

Unusual Polymorphisms in Human Immunodeficiency Virus Type 1 Associated with Nonprogressive Infection

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Factors accounting for long-term nonprogression may include infection with an attenuated strain of human immunodeficiency virus type 1 (HIV-1), genetic polymorphisms in the host, and virus-specific immune responses. In this study, we examined eight individuals with nonprogressing or slowly progressing HIV-1 infection, none of whom were homozygous for host-specific polymorphisms (*CCR5-Δ32*, *CCR2-64I*, and *SDF-1-3'A*) which have been associated with slower disease progression. HIV-1 was recovered from seven of the eight, and recovered virus was used for sequencing the full-length HIV-1 genome; full-length HIV-1 genome sequences from the eighth were determined following amplification of viral sequences directly from peripheral blood mononuclear cells (PBMC). Longitudinal studies of one individual with HIV-1 that consistently exhibited a slow/low growth phenotype revealed a single amino acid deletion in a conserved region of the gp41 transmembrane protein that was not seen in any of 131 envelope sequences in the Los Alamos HIV-1 sequence database. Genetic analysis also revealed that five of the eight individuals harbored HIV-1 with unusual 1- or 2-amino-acid deletions in the Gag sequence compared to subgroup B Gag consensus sequences. These deletions in Gag have either never been observed previously or are extremely rare in the database. Three individuals had deletions in Nef, and one had a 4-amino-acid insertion in Vpu. The unusual polymorphisms in Gag, Env, and Nef described here were also found in stored PBMC samples taken 3 to 11 years prior to, or in one case 4 years subsequent to, the time of sampling for the original sequencing. In all, seven of the eight individuals exhibited one or more unusual polymorphisms; a total of 13 unusual polymorphisms were documented in these seven individuals. These polymorphisms may have been present from the time of initial infection or may have appeared in response to immune surveillance or other selective pressures. Our results indicate that unusual, difficult-to-revert polymorphisms in HIV-1 can be found associated with slow progression or nonprogression in a majority of such cases.

Disease induction following human immunodeficiency virus type 1 (HIV-1) infection, or the ability to control the infection in rare cases, is likely to be regulated by the balance of at least three factors: genetic susceptibility of the host, the ability of the host to elicit effective immune responses, and the sequence of the incoming virus.

Twenty-five to 30% of long-term nonprogressors (LTNPs) who have remained AIDS free for more than 15 years in the absence of antiretroviral drugs express specific mutant forms of the HIV-1 second receptors *CCR5* and *CCR2* (11, 23, 52). Individuals who are homozygous for a 32-bp deletion ($\Delta 32$) in *CCR5*, which encodes the most frequently used second receptor for HIV-1, are resistant to infection by HIV-1 (11, 36). HIV-1-infected individuals who are heterozygous for *CCR5Δ32* (-/+) show a slightly slower disease progression than *CCR5*+/+ homozygotes (11, 23, 36, 49). A V64I polymorphism in the *CCR2* gene, which is linked to a point mutation in the regulatory region of the *CCR5* gene, and a common polymorphism in

the 3'-untranslated region of the stromal derived factor gene (*SDF-1*) are also associated with delayed disease progression (3, 5, 6, 57). *SDF-1* is the natural ligand for CXCR4, the second receptor for syncytium-inducing strains of HIV-1. Intracellular factors may also contribute to the inherent susceptibility of host cells to support HIV replication (13, 22, 35).

HIV-1-infected individuals who are LTNPs also appear in general to have more effective HIV-1-specific immune responses. Perhaps most remarkable is the HIV-specific CD4 helper cell activity seen in nonprogressors controlling their HIV infection (47). This vigorous helper cell response contrasts with the absent or low response typically observed in progressors (47). Nonprogressors also generally exhibit more vigorous cytotoxic T-lymphocyte (CTL) activity than typical progressors (18, 21). The ability to mount effective immune responses may be influenced by the extent of heterozygosity at major histocompatibility complex class I (MHC-I) loci or by the specific MHC-I genotype or both (6). The ability to mount effective immune responses may of course be related in some cases to the virulence of the infecting strain of HIV-1 and/or the inherent susceptibility of the host.

One patient from central Massachusetts (27) and nine from Australia (10) are infected with Nef-deleted forms of HIV-1.

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Infection with this replication-competent, attenuated form of HIV-1 is clearly responsible for slow progression or nonprogression in these individuals (10, 27). This is particularly evident for eight of the individuals in the Australian cohort, who were all infected by a single blood donor, who all have the Nef-defective form of the virus, and who all have been slow progressors or nonprogressors (33, 43). An additional LTNP with consistently deficient forms of Nef has been described (48). One LTNP whose *rev* gene exhibited deficient activity over more than 4.5 years of observation has been described (25), another with a consistently defective p17 sequence (5) and a nonprogressing infant-mother pair containing defects clustered in the C terminus of Vpr have been documented (54).

In this report we describe full-length HIV-1 sequences from eight nonprogressors living in New England. Additional examples of uniform, nonrevertible inactivating deletions in auxiliary genes were not discovered. However, unusual, difficult-to-revert polymorphisms were noted in the HIV-1 present in seven of these eight individuals.

MATERIALS AND METHODS

LTNP HIV-1 cultures. Peripheral blood mononuclear cells (PBMC; 5×10^6) from LTNPs were cocultivated with an equal number of phytohemagglutinin-activated PBMC from seronegative donors. These cultures were monitored for p24 production using an HIV-1 core antigen kit (Coulter, Hialeah, Fla.). If HIV-1 replication was not detected in these cultures, CD8 cells were depleted using immunomagnetic beads (Dynabeads; Dynal Inc., Great Neck, N.Y.) prior to cocultivation (14). Using these protocols, HIV-1 was recovered from seven of the eight LTNPs described in this report.

Isolation of cellular DNA. Infected cultured cells (5×10^6) or LTNP PBMC were lysed in 0.5 ml of lysis buffer (10 mM Tris [pH 8.2], 0.4 M NaCl, 2 mM EDTA [pH 8.2]) that was supplemented with 33 μ l of 10% sodium dodecyl sulfate (SDS) and 10 μ l of proteinase K (10 mg/ml) for 1 h at 56°C. After lysis, 160 μ l of saturated NaCl was added and the tube was inverted to mix the reagents. The mixture was then centrifuged at 14,000 rpm in a microcentrifuge for 10 min. The clear supernatant was removed and placed in a fresh tube, and 700 μ l of isopropanol was added. The mixture was inverted and centrifuged for 10 min at 14,000 rpm. The supernatant was then removed, and the pellet was washed with 70% ethanol and then air dried for 1 h.

Amplification of LTNP HIV-1 sequences. One microgram of cellular DNA served as the template for PCR amplifications. Primers for PCR that annealed to highly conserved regions of HIV-1 were chosen. These primers were designed such that their sequences were rare in both the documented human genome and HIV-1 sequences according to the Right Primer analysis program (Biodisk, San Francisco, Calif.). In addition, they were designed to have annealing temperatures of approximately 70°C as determined by the Oligo primer analysis program (National Biosciences, Plymouth, Minn.). Stringent PCR conditions that had been previously shown to optimize the amplification of large, low-copy-number templates were chosen (3, 8, 58). A 200- μ l reaction volume in a 0.5-ml thin-walled PCR tube (Perkin-Elmer Cetus, Norwalk, Conn.) was used. The volume included 2 U of rTthXL (Perkin-Elmer Cetus), 200 mM deoxynucleoside triphosphates, and a 0.2 μ M concentration of each primer. This PCR mixture was preheated for 60 s at 80°C before 1.0 mM magnesium acetate was added. The sample was then inserted into an Omnigene PCR cyler (Hybaid, Franklin, Mass.) that was preheated to 80°C. Seventy-five cycles/round of PCR was used to amplify LTNP HIV-1 sequences. Each cycle consisted of a 93°C denaturation step followed by a rapid-cooling step to 70°C and a slow-cooling step to 65°C. The 65°C annealing and polymerization step was carried out for 1 min for every 1 kbp of amplified HIV-1 sequence. The complete HIV-1 sequences from the seven LTNPs for which virus was successfully isolated were obtained by amplification of 11 overlapping fragments of approximately 1 kbp each using a single round of PCR for each fragment. HIV-1 from 161J has not been isolated, and thus overlapping fragments of approximately 500 bp were amplified using two rounds of PCR with nested primer sets to complete the HIV-1 sequences. Nested PCR was also employed for amplification of HIV-1 sequences from PBMC samples of LTNPs from whom virus was recovered. Overlapping PCR fragments were treated with T4 DNA polymerase (New England Biolabs, Beverly, Mass.) and inserted into a *Sma*I-digested pUC18 vector (Promega, Madison, Wis.). The DNA sequence of the inserted fragment was then determined with M13 primers using an ABI 377 DNA sequencer (Perkin-Elmer Cetus). Sequence data for LTNP HIV-1 presented in this report represent a consensus of two independent clones from each of two independent PCR amplifications. Nested PCR was used to amplify HIV-1 DNA from the LTNP designated LTNP 5 and the infectious clone NL 4-3 in two approximate halves which could be combined at a common restriction site for transfection into cells. The internal oligonucleotide for the

amplification of each half genome contained an A-to-G change from the consensus sequence which introduces an *Xma*I site (CCCGG) into a highly conserved region (nucleotide 4402 for NL 4-3) (1) without changing the *pol* amino acid coding sequence. 5' PCR fragments included bp 297 to 4402, and 3' fragments included bp 4402 to 9607 (base pairs are based on NL 4-3 sequences).

Phylogenetic analysis of LTNP sequences. The subtype of each LTNP HIV-1 sequence was determined by comparison to HIV-1 subtype reference sequences using the Los Alamos subtyping program (B. Korber, www.ncbi.nih.gov, Los Alamos National Laboratory, 1999). Phylogenetic trees based on neighbor-joining analyses were produced to determine the genetic distance between LTNP and reference *gag* and *nef* sequences using the PAUP phylogenetic analysis software (D. L. Swofford, Phylogenetic analysis using parsimony [and other methods], 4th ed., Sinauer Associates, Sunderland, Mass., 1998). The reference sequences in these analyses, indicated by (in order) code, subgroup, strain, identification number, and accession number(s) are as follows: A1A, U455, 328902, M230; A2, A, 92UG037.1, U51190; B1, B, OYI, 328440, M26727; B2, B, RF, 328565, M17451, M12508, USA; C1, C, ETH2220, 1353860, U46016; C2, C, 92BR025.8, 2194183, U529523; D1, D, NDK, 328154, M27323; D2, D, Z226, 329377, M22639; F1, F, 93BR020.1, 3114544, AF005494; H1, H, 90CR056.1, 3114562, AF005496; AEI, A/E (E in most of *env*, A in *gag* and *pol*, and a mixture of A and E in regulatory genes), CM240, 1537050, U54771; G1, G, SE6165, 3403225, AF061642; E1, MP38; E2, MP59.

Plasmid construction. NL 4-3/LTNP Gag chimeras were engineered by insertion of an *Apa*I-*Bcl*I-digested 320-base fragment that contained the coding sequence for LTNP p6^{gag-pol} into *Apa*I-*Bcl*I-digested p83-2 plasmid which contained NL 4-3 sequences. In order to engineer simian immunodeficiency virus/HIVnef (SHIVnef) recombinants, SIVmac *nef* sequences were deleted from the 3' extent of the SIVmac *env* gene to 120 bases 5' of the NF- κ B binding site in SIVmac239 (45) and HIV-1 sequences derived from the LTNP 1486D were inserted as previously described (2). Thorough DNA sequence analysis verified that all recombinant clones selected for study contained exactly the desired sequences.

HIV-1 and SHIVnef replication assays. All stocks of NL 4-3 or recombinants described in this report were generated by DEAE-dextran transfection of the cell line CEMx174 (41). NL 4-3 or recombinant constructs were transfected into CEMx174 cells, and virus in the cell-free supernatant was harvested at or near the peak of virus production as previously described (15). Transfected or infected CEMx174 cells were grown in RPMI 1640 (Gibco BRL, Grand Island, N.Y.) that was supplemented with 10% fetal calf serum (Gibco BRL). The levels of p27 viral protein that were produced from transfections or infections or that were contained within viral stocks were quantified using a SIV core antigen kit (Coulter).

