looped out (dark line), leading to the formation of a viral circle with two LTRs (hatched boxes) in opposite orientation. Arrows indicate the transcriptional direction of the viral LTRs, and vertical bars depict the target sequence duplication characteristics of HIV-1 integration.

directly from uncultured brain also allowed us to better define HIV-1 integration processes as they occur *in vivo*. Previous studies of cultured viral isolates demonstrated a 5-bp target sequence duplication immediately adjacent to integrated HIV-1 proviruses (6, 38, 67). Moreover, the majority of HIV-1 circle junctions, as amplified by PCR from HIV-1-infected culture DNA, were shown to contain an additional 4 bp between their tandem LTRs (25, 43, 59, 71). Our results demonstrating the same four nucleotides (GTAC) between the two LTRs in YU-32 (Fig. 3B) as well as an identical 5-bp direct repeat (AATAC) immediately adjacent to the self-integrated LTR in YU-4 (Fig. 4) independently confirm the findings of these earlier studies. Most importantly, however, our results extend these conclusions to an *in vivo* situation.

The biological significance of unintegrated HIV-1 circles, including those resulting from self-integration processes, are presently unknown. Circular viral forms are generally believed to represent a by-product of retroviral integration, while linear molecules serve as the direct precursor for proviral integration (19, 64). While this appears to be the case in PBMCs and immortalized T-cell lines, the function of unintegrated viral DNA, including viral circles, may be quite different in macrophages or microglia. Cells of this lineage

are terminally differentiated and do not divide. It is still unknown whether and to what extent HIV-1 integrates into the genomes of these cells. Our finding of an integrated provirus among the 10 brain clones unfortunately provides no further clarification of this point, since the presence of HIV-1-infected blood-derived lymphocytes in the brain specimen cannot be excluded. It is also unclear whether HIV-1 can bypass the requirement of proviral integration for its expression and replication, as has been suggested for other lentiviruses (23), and whether unintegrated viral forms are transcriptionally active in macrophages and related brain cells. If this were the case, the persistence of large amounts of unintegrated DNA, including circular molecules, may be relevant to some aspects of HIV-1-induced pathogenicity or latency in patients with ADC. Independent replication and transcription of circular self-integrated molecules has been hypothesized (44), although there are presently no experimental data to support this speculation. Alternatively, large amounts of unintegrated DNA, in particular unintegrated viral circles, may simply reflect an inefficient integration process in macrophages and microglia and may represent a marker rather than a pathogenic factor of HIV-1 infection in the brain. Further studies focusing on HIV-1 integration and replication processes specific for the human brain are obviously needed to clarify these important questions.

Genetic variability of HIV-1 in infected brain tissue. Sequence analysis of a hypervariable envelope region in 8 brain-derived lambda phage clones and 12 PCR clones revealed a mixture of highly related HIV-1 genotypes which included a predominant viral form as well as a number of minor genotypic variants. This quasispecies nature of HIV-1, first described in virus-infected cell cultures (20, 49) and later in uncultured PBMCs (3, 36, 65), is well recognized as a characteristic feature of all immunodeficiency viruses. Comparison of the brain-derived HIV-1 strains with other unrelated HIV-1 viruses indicated a degree of genetic variability similar to that previously identified for North American isolates. Nucleotide sequence divergence in the selected envelope region ranged between 9% for HIV-1/JRFL (29) and 25% for LAV/ELI (1). The deduced V3 loop sequence of the predominant brain strain differed from the V3 consensus sequence by two amino acid changes, both of which represented less frequent substitutions characteristic for a subset of HIV-1 viruses (31).

In contrast to the usual range of heterogeneity observed in independent HIV-1 isolates, intrastrain variability among the brain-derived clones was surprisingly low. Except for one PCR clone with 42 (8.2%) base pair substitutions, we identified only 0.2 to 1.2% nucleotide sequence differences among all other clones sequenced (Fig. 5). A recent study of Scottish hemophiliacs indicated that intrastrain diversity can vary considerably among individual patients (3). It is thus possible that the greater uniformity of HIV-1 sequences in our brain sample is the result of a lower propensity to genetic changes in one particular individual. Alternatively, a reduced intrastrain variability may also be tissue specific and may reflect less stringent immune pressures, lower rates of replication, the persistence of one particular viral variant, or other tissue-specific constraints that affect HIV-1 genotypic variability. Additional brain samples from other patients with ADC will have to be examined to answer these questions.