Analysis of Vpr incorporation. Virus from NL 4-3 or HIV-1 Δ vpr or recombinants with LTNP p6^{gag} sequences that contained 500 ng of p24 antigen were centrifuged at 13,000 \times g for 2 h at 4°C. The supernatant was removed, and the virus pellet was resuspended in Laemmli sample buffer prior to electrophoresis through an SDS-12% polyacrylamide gel electrophoresis gel. The proteins were electroblotted onto an Immobilon-P membrane (Millipore, Bedford, Mass.) which was then blocked with 5% skim milk in phosphate-buffered saline-0.05% Tween 20 (PBST). The blot was incubated overnight at 4°C in blocking solution that contained the anti-HIV-1 Vpr-specific polyclonal antibody (NIH AIDS Research and Reference Reagent Program) at a dilution of 1:2,000. Primary antibodies were removed by washing the membranes three times for 30 min with PBST at room temperature. The dilution of the secondary antibody and the detection of Vpr were performed according to the protocol of the enhanced chemiluminescence system (Amersham, Chicago, Ill.).

Vif functional assay. The Vif genes of LTNP 5 virus clones 2 and 3 were isolated as *Xba*I-*Xho*I fragments using PCR and inserted into the HIV-1_{HXB-3} Vif expression vector pgVIF. Each Vif expression vector as well as the negative control (pg Δ vif) was cotransfected into H9 cells with the vif-deficient proviral vector pIII Δ vif. At 24 h posttransfection, viruses were harvested from the culture supernatants, normalized according to reverse transcriptase activity, and used in single-cycle infections of the indicator cell line C8166/HIV-CAT. After an additional 30 h, the levels of chloramphenicol acetyltransferase (CAT) expression were measured from whole-cell lysates and used to determine relative levels of infectivity. Whole-cell lysates of transfected H9 cultures were also evaluated for Vif accumulation by Western blotting using a Vif-specific monoclonal antibody as previously described (51).

Experimental infection of rhesus monkeys. Virus diluted to contain 50 ng of p27 antigen was inoculated intravenously into juvenile rhesus monkeys (*Macaca mulatta*) (2). At various time points postinoculation, blood samples were collected as previously described (15).

Determination of viral RNA and infectious cell loads. Cell-associated virus loads in infected monkeys were determined by quantitative cocultivation of PBMC with CEMx174 cells (12). PBMC were purified, counted in a hemocytometer, and cocultured with CEMx174 cells in various numbers. On day 21, the presence of SIV p27 antigen was determined and the numbers of PBMC needed to recover SIV were calculated. The results described in this report represent averages of duplicate determinations. Virion-associated SIV RNA in plasma samples was quantified using a reverse transcription-PCR assay on an Applied Biosystems Prism 7700 sequence detection system (Perkin-Elmer Cetus) (53).

Determination of the percentages of CD4⁺ cells in blood of SHIVnef-infected animals. Whole blood was drawn from SHIV nef-inoculated animals at various

TABLE 1. Clinical history of LTNPs

LTNP	Date of first seropositivity detection ^a (mo-day-yr or yr)	Date of last visit ^b (mo-day-yr or mo-yr)	Absolute CD4 T-cell counts/ml at last visit	Plasma viral RNA level at last visit (copies/ml)
LTNP 1	12-21-83	2-11-99	257 ^c	<50
LTNP 2	3-7-84	9-16-98	589	58
LTNP 3	12-7-83	4-21-99	530	180
LTNP 5	2-1-84	3-2-95	464	644
LTNP 6	5-16-84	3-24-99	1,122	5,000
LTNP 7	2-29-84	1-6-99	606	97
161J	1979	6-15-99	843	<50
1486D	1991	12-98	630	8,360

^a LTNP 1, LTNP 2, LTNP 3, LTNP 6, and LTNP 7 were all seropositive at their first visit. LTNP 5, 161J, and 1486D seroconverted between regularly scheduled visits.

^b The date shown is the date of the last visit. LTNP 5 died from complications of hepatitis C infection in June 1997. All other patients are healthy as of their last visit.

^c CD4 T-cell counts in LTNP 1, who is infected with Nef-deleted HIV-1, began declining around 1996 to 1997 despite consistently undetectable viral loads (19).

times postinoculation and stained with OKT4, a fluorescein isothiocyanate-conjugated murine monoclonal antibody that reacts with rhesus macaque CD4. The stained samples were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.).

CCR5, CCR2, and SDF-1 genetic analysis. Genomic DNA was extracted from patient samples using a DNA/RNA extraction kit (Amersham Life Science, Piscataway, N.J.). *CCR5*, *CCR2*, and *SDF-1* genetic polymorphisms were amplified using previously described oligonucleotides and procedures (11, 52, 57).

RESULTS

Introduction to patient population. The patients presented here are HIV-1-infected individuals from the Massachusetts area who have remained AIDS free with low viral loads without antiretroviral intervention (Table 1). Seven of the eight (LTNP 1, LTNP 2, LTNP 3, LTNP 5, LTNP 6, LTNP 7, and 161J) are individuals with hemophilia who were likely infected by contaminated blood products. 1486D is a homosexual male. Of the eight LTNPs, seven continue to be monitored for CD4 concentrations and viral load (Table 1) (17). LTNP 5 died in June 1997 from complications of hepatitis C infection. At the time of his last visit, LTNP 5 exhibited a CD4 concentration of 464/ml and maintained low but detectable viral RNA loads (Table 1). In recent evaluations, CD4 concentrations from LTNP 1 have declined although viral RNA loads remained below the level of detection (Table 1) (19). This individual has maintained a large deletion in Nef and 3' LTR sequences over the entire course of infection, and no reversion of Nef sequence was observed during the period when CD4 concentrations have declined (19, 27). Except for the recent treatment of LTNP 1, none of the LTNPs in this report have received any antiretroviral therapy for their HIV-1 infection despite infection in most cases for over 15 years (Table 1). 161J continues to maintain detectable CD4 helper T-cell responses previously shown to be associated with nonprogression (47). Detectable proliferation responses to p24 antigen have been observed in LTNPs 1, 2, and 3 but not in LTNPs 6 and 7 (17). LTNP 6 was previously reported to contain a polymorphism in Sp1 binding site III which resulted in suboptimal Tat-mediated transactivation (28), consistent with the independent analysis described in this report. LTNP 6 isolates were also shown to be defective in a single cell killing assay in comparison to isolates from progressors (44). LTNP 2, LTNP 3, and LTNP 6 Nef sequences have been previously determined and were shown to be functional in a CD4 down-regulation assay (38).

TABLE 2. LTNP *CCR5*, *CCR2*, and *SDF-1* genotypes^a

LTNP	Genotype ^b of:		
	<i>CCR5</i>	<i>CCR2</i>	<i>SDF-1</i>
LTNP 1	+/+	+/+	+/3'A
LTNP 2	+/+	+/+	+/+
LTNP 3	+/+	+/+	+/+
LTNP 5	+/ Δ 32	+/+	+/+
LTNP 6	+/+	+/+	+/+
LTNP 7	+/+	+/ Δ 64I	+/ Δ 3'A
161J	+/+	+/+	+/ Δ 3'A
1486D	+/+	+/+	+/ Δ 3'A

^a Patient samples were analyzed for HIV-1 second-receptor (*CCR5* and *CCR2*) and chemokine (*SDF-1*) polymorphisms associated with slower progression.

^b +, most common allele for each gene; Δ 32, Δ 64I, and 3'A, *CCR5*, *CCR2*, and *SDF-1* polymorphisms, respectively, associated with slower Δ 64I and 3'A progression in the homozygous state; and Δ 32 and Δ 64I in the heterozygous state.

Analysis of HIV-1 second-receptor genotypes. Specific mutations in the genes for HIV-1 second receptors *CCR5* (Δ 32) and *CCR2* (Δ 64I) and for the chemokine *SDF-1* (3'A) that is the ligand for the CXCR4 second receptor have been associated with slower progression to AIDS (11, 23, 30, 39, 49, 52, 57). We investigated the extent to which these polymorphisms were present in the eight LTNPs studied in this report. LTNP 5 and LTNP 7 carried +/ Δ 32*CCR5* and +/ Δ 64I*CCR2* protective genotypes, respectively, and the other six patients contained more common, nonprotective genotypes (Table 2). No patient was homozygous for *CCR5*- Δ 32, for *CCR2*- Δ 64I, or for *SDF-1*-3'A (Table 2).

HIV-1 sequences of LTNPs. Replication-competent HIV-1 was successfully isolated from seven of the eight LTNPs described in this report by cocultivation of uninfected donor PBMC with PBMC samples obtained in 1994 for LTNP 1,

TABLE 3. Summary of LTNP HIV-1 genotypic and phenotypic characteristics

LTNP	Virus isolation ^a	In vitro growth ^b	Unusual polymorphisms ^c	Frequency (%) of polymorphism in the database ^d
LTNP 1	Yes	Good	Δ Nef (>100 aa)	0
LTNP 2	Yes	Good	None	
LTNP 3	Yes	Good	Δ 112p17 ^{gag}	0
LTNP 5	Yes	Poor	Δ 23TM ^{env}	0
LTNP 6	Yes	Good	4-base insertion 324U3LTR 4-aa insertion Vpu Δ 9-11TM ^{env} Δ 49-50nef 1-aa deletion in Nef C terminus	4 2 9 0
LTNP 7	Yes	Good	Δ 466-467p6 ^{gag-pol}	0
161J	No	NA	Δ 483-484p6 ^{gag-pol} Δ 48-49Nef	0 0
1486D	Yes	Good	Δ 370p2 ^{gag} Δ 465-466p6 ^{gag-pol} Δ 48-49Nef	2 0 0

^a For virus isolation LTNP PBMC were cocultivated with either bulk or CD8-depleted, PHA-activated PBMC from seronegative donors.

^b Good production of >100 ng of p24 antigen/ml in cocultivation assays. LTNP 5 isolates produced a maximum of 5 ng of p24 antigen/ml (poor growth). NA, not applicable.

^c Includes those that are difficult to revert and those that were consistently observed in cultured virus and in patient PBMC.

^d Frequency of polymorphism in the Los Alamos sequence database.

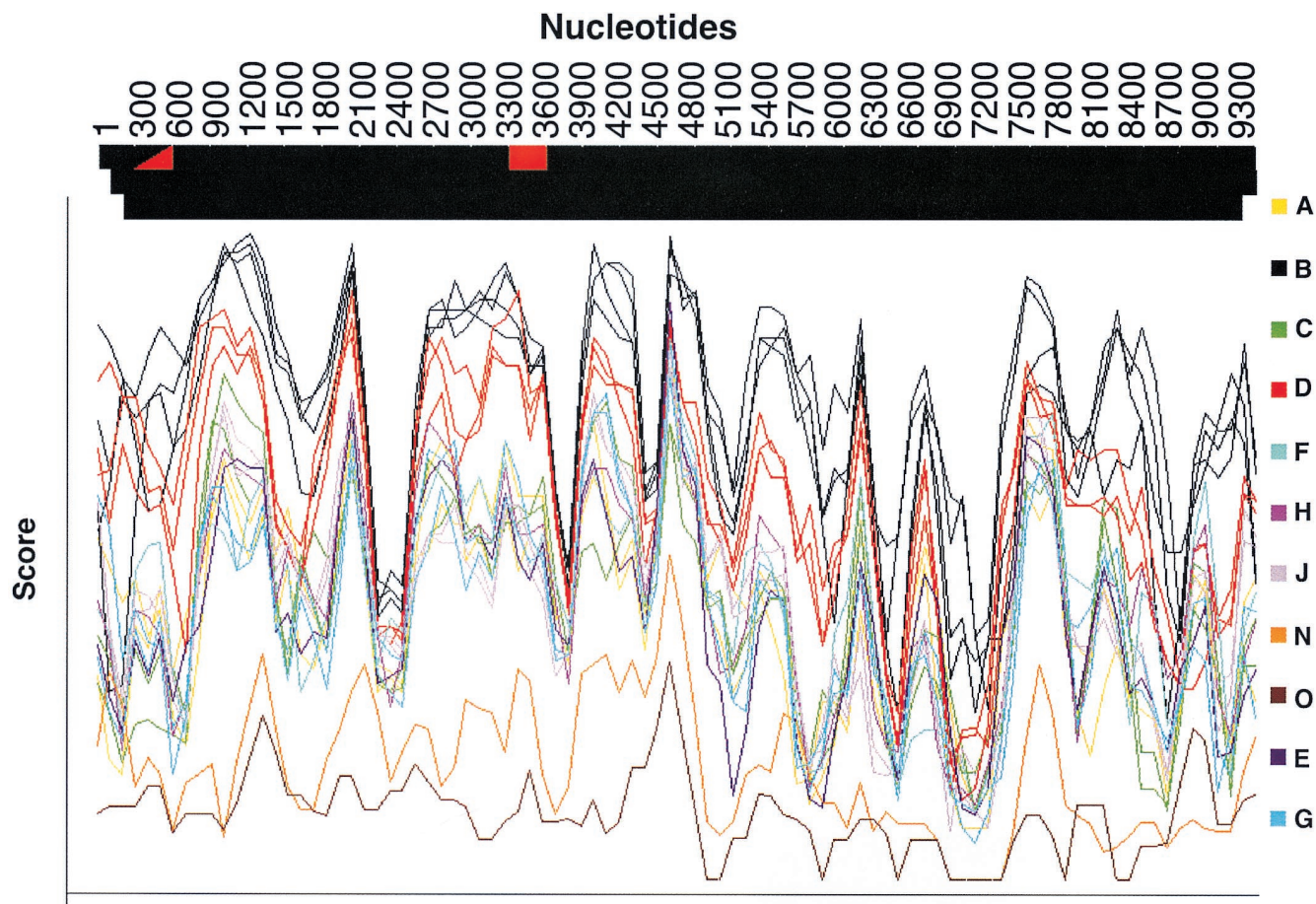


FIG. 1. Subtyping of the HIV-1 sequence of LTNP 1486D. 1486D sequences from bp 297 to 9607 (numbers based on NL 4-3 sequences) were analyzed using the Los Alamos subtyping program and subtyping reference sequences. The score value indicates the relative relatedness of 1486D sequences at different loci throughout the genome. The color of the bar at the top indicates the subtype that 1486D sequences are most closely related to at loci throughout the HIV-1 genome.

LTNP 2, LTNP 3, LTNP 5, LTNP 6, and LTNP 7 as well as in 1995 for 1486D (Table 3) (17). DNA from cells infected with these isolates served as the template for the amplification of HIV-1 sequences. HIV-1 was not successfully isolated from 161J (Table 3); we used DNA from a PBMC sample obtained in 1994 from 161J to serve as template for PCR.

The laboratories in which cellular DNA was purified and HIV-1 sequences were amplified for this study are not otherwise used for HIV-1 studies. All of the sequences from the eight LTNPs described in this report were uniquely different from one another and uniquely different from sequences of laboratory strains of HIV-1 in the Los Alamos HIV-1 sequence database (B. Korber, <http://hiv-web.lanl.gov>, Los Alamos National Laboratory, 1999). Furthermore, as described below, the examination of unusual HIV-1 polymorphisms in PBMC taken at different times affirmed the authenticity of each sequence. Thus, the sequences reported here are authentic and cannot be due to contamination.

All eight LTNPs described here lived, and were likely infected, in the United States, where group M, subtype B, isolates vastly predominate. Full-length sequences from all eight LTNPs were analyzed using the Los Alamos subtyping program and subtype reference sequences (B. Korber, www.ncbi.nlm.nih.gov). Nucleotide sequences of all eight LTNPs had the highest relatedness to subgroup B sequences across the entire length of the HIV-1 genome. The relatedness of 1486D

sequences to sequences of different subgroups is shown as an example in Fig. 1. In addition, dendrograms based on alignments of LTNP Gag and Nef amino acid sequences were generated (Fig. 2). Neighbor-joining analyses demonstrated that the LTNP Gag (Fig. 2A) (confidence value of 99) and Nef sequences (Fig. 2B) (confidence value of 77) were most closely related to subgroup B sequences. Trees using parsimony analysis revealed identical intersubtype relationships among the LTNP and reference Gag and Nef sequences.

Analysis of Gag sequences revealed that five of the eight LTNPs contained 1- or 2-amino-acid (aa) deletions in Gag compared to subgroup B consensus sequences (Fig. 3B and Table 3). HIV-1 from LTNP 3 had a deletion in p17^{gag} (1 aa); HIV-1 from LTNP 1 had a deletion in p2^{gag} (1 aa); HIV-1 from LTNP 7 had a deletion in the p6^{gag-pol} overlap region (2 aa); HIV-1 from 1486D had deletions in both p2^{gag} (1 aa) and p6^{gag-pol} (2 aa); and 161J had two separate deletions in p6^{gag-pol} (1 and 2 aa) (Fig. 3B and Table 3). None of the Gag deletions were independently observed in different subjects. To ensure that these deletions were representative of sequences maintained in these subjects, DNA was isolated from LTNP PBMC obtained at distinct times and used as the template for PCR. These experiments revealed that the polymorphisms in Gag sequences described here were consistently observed for PBMC samples obtained in 1985 and 1994 for LTNP 3, 1983 and 1994 for LTNP 1 and LTNP 7,

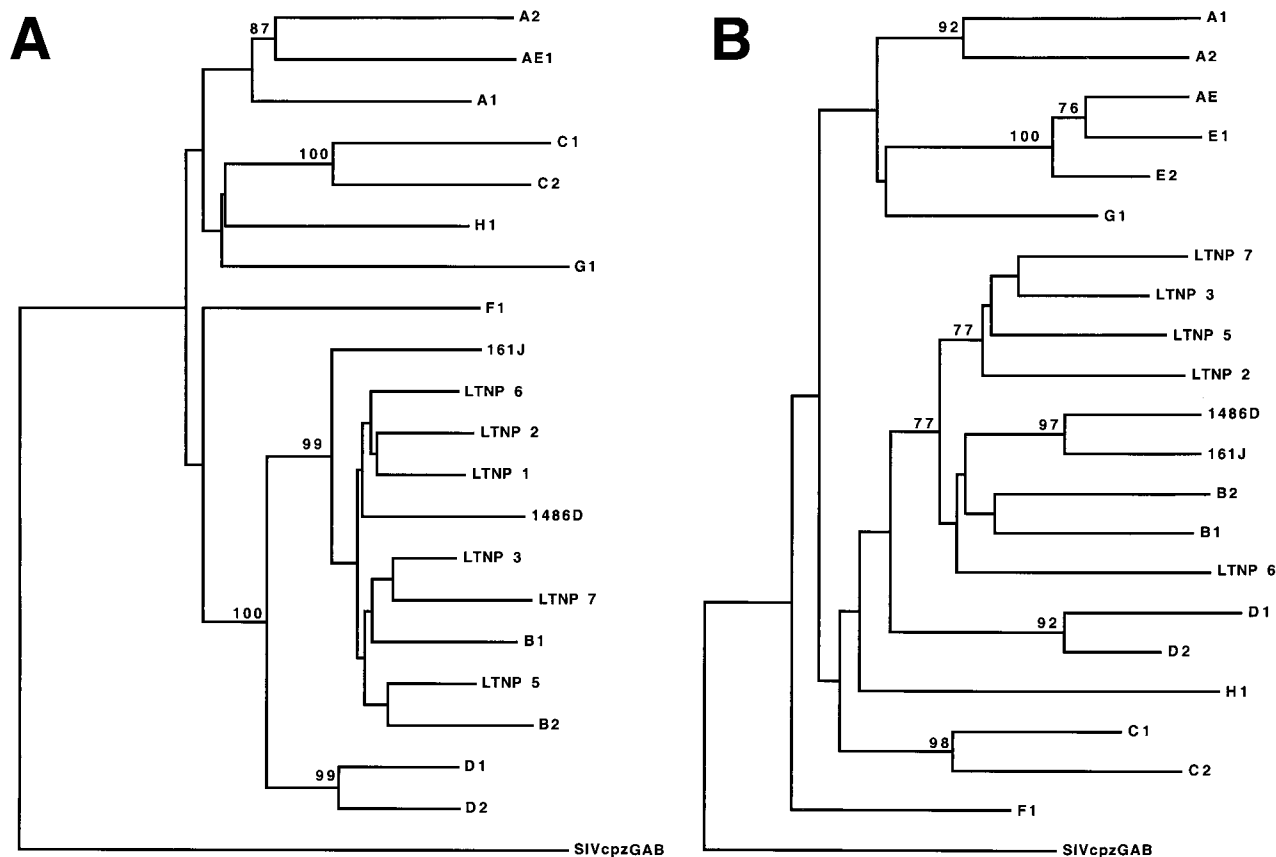


FIG. 2. Dendrograms of reference sequences of HIV-1 isolates of different subtypes and LTNP sequences for Gag (A) and Nef (B). These analyses were performed using PAUP. Bootstrap confidence values were assigned when a minimum value of 70 was achieved for a particular branch. The dendrogram was rooted to the SIVcpzGAB sequences. The identification of the subtype isolates which are symbolized here is described in Material and Methods.

1992 and 1995 for 1486D, and 1994 and 1998 for 161J (data not shown). The Los Alamos HIV-1 sequence database (B. Korber, <http://hiv-web.lanl.gov>) containing Gag sequences from 93 group M, group O, and SIVcpz isolates was examined for the Gag polymorphisms observed in our LTNP sequences. The p17^{gag} Δ112 polymorphism contained in HIV-1 from LTNP 3 was not observed in any of the isolates in the database. The 2-aa p6^{gag} polymorphisms contained in HIV-1 from 161J (Δ483-484), LTNP 7 (Δ466-467), and 1486D (Δ465-466) were also not observed in any of the isolates in the database, although Δ467 was observed for four subgroup A sequences (B. Korber, <http://hiv-web.lanl.gov>). The p2^{gag} Δ370 polymorphism contained in HIV-1 from 1486D was observed in two database sequences: one from subgroup B (GA.OYI) and one from subgroup F (FI.FIN9363). We independently derived Gag sequences from four Massachusetts subjects who had progressed to AIDS. In contrast to what was observed in the LTNP Gag sequences, we observed no unusual polymorphisms in the Gag sequences from these four progressors (data not shown). In addition, we scanned 90 group M Gag sequences in the Los Alamos database and did not find a single example of a deletion unique to a single individual. Thus, the polymorphisms observed in four of the LTNPs are unique among determined HIV-1 Gag sequences and their occurrence appears to be non-random in nature.