Whereas all brain-derived HIV-1 sequences were highly related to each other, mutations among the lambda phage-derived clones were found to be less frequent than for PCR-derived clones. Of 4,080 bp of total sequence, only five base-

pair differences were detected among the eight lambda phage clones (Fig. 5). PCR sequences exhibited more sequence changes per individual clone and a greater number of distinguishable variants. In addition, only PCR clones contained single-base-pair deletions as well as G-to-A changes, which resulted in frameshift mutations and premature stop codons. Given the small number of PCR and lambda phage clones available and the limited amount of envelope sequence determined, an interpretation of these results is difficult. However, it appears that HIV-1 circles comprise a relatively more homogeneous population of viral sequences than do the PCR-derived clones. Since virtually every section of the brain tissue used for the cloning study expressed HIV-1-specific antigens (37), it is conceivable that the unintegrated DNA circles represent a subgroup of transcriptionally active viral genomes which are more limited in sequence variability. PCR, on the other hand, would be expected to amplify all HIV-1 sequences, including defective and latent viruses. It will be important to determine whether the greater spectrum of genetic variability as determined by PCR analysis is biologically relevant or whether it represents, at least to some extent, a "graveyard" of viral sequences with little contribution to viral pathogenicity.

The identification of clustered G-to-A mutations in 1 of the 12 PCR clones is of interest, particularly in light of recent results of other investigators (38a, 65). G-to-A hypermutation is frequently found in PCR-derived clones from cultured as well as uncultured material and appears to represent a characteristic feature of lentiviral genomes. Control experiments with cloned DNA molecules (20, 36) as well as sequence analysis of lambda phage clones (42) have ruled out *Taq* polymerase errors as the source of these sequence changes. A mutant reverse transcriptase or suboptimal conditions within an infected cell during reverse transcription are likely responsible for the observed changes (65). The identification of such molecules in primary brain tissue indicates that G-to-A hypermutation is not restricted to PBMCs but rather represents a general phenomenon of HIV-1 replication in vivo.

Biological properties of HIV-1 genomes cloned directly from uncultured brain. Isolation and propagation of HIV-1 in tissue culture has thus far been the only method feasible for obtaining sufficient quantities of viral DNA for the molecular cloning of transfection-competent HIV-1 genomes. PCR has facilitated the amplification of up to 5,000 bp of viral sequences from primary tissue (38a), although cloning of full-length HIV-1 proviruses has not yet been accomplished. Since tissue culture is highly selective for viruses with an in vitro growth advantage, molecular clones derived from cultured cell DNA might not be representative of the biologically most relevant virus. Viral properties, including cell tropism, replication potential, and cytopathic activity, are known to differ in various target cells and are thus particularly prone to in vitro selection pressures (8, 12, 13, 29, 73). HIV-1 genomes obtained without interim culture might therefore be superior to tissue culture-derived clones for the evaluation of such viral properties.

Preliminary biological analysis of four full-length HIV-1 genomes cloned from uncultured brain identified one (YU-2) as replication competent. Virus derived from this clone replicated in primary T cells and Molt-4 (clone 8) cells but was restricted in its cell tropism for SupT1 and CEMx174 cells. Most importantly, the brain-derived virus was able to grow and replicate to high titers in primary macrophages. In fact, comparative studies indicated that its growth characteristics and replication kinetics were comparable to or

exceeded those of previously identified macrophage tropic prototype strains (47a, 69). More detailed *in vitro* studies are needed to determine the full spectrum of YU-2's host cell range, replication rate, and cytopathic potential. However, the preliminary data obtained thus far point toward the potential importance of this molecular clone for future studies of macrophage and neurotropism. The availability of HIV-1 brain clones will also be instrumental for studies of gene structure-function relationships in viral genomes not previously subjected to *in vitro* selection pressures. In particular, the analysis of brain-derived envelope glycoproteins might reveal interesting information, since their biophysical properties, including host cell tropism, fusogenic properties, and CD4 binding affinity, have been selected by nature and not by tissue culture passage and amplification.

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