In addition to the Gag deletions, small deletions and insertions were also observed in other LTNP HIV-1 sequences that have not previously been seen in any subgroup B sequence in

the database (B. Korber, <http://hiv-web.lanl.gov>). LTNP 5 contained a single amino acid deletion in the transmembrane (gp 41) protein encoded by the *env* gene (Fig. 3E and Table 3). This mutation was consistently contained in PBMC samples obtained from LTNP 5 in 1987 and 1994 and was not observed in the sequences of the 131 isolates in the Los Alamos HIV-1 sequence database (B. Korber, <http://hiv-web.lanl.gov>). LTNP 6 HIV-1 cultures from virus obtained in 1984 and 1995 had a 3-aa deletion in gp41 that was previously observed in only three subgroup C isolates (C.DJ.DJ259A, C.DJ.DJ373A, and C.ET.ETH2220). Cultured virus from LTNP 6 from 1984 and 1995 also retained a 4-bp insertion in *nef* and 3'-LTR sequences (Fig. 3A) that resulted in truncation of LTNP 6 Nef by 1 aa short of the consensus C-terminal cysteine (Fig. 3J). This polymorphism has not been previously noted in any of the 256 Nef sequences in the Los Alamos HIV-1 sequence database (B. Korber, <http://hiv-web.lanl.gov>). HIV-1 from LTNP 6 obtained in 1984 and 1995 also had a 2-aa deletion in Nef (Δ49-50; Fig. 3J and Table 3) which appears in about 9% of subgroup B Nef sequences and represents the consensus for subgroup E Nef. LTNP 6 isolates also contained a 4-aa insertion near the N terminus of Vpu (Fig. 3G; Table 3) which is similar to a 4-aa insertion contained in four subgroup B isolates in the database. The 4 aa insertion was not detected in LTNP 6 PBMC samples obtained in 1984 but was detected in samples obtained in 1987 and at later times (Fig. 4). Cultured LTNP 1 virus maintained a large deletion in the 3'-LTR and Nef sequences (Fig. 3A and Table 3), similar to what has been

A

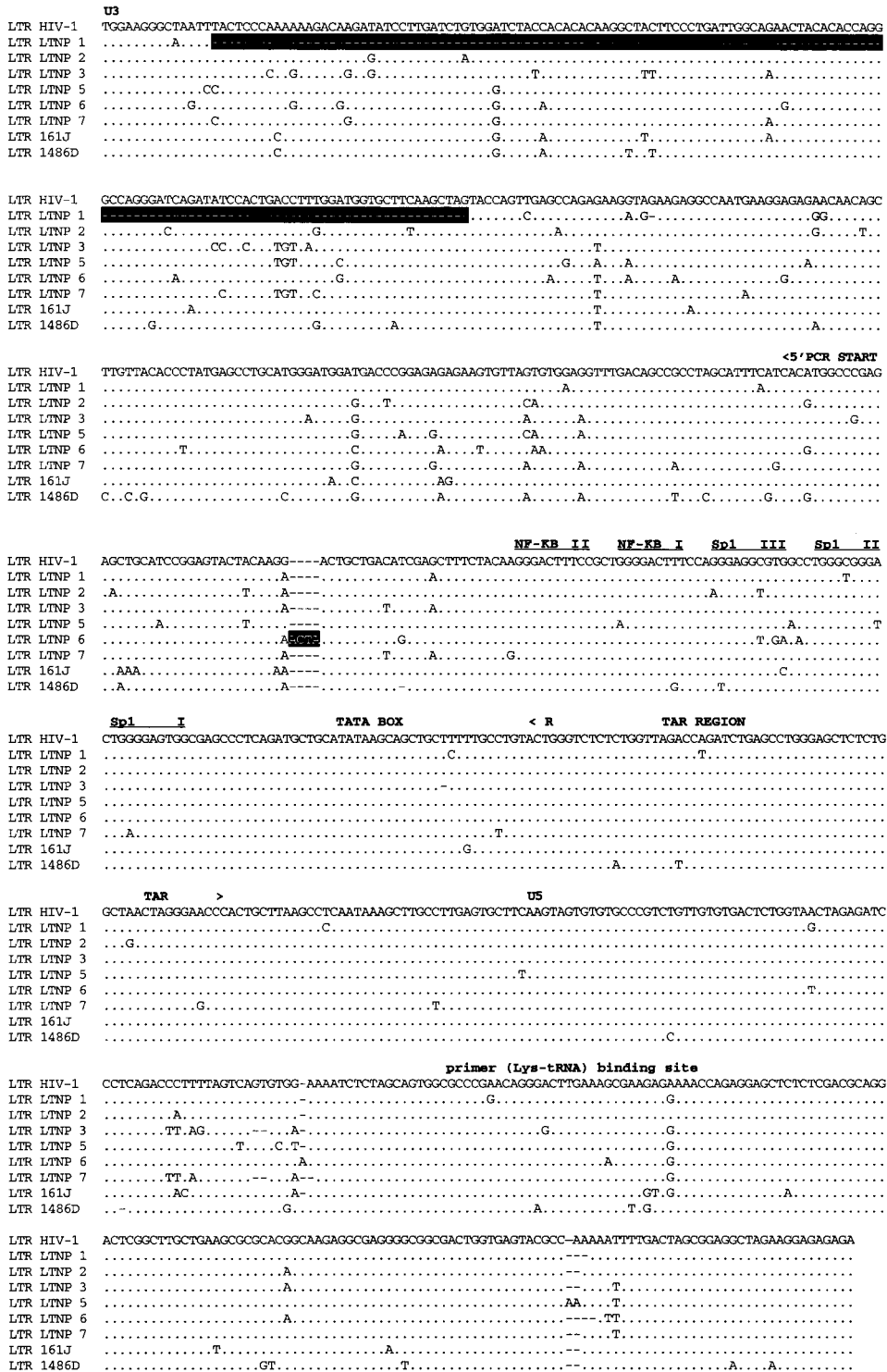


FIG. 3. Alignments of LTNP sequences. Nucleotide (A) and amino acid (B to J) sequences of the indicated HIV-1 genetic elements and proteins were aligned to the group M, subgroup B, consensus sequences (HIV-1). Periods indicate conservation between LTNP and consensus B sequences. A dash indicates that a base or amino acid is not contained in a particular sequence. Unusual polymorphisms consistently observed in HIV-1 sequences from LTNPs are highlighted in black. The data presented represent consensus sequences from two clones from each of two independent PCRs.

observed previously (27). The PBMC sample obtained from 161J in 1994 revealed a 2-aa deletion in Nef (Δ 48-49) (Fig. 3J and Table 3) that was also retained in PBMC obtained in 1998 from 161J. Cultured 1486D HIV-1 from 1995 maintained

Δ 48-49 Nef and also maintained a 1-aa insertion in Nef (61Q; Fig. 3J and Table 3). These polymorphisms were retained in 1486D 1995 PBMC (Fig. 5). However, the 61Q insertion was not observed in 1486D 1992 PBMC, suggesting that the addi-

C

PROTEASE

POL HIV-1 PFREDLAPQGGKAREFSSBQTRANSPTRELVWGRDNNLSSEAGADRQGTVSPFPQITLWQRPLVTKIKGGQLKEALLDTGADDTVLEEMNLPGRWKPF
POL LTNP 1SL.....L.....I.....I.....
POL LTNP 2E.K.....P.....A.....
POL LTNP 3P.....P.....FF.K.E.....I.....I.....V.....
POL LTNP 5G.....D.....N.....VR.....S.....
POL LTNP 6V.....G.....N.....R.I.....G.....D.S.....K.....
POL LTNP 7L.....G.....A.N.....
POL 161JN.....G.....PK.....T.....R.....T.BG.....L.....D.....
POL 1486DN.....G.....PK.....T.....R.....T.BG.....L.....D.....

RT

POL HIV-1 KMIGGIGGPIKVRQYDQILIEICGKHAIGTVLVGGTPTFVNIIGRNLLTQIGCTLNFPISPIETVPVKLKPMDGPKVKQWPLTEEKIKALVEICTEMREKES
POL LTNP 1K.....
POL LTNP 2K.....
POL LTNP 3K.....
POL LTNP 5K.....
POL LTNP 6K.....
POL LTNP 7S.....K.....V.....
POL 161JK.....P.....I.....K.....I.....
POL 1486DK.....P.....I.....K.....I.....

POL HIV-1 KISKIGPENFYNTFVFAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIHPHAGLKKKKSVTVLDVGDAYFVPLDKDFRKYTAFTIPSIINNETPGIRYQ
POL LTNP 1G.....K.....N.....
POL LTNP 2G.....K.....N.....
POL LTNP 3G.....K.....N.....
POL LTNP 5G.....K.....N.....
POL LTNP 6G.....K.....N.....
POL LTNP 7G.....K.....N.....
POL 161JG.....K.....N.....
POL 1486DG.....K.....N.....

POL HIV-1 YNVLPGQWGGSPAIPQSSMTKILEPFRKQNPDIIVYQVMDLTVGSDLEIGQHRTKIEELRQHLLRWGPTTTPDKKHQKBPFFLWGMGYLHPDKWTVQPIV
POL LTNP 1M.....
POL LTNP 2V.....L.....
POL LTNP 3V.....L.....
POL LTNP 5L.....
POL LTNP 6S.....P.....E.....
POL LTNP 7D.....E.....K.....
POL 161JD.....E.....K.....
POL 1486DD.....E.....K.....

POL HIV-1 LPEKDSWTVNDIQKLVGKLNWASQIYAGIKVKQLCKLLRGTALTEVPLTEEAELAEABNREILKEPVGHVYDPSKDLIAETIKQGGQGWYQIYQEP
POL LTNP 1L.....D.....V.....E.....
POL LTNP 2N.....V.....E.....
POL LTNP 3G.....P.....R.....C.....
POL LTNP 5R.....L.....
POL LTNP 6D.....A.S.....K.....L.....
POL LTNP 7P.....R.....L.I.....H.....Y.....
POL 161JN.....R.....K.....V.....V.....
POL 1486DN.....R.....K.....V.....V.....

RNase H

POL HIV-1 FKNLKTGKYARMGAHTNDVQQLTEAVQKLADESIVIVGKTKPKFKLPQKETWEAMWTEYQATWIPWEPFVNTPPVLKLVQLEKEPVGATPFYVDGA
POL LTNP 1A.....T.....T.....
POL LTNP 2A.....T.....D.....
POL LTNP 3T.....A.....I.....I.....
POL LTNP 5T.....L.....I.....
POL LTNP 6I.....D.....I.....
POL LTNP 7T.....I.....I.....
POL 161JR.....I.....G.....
POL 1486DT.....I.....I.....

POL HIV-1 ANRETKLKGAGYVTRGRQKVVSLTDTTINQKTELQAIHLALQDSGLEVINIVTDSQYALGITQAQFDKSESELVSQITBQLIKKEKVLAWVPAHGIGGN
POL LTNP 1N.....P.....S.....L.....N.....
POL LTNP 2P.....S.....L.....N.....
POL LTNP 3P.....S.....L.....N.....
POL LTNP 5P.....S.....L.....N.....
POL LTNP 6N.....S.....L.....N.....
POL LTNP 7S.....I.....E.....
POL 161JI.....P.....I.....E.....
POL 1486DI.....P.....I.....E.....

INTEGRASE **5'/3' PCR**

POL HIV-1 EQVDKLVASAGIRKVLFLDGDIDKAQEERHEKXHSNWRAMASDFNLPPVAKETVASCDCQKLGKGEAMIGQVDCSPGIWQLDCTHLEKGVILVAVHVASGYIE
POL LTNP 1I.....I.....
POL LTNP 2A.....V.R.I.....D.....C.....I.....
POL LTNP 3A.....V.R.I.....D.....C.....I.....
POL LTNP 5D.....C.....T.....I.....
POL LTNP 6V.R.I.....D.....G.....E.....I.....
POL LTNP 7D.....G.....E.....G.....
POL 161JD.....G.....E.....G.....
POL 1486DD.....G.....E.....G.....

POL HIV-1 AEWIIPAETQETAYPFLKLAGRWPKVTKHTDNGSNFTSTVKAACWAGIKQEPGIFYNPQSGVSVESMKNELKKITIGQVRDQAEHLKTVAVQMAVFIHNF
POL LTNP 1L.....L.....
POL LTNP 2A.....L.....
POL LTNP 3T.....Y.I.A.....V.....
POL LTNP 5A.....G.....NV.....F.....R.....L.....D.....
POL LTNP 6V.....G.....NV.....F.....R.....L.....D.....
POL LTNP 7A.....G.....NA.....
POL 161JA.....G.....NA.....
POL 1486DT.....I.....N.....

POL HIV-1 KRKGGIGGYSAGERIVDIIATDIQTKELQKQTKIQNFRVYRDSRDLWKGPKALLWKGEGAVVIQDNSDIKVVPRRKAIIIRDYKQMGAGDDCVASRQDED
POL LTNP 1V.....V.....N.....
POL LTNP 2K.....N.....K.....N.....E.....V.....N.....
POL LTNP 3K.....N.....K.....N.....E.....V.....N.....
POL LTNP 5S.....L.....I.....V.....
POL LTNP 6N.....K.....N.....E.....V.....R.....
POL LTNP 7N.....K.....N.....E.....V.....R.....
POL 161JN.....K.....N.....E.....V.....R.....
POL 1486DN.....K.....N.....E.....V.....R.....

FIG. 3—Continued.

D

VIF HIV-1	MENRWQVMIVWQVDRMRIRITWKSLSVKHHMYISGRKAKGWFYRHHYESTHPRISSSEVHIPLGDARLVITTYWGLHTGERDWHLGGQVSIIEWRKKRYSTQVDP
VIF LTNP 1Y.....R.....
VIF LTNP 2A.....A.....T.....D.P..K.....P.....R.....
VIF LTNP 3A.....S.....KL.....
VIF LTNP 5V.....R.A.V.....FN.T.....K.....R.....
VIF LTNP 6N.....R.T.....N.....V.....K.....R.....
VIF LTNP 7D.....A.....E.....
VIF 161JN.....R.K..E.V.....K.....K.....RK.....
VIF 1486DA.....K..K.T..V.....K.....IR.R.....
VIF HIV-1	DLADQLIHLYYFDFCFSESAINRAILGHIVSPRCEYQAGHNKVGSLQYLALALITPKKIKPPLPSVTKLTEDRWKPKQTKGHRGSHTMNGH
VIF LTNP 1	N.....L..R..G.....R.....
VIF LTNP 2	N.....S.....S.....P.....
VIF LTNP 3	S.....K.....S.....V..R.....R.....R.....R.....
VIF LTNP 5	G..R.....V.R..R.....Q.....
VIF LTNP 6	N.....S.....R.....
VIF LTNP 7	S.....N.S.....V.....R.....R.....H.....
VIF 161JK..V.HR.....R.....
VIF 1486D	T..A..QH.....G..V.H..I.....T.....R.T.....A.....Q.....

FIG. 3—Continued.

plasma antigenemia, CD4 counts, numbers of infectious cells in PBMC, and viral RNA loads with blood samples obtained at intervals following experimental infection of these monkeys. These analyses revealed that Mm 32-97 and Mm 33-97 did not maintain consistently measurable numbers of infectious cells in PBMC or RNA loads in a readily measurable range (Fig. 7C and D). Conversely, Mm 34-97 and Mm 35-97 displayed persistently high numbers of infectious cells in PBMC and high RNA loads. We investigated if Nef sequences in Mm 32-97 and Mm 33-97 were under selective pressure to acquire the 61Q insertion. At week 44 postinoculation we did not observe this polymorphism in Mm 32-97 and Mm 33-97. The 61Q polymorphism was retained in Mm 34-97 and Mm 35-97 at the same time point (Fig. 5). We also examined if the Δ48-49 deletion was stable in the 1486D Nef sequences and found that this polymorphism had been maintained in all four animals at 44 weeks postinoculation (Fig. 5).

Determinants for poor growth of HIV-1 from LTNP 5. Of the seven LTNP HIV-1 isolates that were successfully cultured, six grew well in infected PBMC cultures (Table 3). Only LTNP 5 HIV-1 grew poorly (Table 3), reaching a maximum p24 concentration of 5 ng/ml in PBMC obtained from several seronegative donors (17). We investigated potential determinants of this slow- or low-growth phenotype. Chimeric LTNP 5/NL 4-3 HIV-1 was created to assess the effect of LTR, Gag, and Pol sequences on LTNP 5 replication. LTNP 5 and NL 4-3 sequences from bp 297 to 4402 (5') and NL 4-3 sequences from bp 4402 to 9607 (3') (Fig. 3) were amplified separately. An *Xma*I (CCCGGG) site was introduced at the junction of the PCR fragments (bp 4402) as described in Materials and Methods. *Xma*I-digested 5' LTNP 5 and 5' NL 4-3 fragments were cotransfected with 3' NL 4-3 fragments into permissive CEMx174 cells. Supernatants were collected starting at day 3 posttransfection and assayed for p27 antigen concentration. Production of both wild-type NL 4-3 and recombinant 5' LTNP 5-3' NL 4-3 peaked at day 9 posttransfection with similar peak yields (Fig. 8). Thus, the 5' sequences in LTNP 5 to bp 4402 seem suitably competent for viral replication.

Previous studies have shown that *vpu*, *vpr*, and *nef* can be deleted from HIV-1 with little or no effect on replication in lectin-stimulated PBMC cultures (16, 26). Therefore, *vpu*, *vpr*, and *nef* are unlikely to be responsible for the poor growth of HIV-1 from LTNP 5. Among additional LTNP 5 sequences contained in the 3' LTNP 5 sequences that could contribute to slow growth, no unusual, difficult-to-revert mutations were observed in *integrase*, *vif*, *tat*, *rev*, and U3 LTR sequences (Fig. 3 and Table 3). Among these sequences, LTNP 5 *vif* sequences

displayed the greatest divergence from the subgroup B consensus as well as from the LTNP *vif* sequences presented here (Fig. 1D). LTNP 5 *vif* allele 2 and LTNP 5 *vif* allele sequences as well as HBX-3 *vif*-positive control sequences were inserted into an expression vector and were cotransfected with a *vif*-deficient proviral vector pIIIIB/Δ*vif* into H9 cells. Western blots of whole-cell lysates from transfected H9 cultures indicated that the vectors containing LTNP 5 *vif* alleles 2 and 3 and HBX-3 *vif* sequences expressed comparable levels of Vif (Fig. 10B). HIV-1 produced from these cells was used in single-cycle challenges of the indicator cell line C8166/HIV-CAT to assess Vif function. Cells transfected with LTNP 5 *vif* alleles 2 and 3 and HXB-3 *vif* produced similar levels of CAT (Fig. 10A) in this assay, indicative of the wild-type-like activity of these LTNP 5 *vif* genes.

Our analysis of LTNP 5 isolate sequences revealed a difficult-to-revert polymorphism in LTNP 5 *env* sequences (Fig. 3E and Table 3) that was also contained in the 3' LTNP 5 PCR fragments. A single amino acid deletion in the fusion peptide region of the transmembrane domain (Fig. 3E and Table 3) was consistently retained in LTNP 5 virus isolates and in PBMC samples obtained in 1987 and 1994. This polymorphism is not observed in any of the 131 group M, group O, or SIVcpz Env sequences in the Los Alamos database (B. Korberg, <http://hiv-web.lanl.gov>) and represents the only deletion or insertion contained in LTNP 5 HIV-1 sequences (Fig. 3 and Table 3).

DISCUSSION

HIV-1 gene sequences from LTNPs have been investigated extensively in order to determine if inactivating polymorphisms are associated with nonprogression. Nucleotide and amino acid substitutions have been associated with defective p17 and Rev activities (5, 25). Among the auxiliary genes, several examples of large deletions in *nef* have been documented (10, 27) but have not been observed in LTNP *vpu* or *vpr* sequences. Thus, the types of polymorphisms described here are unusual in that they are difficult to revert and in many cases without precedent in the Los Alamos HIV-1 sequence data base (B. Korber, <http://hiv-web.lanl.gov>).

Seven of the eight LTNPs contained a total of 13 unusual, difficult-to-revert polymorphisms in HIV-1 sequences. The rate of misincorporation during the process of reverse transcription has been estimated at approximately 3×10^{-4} bases or approximately one misincorporation per genome transcribed (4, 40, 46). Amino acid polymorphisms that are detrimental to

E

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ENV HIV-1 MRVKGIRKNYQHLWRWGTMLLGLMLICSAAEKLVVTVVYGVVWKEATTLFCASDAKAYDTEVHNWVWTHACVPTDPNPQEVVLENVTENFNMWK
ENV LTNP 1 . . . . . C . . . . . I . . . . . GN . . . . . MP . . . . . P . . . . . P . . . . .
ENV LTNP 2 . . . . . ME . . . . . N . . . . . N . . . . . G . . . . . D . . . . .
ENV LTNP 3 . . . . . K . . . . . R . . . . . I . . . . . GQ . . . . . T . . . . . D . . . . . G . . . . .
ENV LTNP 5 . . . . . EL . . . . . SG . . . . . VEMGHHPWDIDD . . . . . QS . . . . . T . . . . . E . . . . . I . . . . . D . . . . . E
ENV LTNP 6 . . . . . E . . . . . R . . . . . C . . . . . R . . . . . VL . . . . . T . . . . . R . . . . . KA . . . . . L . . . . . I . . . . .
ENV LTNP 7 . . . . . A . . . . . E . . . . . HW . . . . . YW . . . . . K . . . . . GSI . . . . . AN . . . . . K . . . . . G . . . . .
ENV 161J . . . . . MK . . . . . HW . . . . . YW . . . . . AG . . . . . IL . . . . . V . . . . . V . . . . . A . . . . . D . . . . . IA . . . . .
ENV 1486D . . . . .

< V1 > < V2 >
ENV HIV-1 NNMVQEMHEDIISLWDQSLKPCVKLTPLCVTLNCTD?????N?????????MEK-GEIKNGSFNITTSIRDKVQKRYALFYKLDVVPID
ENV LTNP 1 . . . . . LR . . . . . AT . . . . . N . . . . . T . . . . . S . . . . . S . . . . . G . . . . . T . . . . . G . . . . .
ENV LTNP 2 . . . . . NL . . . . . KN . . . . . AT . . . . . T . . . . . S . . . . . E . . . . . G . . . . . T . . . . . KE . . . . . M . . . . .
ENV LTNP 3 . . . . . LE . . . . . AT . . . . . N . . . . . T . . . . . T . . . . . I . . . . . N . . . . . S . . . . . K . . . . . R . . . . .
ENV LTNP 5 . . . . . H . . . . . VEL . . . . . AT . . . . . N . . . . . T . . . . . S . . . . . N . . . . . E . . . . . T . . . . . C . . . . . L . . . . .
ENV LTNP 6 . . . . . AK . . . . . N . . . . . T . . . . . T . . . . . DS . . . . . K . . . . . SP . . . . . N . . . . . T . . . . . S . . . . . S . . . . .
ENV LTNP 7 . . . . . Q . . . . . LEN . . . . . AN . . . . . NT . . . . . ST . . . . . ES . . . . . KN . . . . . ST . . . . . V . . . . . S . . . . . R . . . . .
ENV 161J . . . . . N . . . . . LR . . . . . N . . . . . LR . . . . . GT . . . . . GT . . . . . K . . . . . G . . . . . T . . . . . S . . . . .
ENV 1486D . . . . . T . . . . . L . . . . . KN . . . . . AT . . . . . I . . . . . TH . . . . . NS . . . . . G . . . . . K . . . . . AI . . . . .

V2 >
ENV HIV-1 N????????YRLISCNSTSVITQACPKVSEFPIPIHYCAPAGFAILKCNCKFNGTGPCNTVSTVQCTHGIRPVVSTQQLLNGSLAAEEVVRSEN
ENV LTNP 1 . . . . . D . . . . . NT . . . . . TS . . . . . K . . . . . Q . . . . .
ENV LTNP 2 . . . . . D . . . . . NN . . . . . ASE . . . . . M . . . . . V . . . . . Y . . . . . E . . . . . D . . . . . K . . . . . Q . . . . . A . . . . .
ENV LTNP 3 . . . . . S . . . . . N . . . . . TS . . . . . K . . . . .
ENV LTNP 5 . . . . . E . . . . . N . . . . . TS . . . . . A . . . . . T . . . . . N . . . . . T . . . . . D . . . . . K . . . . . Q . . . . . K . . . . .
ENV LTNP 6 . . . . . D . . . . . T . . . . . S . . . . . S . . . . . A . . . . . N . . . . . Y . . . . . T . . . . . N . . . . .
ENV LTNP 7 . . . . . S . . . . . N . . . . . TS . . . . . K . . . . .
ENV 161J . . . . . D . . . . . NH . . . . . DN . . . . . SS . . . . . N . . . . . T . . . . . S . . . . . G . . . . .
ENV 1486D . . . . . D . . . . . NN . . . . . TS . . . . . T . . . . . R . . . . . L . . . . . R . . . . . K . . . . . S . . . . . G . . . . . I . . . . . K . . . . .

< V3 >
ENV HIV-1 FTDNAKTIIVQLNESVEINCTRPNNTTRKSIHIGPGRAPYTTG?-IIGDIRQAHNCNISRAKWNNTLKQIVKQLRQFRNKTIIVFNQSSGGDPETVM
ENV LTNP 1 . . . . . M . . . . . I . . . . . EIN . . . . . REIL . . . . . R . . . . .
ENV LTNP 2 . . . . . K . . . . . I . . . . . T . . . . . R . . . . . V . . . . . V . . . . . D . . . . . S . . . . . Q . . . . . A . . . . .
ENV LTNP 3 . . . . . K . . . . . K . . . . . D . . . . . P . . . . . K . . . . . S . . . . . A . . . . . D . . . . . N . . . . . L . . . . . S . . . . . D . . . . . K . . . . .
ENV LTNP 5 . . . . . H . . . . . A . . . . . A . . . . . A . . . . . N . . . . . R . . . . . A . . . . . R . . . . . T . . . . . K . . . . . G . . . . . I . . . . .
ENV LTNP 6 . . . . . S . . . . . K . . . . . TEN . . . . . G . . . . . H . . . . . I . . . . . G . . . . . I . . . . . E . . . . . F . . . . . G . . . . . N . . . . . T . . . . . E . . . . . S . . . . . R . . . . .
ENV LTNP 7 . . . . . K . . . . . D . . . . . P . . . . . T . . . . . A . . . . . G . . . . . T . . . . . L . . . . . D . . . . . K . . . . . A . . . . . G . . . . . E . . . . . H . . . . . N . . . . . F . . . . .
ENV 161J . . . . . K . . . . . P . . . . . T . . . . . R . . . . . N . . . . . S . . . . . L . . . . . E . . . . . T . . . . . Q . . . . . N . . . . . K . . . . . P . . . . .
ENV 1486D . . . . . N . . . . . Q . . . . . A . . . . . R . . . . . S . . . . . M . . . . . T . . . . . I . . . . . A . . . . . D . . . . . R . . . . . D . . . . . R . . . . . T . . . . . D . . . . . V . . . . . E . . . . . K . . . . . P . . . . . R . . . . . T . . . . .

< V4 > < V5 >
ENV HIV-1 HSFNCGEFFYCNMTQLFN----STWNTNT?????????ITLPCRKIQIINMWEVKGAMYAPPPIRGQIRCSNNTIGLLLTRDGN????
ENV LTNP 1 . . . . . S . . . . . ST . . . . . W . . . . . SV . . . . . V . . . . . D . . . . . GN . . . . . N . . . . . TE . . . . .
ENV LTNP 2 . . . . . DS . . . . . I . . . . . VA . . . . . TG . . . . . NV . . . . . TG . . . . . S . . . . . EN . . . . . T . . . . . K . . . . . M . . . . .
ENV LTNP 3 . . . . . A . . . . . V . . . . . NG . . . . . ER . . . . . ED . . . . . GN . . . . . I . . . . . GN . . . . . NT . . . . .
ENV LTNP 5 . . . . . A . . . . . S . . . . . G . . . . . TE . . . . . GS . . . . . D . . . . . N . . . . . A . . . . . T . . . . . E . . . . . D . . . . .
ENV LTNP 6 . . . . . S . . . . . D . . . . . S . . . . . T . . . . . EN . . . . . EL . . . . . S . . . . . CT . . . . . G . . . . . EN . . . . . K . . . . . L . . . . . E . . . . . R . . . . . K . . . . . I . . . . . A . . . . . N . . . . . T . . . . . G . . . . .
ENV LTNP 7 . . . . . K . . . . . AS . . . . . ST . . . . . LD . . . . . G . . . . . TE . . . . . ES . . . . . D . . . . . NN . . . . . ST . . . . . L . . . . . S . . . . . P . . . . . N . . . . . D . . . . . S . . . . . G . . . . . K . . . . . K . . . . .
ENV 161J . . . . . S . . . . . B . . . . . S . . . . . NN . . . . . T . . . . . E . . . . . G . . . . . T . . . . . L . . . . . V . . . . . G . . . . . K . . . . . K . . . . . N . . . . . M . . . . .
ENV 1486D . . . . . T . . . . . E . . . . . Y . . . . . M . . . . . E . . . . . T . . . . . ER . . . . . K . . . . . N . . . . . T . . . . . I . . . . . R . . . . . ED . . . . . SN . . . . . II . . . . . T . . . . . T . . . . .

< TM >
ENV HIV-1 ??TEIPRPGGDMRDNWRSELYKYKVVKI EPLGVAPTAKRRVVQREKRAVG-MGAMFLGFLGAAGSTMGAAS?TLTVQARQLLSGIVQQNLLRAIE
ENV LTNP 1 . . . . . SE . . . . . V . . . . . L . . . . . M . . . . .
ENV LTNP 2 . . . . . NES . . . . . T . . . . . L . . . . . V . . . . . L . . . . . L . . . . .
ENV LTNP 3 . . . . . NE . . . . . R . . . . . I . . . . . V . . . . . M . . . . . L . . . . .
ENV LTNP 5 . . . . . SSP . . . . . R . . . . . R . . . . . I . . . . . L . . . . . L . . . . . Q . . . . . L . . . . .
ENV LTNP 6 . . . . . NE . . . . . I . . . . . V . . . . . V . . . . . L . . . . .
ENV LTNP 7 . . . . . NE . . . . . V . . . . . V . . . . . I . . . . . V . . . . . M . . . . . L . . . . .
ENV 161J . . . . . ET . . . . . R . . . . . TI . . . . . V . . . . . A . . . . . L . . . . . K . . . . . G . . . . .
ENV 1486D . . . . . ND . . . . . T . . . . . R . . . . . L . . . . . GI . . . . . V . . . . . M . . . . .

AQHLLQLTVWGIKQLQARVLAVERYLKDQQLLIGWCSGKLCITAVPWNASWSNKL??IWN?MTWMEWEREIDNYT?LIYTLIEESQNOQEKNEQE
ENV HIV-1 . . . . . T . . . . . DE . . . . . N . . . . . N . . . . . S . . . . .
ENV LTNP 1 . . . . . T . . . . . T . . . . . DK . . . . . N . . . . . D . . . . . N . . . . . S . . . . .
ENV LTNP 2 . . . . . Q . . . . . T . . . . . R . . . . . DN . . . . . N . . . . . D . . . . . N . . . . . S . . . . .
ENV LTNP 3 . . . . . M . . . . . E . . . . . F . . . . . S . . . . . D . . . . . DQ . . . . . N . . . . . Q . . . . . DK . . . . . S . . . . . N . . . . . N . . . . . L . . . . . Q . . . . .
ENV LTNP 5 . . . . . Q . . . . . T . . . . . N . . . . . DE . . . . . GN . . . . . K . . . . . N . . . . . S . . . . . N . . . . . K . . . . . I . . . . .
ENV LTNP 6 . . . . . Q . . . . . R . . . . . DN . . . . . N . . . . . D . . . . . N . . . . . S . . . . . K . . . . .
ENV LTNP 7 . . . . . NT . . . . . T . . . . . DE . . . . . K . . . . . K . . . . . G . . . . .
ENV 161J . . . . . M . . . . . T . . . . . T . . . . . DQ . . . . . H . . . . . DT . . . . . K . . . . . G . . . . . A . . . . .
ENV 1486D . . . . .

LLELDKWSLWVWFDITNWLWYKIFIMIVGGLVGLRIVFAVLSIVNRVROGYSPLSFQTRLPAPRGPDRPGIEEHEGGERDRDRSRLVDGFLALIVW
ENV HIV-1 . . . . . K . . . . . I . . . . . L . . . . . Q . . . . . D . . . . . E . . . . . F . . . . .
ENV LTNP 1 . . . . . S . . . . . S . . . . . I . . . . . A . . . . . I . . . . . H . . . . . G . . . . . G . . . . . S . . . . . N . . . . . E . . . . .
ENV LTNP 2 . . . . . A . . . . . S . . . . . A . . . . . I . . . . . H . . . . . G . . . . . G . . . . . S . . . . . N . . . . . E . . . . .
ENV LTNP 3 . . . . . N . . . . . T . . . . . K . . . . . A . . . . . S . . . . . IP . . . . . P . . . . . I . . . . .
ENV LTNP 5 . . . . . A . . . . . K . . . . . P . . . . .
ENV LTNP 6 . . . . . MA . . . . . G . . . . . N . . . . . E . . . . .
ENV LTNP 7 . . . . . S . . . . . E . . . . . S . . . . . K . . . . . V . . . . . V . . . . . Q . . . . . K . . . . . K . . . . . H . . . . . L . . . . . D . . . . .
ENV 161J . . . . . K . . . . . I . . . . . T . . . . . TC . . . . . S . . . . . H . . . . . I . . . . .
ENV 1486D . . . . .

DLRSLCLFSPYHRDLILLIVARIVELLRRGWEALKYWNLLQYWSQELKNSAVSLLNATAIAVAEGTDRVIEVQRA?RAILHIP?PIRQGLERALL
ENV HIV-1 . . . . . T . . . . . I . . . . . P . . . . . C . . . . . V . . . . . T . . . . .
ENV LTNP 1 . . . . . T . . . . . L . . . . . Y . . . . . T . . . . .
ENV LTNP 2 . . . . . I . . . . . DG . . . . . S . . . . . V . . . . . IL . . . . . P . . . . . V . . . . .
ENV LTNP 3 . . . . . T . . . . . N . . . . . L . . . . . P . . . . . R . . . . . S . . . . .
ENV LTNP 5 . . . . . V . . . . . S . . . . . A . . . . . L . . . . . VA . . . . . TT . . . . .
ENV LTNP 6 . . . . . IH . . . . . AT . . . . . I . . . . . S . . . . . TL . . . . . F . . . . . V . . . . .
ENV LTNP 7 . . . . . L . . . . . T . . . . . P . . . . . I . . . . . P . . . . . I . . . . . IA . . . . . IL . . . . . FF . . . . . TK . . . . .
ENV 161J . . . . . G . . . . . T . . . . . I . . . . . K . . . . . TC . . . . . R . . . . . I . . . . .
ENV 1486D . . . . .

```

FIG. 3—Continued.

the virus rapidly revert (26, 32). Conversely, the elimination of insertion or deletion polymorphisms requires slippage or stuttering of reverse transcriptase, which is a much rarer occurrence. A 4-aa deletion in SIV Nef has been repaired by dupli-

cation of adjacent sequences and subsequent reversion to wild-type sequences (56). However, reversion of this deletion is rare and is not observed in most infected monkeys. In all cases presented here, with the exception of the 4-aa insertion in

F

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VPR HIV-1  MEQAPEDQGPQREYNEWLELLEELKNEAVRHFPRIMLHSLGQHIVYETYGDWAGVEAIIIRILQQLFIHFRIGCHSRIGITRQRANRASRS
VPR LTNP 1  .....T.....V.....Q.....
VPR LTNP 2  .....T.....G.....Q.....G.....
VPR LTNP 3  .....M.....G.....Q.....
VPR LTNP 5  ..P.....F.....M.....
VPR LTNP 6  .....G.....M.....V.....P.....
VPR LTNP 7  .....A.....S.....M.....G.....VQ.....
VPR 161J   .....A.....S.....Y.....IQR.....T.....
VPR 1486D  .....N.....N.....G.....IQR.....
```

G

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VPU HIV-1  MQSLQILAIVALVVA---AII-AIVVWTVIVFIEYRKILRQRKIDRLIDRIRERAEDSGNESEGDQEELSALVEMGHAPWDVDDL
VPU LTNP 1  .....L.....I.....S.....D.....IN.....
VPU LTNP 2  .....H.....I.....I.....S.....L.....D.....G.....I.....
VPU LTNP 3  .....Y.....T.....F.....I.....S.....V.....I.....
VPU LTNP 5  .....P.....T.....A.....S.....L.....R.....K.....I.....E.....G.....I.....
VPU LTNP 6  .....PI.....TP.....A.....I.....K.....I.....E.....RPP.....G.....IN.....
VPU LTNP 7  .....H.....T.....F.....I.....V.....S.....I.....
VPU 161J   .....A.....V.....T.....S.....L.....E.....E.A.AV.....GEY.....
VPU 1486D  .....V.....L.....L.....E.A.AV.....GR.....L.....N.....
```

H

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REV HIV-1  MAGRSGDSDEELKLTVRLIKFLYQSNPPSPGTRQARRNRWRERQQRIRISSEWILSTYLGSAEPVPLQLPPLERLTLDCSEDCGTSQTQGVGSPQILVESPAVLESGTKE
REV LTNP 1  .....S.....E.....L.....GRV.....F.....N.....N.....F.....T.....
REV LTNP 2  .....D.....S.....G.....N.....
REV LTNP 3  .....G.....K.....S.....R.....M.....Q.....R.H.....P.....H.....N.....R.....ST.....
REV LTNP 5  .....D.....V.T.G.....N.....P.....N.....A.....
REV LTNP 6  .....A.....I.....GS.....Q.L.G.....S.....P.K.....T.....
REV LTNP 7  .....R.....K.....S.....R.H.....HP.....N.....A.....N.....ST.....
REV 161J   .....R.....A.....I.....S.....T.....L.T.I.H.....P.....P.....N.....A.....
REV 1486D  .....I.....K.....S.....N.....A.....Q.....G.....N.....PT.....G.....
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I

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TAT HIV-1  MEFVDPRLPEPWKHPGSQPKTACTNICYCKKCCFHCQVCFTTKGLGJSYGRKKRRRQRRAPQDSQTHQVLSLQKQASQPRGDPDTPGPK
TAT LTNP 1  .....R.....T.....LN.....
TAT LTNP 2  .....T.....
TAT LTNP 3  .....P.....I.....T.A.....
TAT LTNP 5  .....R.....P.....Q.....I.....P.....
TAT LTNP 6  .....R.....T.....H.S.N.....PR.....P.....
TAT LTNP 7  .....R.....P.....Y.....I.....TS.G.N.....L.....H.....Q.....
TAT 161J   .....L.....
TAT 1468D  .....R.....H.....AN.....
```

J

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NEF HIV-1  MGGKWSKRSMGWFTVREMRRAEPAADGVGAVSRDLEKHGAISSNTAATNAACAWLEA-Q-EEBEEVGFPPVRPQVPLRPMYKAAIDLSHFLKEKGGLEGL
NEF LTNP 2  .....RV-E.....E.....P.N.....A.-K.....G.L.....
NEF LTNP 3  .....IVV.....Q.....TN.....V.....
NEF LTNP 5  .....L.....A.....TTS.....I.....
NEF LTNP 6  .....S.LI.....A.....N.....V.....G.....V.....
NEF LTNP 7  .....NVVE.....TN.....T.....D.....S.....L.T.K.....
NEF 161J   .....SKA.....HN.....L.....LN.T.....E.....K.....G.V.....K.....
NEF 1486D  .....VP.....S.....A.....LN.G.T.....Q.....G.F.....D.....
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NEF HIV-1  IYSQRRODILDLVVYHTQGYFPDQNYTTPGFGIRYPLTFGWCFKLVPEPDKVEEANEENSLHHPMSLHGMDDEPEKEVLEWKFDSTLAFHHMARELHPEYYKDC
NEF LTNP 2  V.....R.....N.....S.....I.K.....L.....L.....R.....R.....V.....Q.....F.N.....
NEF LTNP 3  .....P.....E.....I.....F.....S.....CY.....E.....R.....M.....R.....N.....
NEF LTNP 5  .....H.K.....E.....I.....C.....E.I.....E.....G.....Q.....R.....F.....
NEF LTNP 6  .....L.....K.....E.....I.....T.....L.....D.C.....E.S.R.....Q.....R.....V.....N.....
NEF LTNP 7  .....H.K.....E.....I.....C.....TE.....K.....D.C.....E.....G.....M.....H.....Q.....N.....
NEF 161J   .....Q.....N.....K.....L.....I.....V.R.....R.....V.....K.....N.....
NEF 1486D  .....Q.....N.....V.....L.....Y.....Q.....M.....L.....R.....V.....M.....N.....
```

FIG. 3—Continued.

LTNP 6 Vpu, difficult-to-revert polymorphisms were consistently present in historical samples from the LTNPs.

Five of the eight LTNPs in this study had deletions of 1 or 2 aa in p17^{gag}, p2^{gag}, and/or p6^{gag} (Fig. 3B and Table 3). Four had

deletions that did not appear in 93 group M, group O, or SIVcpz isolates in the Los Alamos HIV-1 sequence database (B. Korber, <http://hiv-web.lanl.gov>). In contrast, we did not observe any difficult-to-revert polymorphisms in the Gag se-

```
VPU HIV 1      MQSLQILAIVALVVA---AII-AIVVWTVIVFIEYRKILRQRKIDRLIDRIRERAEDSGNESEGDQEELSALVEMGHAPWDVDDL*
VPU LTNP 6 P84 .....V.....S.....L..I...
VPU LTNP 6 P87 ...I...AP...LVVV...I...I...E.....R.P.G.IN.>
VPU LTNP 6 P94 ...I...TQE..AI.LVVV.V.I.V.....I...E.....RPP.G.IN.>
VPU LTNP 6 P95 ...I...TQE..AI.LVVV.V.IIV.....I...E.....RPP.G.IN.>
VPU LTNP 6 V95 ...I...TP...A...LVVV...I...K...I...E.....RP-.G.IN.>
```

FIG. 4. Alignment of LTNP 6 Vpu sequences. LTNP 6 amino acid sequences from the time points indicated (e.g., P84, PBMC sample obtained in 1984) were aligned to the group M, subgroup B, consensus sequences (HIV-1). V, sequences were obtained from isolated HIV-1. Each sequence represents a consensus of two clones from each of two independent PCRs. Periods indicate conservation between LTNP 6 and the consensus B sequences. A dash indicates that an amino acid is not contained in a particular sequence. The 4-aa insertion observed in Vpu sequences from LTNP 6 is highlighted in black.

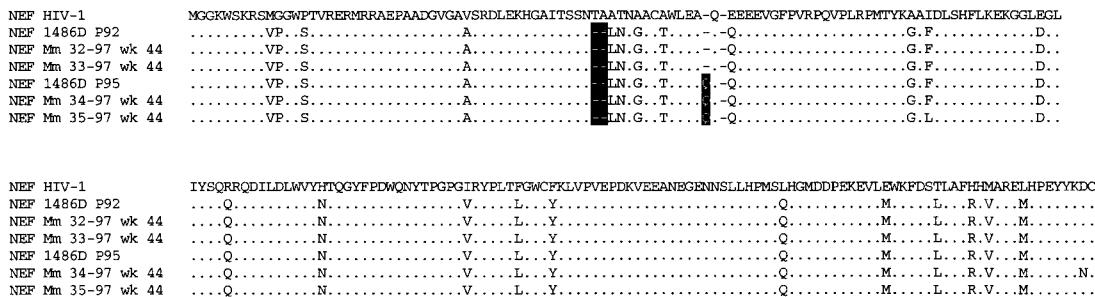


FIG. 5. Amino acid sequences of 1486D Nef after infection into rhesus monkeys with SHIVnef recombinants. A SHIVnef recombinant containing 1486D Nef sequences from PBMC obtained in 1992 (P92) was inoculated into Mm 32-97 and Mm 33-97, and a recombinant containing 1486D Nef sequences obtained in 1995 was inoculated into Mm 34-97 and Mm 35-97. The sequences in the inoculum and sequences obtained from the infected animal 44 weeks postinoculation were aligned to the group M, subgroup B, consensus (HIV-1) sequences. Each sequence represents a consensus of two clones from each of two independent PCRs. Periods indicate conservation between 1486D and the consensus B sequences. A dash indicates that an amino acid is not contained in a particular sequence. The deletion and insertion polymorphisms observed in Nef sequences from 1486D are highlighted in black.

quences isolated from four individuals that progressed to AIDS and we did not observe a single example of a unique deletion in Gag among 90 group M sequences in the Los Alamos database. These results suggest that the deletions in Gag that we observed occurred in a nonrandom fashion in our group of LTNPs. HIV-1 isolated from the LTNPs containing these polymorphisms was able to replicate in culture (Table 3) (17), indicating that they do not dramatically debilitate the virus. However, they could possibly have subtle effects on Gag function that may lower replicative capacity or result in attenuation. Three of the four unique Gag deletions occurred in p6 sequences (161J Δ 483-484, LTNP 7 Δ 466-467, and 1486D Δ 465-466). Vpr incorporation into virions was not detectably affected by the p6 deletions (Fig. 6).

The Nef sequences from the LTNPs belonged to group M, subgroup B (Fig. 2), as would be expected since these individuals live and were likely infected in the United States where subgroup B sequences predominate. The polymorphism seen in Nef sequences isolated from LTNP 6 (Δ 49-50) (Fig. 3J) is found in about 9% of subgroup B sequences although it represents the subgroup E consensus, which is predominant in Asia. Conversely, the Nef polymorphism (Δ 48-49) observed in 161J and 1486D isolates (Fig. 3J) was not observed in any of the 256 Nef sequences in the Los Alamos HIV-1 sequence database (B. Korber, <http://hiv-web.lanl.gov>). Monkeys infected with SHIVnef that contained 1486D sequences from 1992 (Δ 48-49) maintained low viral loads in contrast to monkeys infected with 1486D sequences from 1995 (Δ 48-49 and 61Q insertion), which maintained persistently high viral loads (Fig. 7). These results are consistent with 1486D 1992 Nef sequences being less than optimal for Nef function and with the 61Q insertion resulting in a more functional Nef. However,

these results are based on only four animal infections and are not statistically significant (2).

The extreme slow/low growth phenotype observed for HIV-1 isolates from LTNP 5 (17) appears to map to the *env* region. LTNP 5 Env contains a polymorphism in the fusion peptide region of gp41 which was not observed in any of the 131 isolates in the Los Alamos HIV-1 sequence database (B. Korber, <http://hiv-web.lanl.gov>). The fusion peptide of gp41 inserts into target cell membranes, which is critical to the fusion process between cellular and viral membranes (7). The LTNP 5 Env polymorphism could affect this or other Env functions, resulting in the observed slow/low growth phenotype. Sequences responsible for a slow/low growth phenotype in other isolates have previously been mapped to Env amino acid substitutions in the V3 and C2-V4 regions (9, 24, 42, 50, 55) and thus could easily revert. The polymorphism seen in LTNP 5 Env is not an amino acid substitution, is maintained through long-term passage in cell culture, and is not likely to revert easily to a more rapid/high phenotype.

The origins of the unusual, difficult-to-revert polymorphisms observed in seven of our eight patients cannot be definitively determined with the samples available. As was clearly the case with eight Australians infected with Nef-deleted HIV-1 from a single blood donor (33), it is possible that many of the polymorphisms in our seven subjects were present in the infecting inoculum. Since the noted polymorphisms would be difficult to revert, they could have long-term attenuating effects on the virus. Alternatively, the unusual polymorphisms could have arisen in the infected hosts in response to selective pressures. Two types of selective pressures can be envisioned: the host immune response and polymorphisms in host cell proteins that

TABLE 4. Comparison of LTNP Gag and Nef deletions and HLA types

LTNP	Gag deletions ^a	Nef deletions ^a	HLA type ^b
LTNP 1	373	>100 aa	A2, B13, B62, C2, C5
LTNP 2	112	None	A2, B44, C2, C5
LTNP 6	None	49-50	A1, A2, B44, B57, C5, C6
LTNP 7	466-467	None	A3, A31, B38, B64, C5, C12
161J	471, 483-484	48-49	A2, A3, B7, B60, C3
1486D	370, 465-466	48-49	A1, A24, B57, B65, C4, C6

^a Amino acid(s) consistently absent in LTNP sequences.

^b Designation of a single A, B, or C allele indicates that a patient is homozygous at that locus.

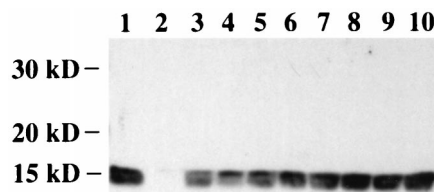


FIG. 6. LTNP p6^{gag}-mediated Vpr incorporation. Recombinant HIV-1 containing LTNP p6^{gag} sequences were purified by centrifugation. Virally associated proteins were separated by SDS-PAGE and electroblotted onto a membrane filter. HIV-1 Vpr was detected using a Vpr-specific polyclonal antiserum. Lane 1, NL 4-3 parental virus; lane 2, NL 4-3 Δ Vpr; lanes 3 to 10, recombinant HIV-1 containing LTNP p6^{gag} sequences: lane 3, LTNP 2; lane 4, LTNP 1; lane 5, LTNP 3; lane 6, LTNP 5; lane 7, LTNP 6; lane 8, LTNP 7; lane 9, 161J; lane 10, 1486D.

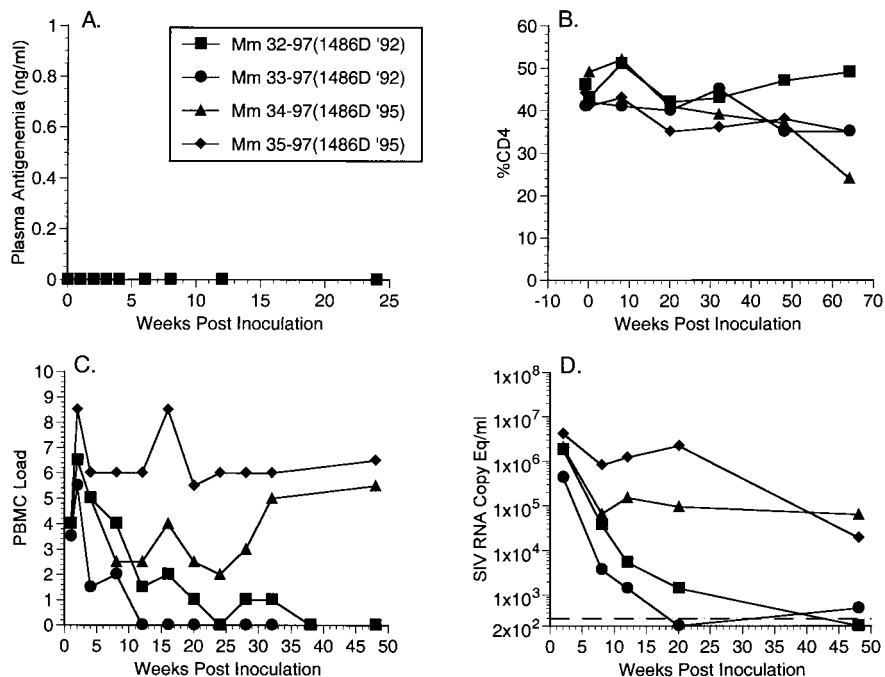


FIG. 7. Plasma antigenemia, CD4 percentage, PBMC load, and RNA copy (equivalents per milliliter) measurements for animals infected with SHIVnef recombinants containing 1486D Nef sequences. (A) Plasma antigenemia in SHIVnef-infected rhesus monkeys. p27 concentrations in plasma were determined at the time points indicated. The limit of detection is approximately 0.05 ng/ml. The week 0 sample is a preinfection sample taken immediately before inoculation with SHIVnef. (B) CD4 percentages in SHIVnef-infected rhesus monkeys. Whole blood was drawn from SHIVnef-inoculated animals at various times postinoculation and stained with OKT4, a fluorescein isothiocyanate-conjugated murine monoclonal antibody that was raised against rhesus macaque CD4 (American Type Culture Collection). The stained samples were analyzed using a FACScan flow cytometer (Becton Dickinson). (C) Frequencies of infectious cells in PBMC of SHIVnef-infected rhesus macaques. Viral loads were graded on a scale from 0 to 10 indicating the number of PBMC needed to recover SIV. A value of 0 denotes that no virus was recovered using 10^6 cells, 1 denotes successful virus recovery from 10^6 cells, and 2 to 10 denote successful virus recovery from 333,333, 111,111, 37,037, 12,345, 4,115, 1,371, 457, 152, or 51 cells, respectively. (D) Plasma SIV RNA levels at the indicated weeks postinoculation for animals infected with SHIVnef recombinants. The dashed line indicates the threshold sensitivity of the assay, 300 copy eq/ml. A value of 0 was assumed for week 0.

must partner with HIV-1 proteins. With respect to selective pressures from host immune responses, strong CTL responses have been measured in all eight of these LTNP. These strong cellular responses could put significant pressure on the virus to escape immune surveillance. However, deletions and insertions within ordinarily conserved sequences would be an unusual way for the virus to avoid immune recognition and would not be obviously advantageous over simple point mutations. Furthermore, we did not observe any consistent pattern of MHC-I haplotypes associated with similarly located deletions in the HIV-1 sequence. MHC-II-restricted CD4 helper cell activity could also conceivably be a selective force for driving sequence changes in the virus. With respect to possible polymorphisms in host cell proteins that must partner HIV-1 proteins, it is curious to note that heterozygosity for the *SDF-1-3'A* polymorphism seemed to be associated with similarly located Gag and Nef deletions in 1486D, 161J, and LTNP 7.

For the LTNP in this study, we did not observe homozygous polymorphisms in second-receptor or chemokine genes (Table 2) that have been previously documented to be associated with nonprogression (11, 23, 39, 52, 57). We have observed unusual, difficult-to-revert polymorphisms in HIV-1 sequences from LTNP that were not observed in the evaluated sequences from individuals who progressed to AIDS. Furthermore, most of these unusual polymorphisms are without precedent among observed HIV-1 sequences. Although we have not directly demonstrated that these deletions have contributed to their rate of disease progression, they appear to be associated with the unusual clinical status of these individuals in an apparently nonrandom fashion. Additional studies using monkeys and

SIV or SHIV recombinants with deletions analogous to those observed in the LTNP will be required to document any contribution to lowering viral load or attenuation of disease progression.

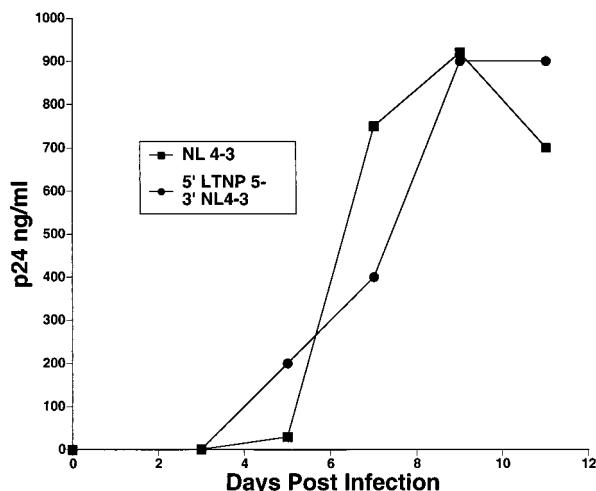


FIG. 8. Growth of recombinant HIV-1 containing 5' LTNP 5 sequences. LTNP 5 and NL 4-3 sequences from bp 297 to 4402 (5') and NL 4-3 sequences from bp 4402 to 9607 (3') were amplified separately. An *Xma*I (CCCGGG) site was introduced at the junction of the PCR fragments (bp 4402) as described in Materials and Methods. *Xma*I-digested 5' LTNP 5 and 5' NL 4-3 fragments were cotransfected with 3' NL 4-3 fragments into permissive CEMx174 cells. Supernatants were collected and assayed for p27 antigen concentration.

```

VIF HIV-1      MENRWQVMIVWQVDRMRIRITWKSIVKHHMYISGKAKGWFYRHHYESTHRISSEVHIFLGDARLVITTYWGLHTGERDWHLGGQVSI EWRRKRYSTQVDP
VIF LTNP 5 #2  .....V.....R..A..V.....EN..T.....K.....R.....
VIF LTNP 5 #3  .....V.....I.....R..A..V.....EN..T.....K.....T.....R.....

VIF HIV-1      DLADQLIHLHYFFDCFSESAIRNAILGHIVSPRCEYQAGHNKVGSLQYLALALITPKKIKPPLPSVTKLITEDRWNKPKQTKGHRGSHTMNGH
VIF LTNP 5 #2  G...R.....V.....R.....Q.....
VIF LTNP 5 #3  G...R.....P..V.R...R.....Q.....R.....
    
```

FIG. 9. Alignment of LTNP 5 Vif sequences. The product of LTNP 5 *vif* allele representing the consensus (allele 2) and the product of an allele representing the most divergent sequences from the group M, subgroup B, consensus (allele 3) were aligned to subgroup B consensus sequences (HIV-1). Each sequence represents a consensus of two clones from each of two independent PCRs. A period indicates conservation between subgroup B and LTNP 5 sequences.

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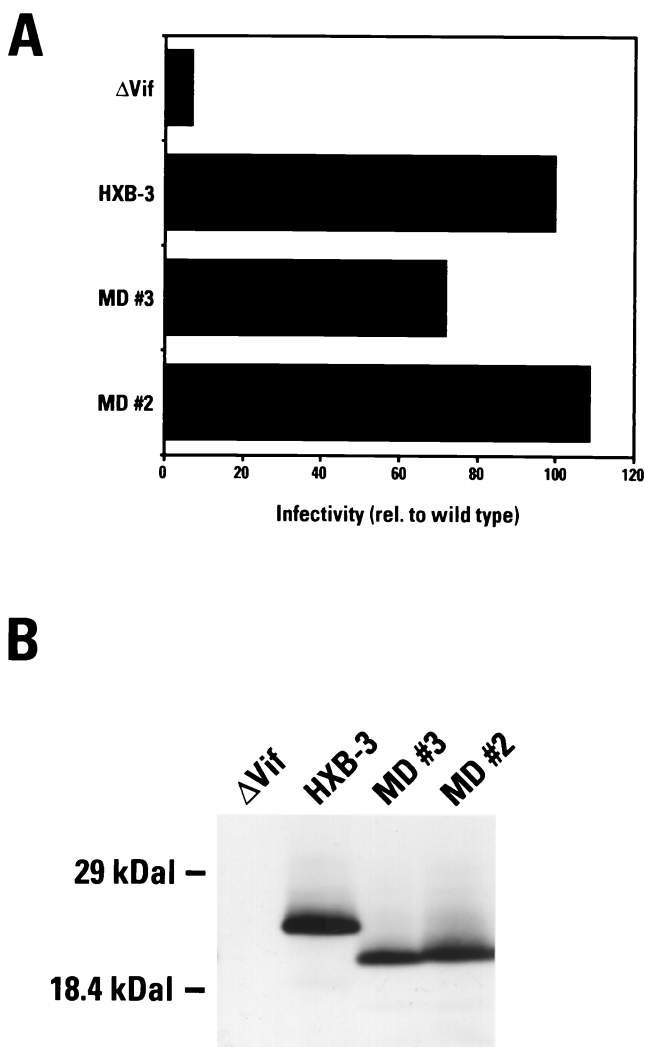


FIG. 10. Vif functional assay. (A) LTNP 5 (MD) *vif* sequences 2 and 3 as well as HXB-3 *vif* were cotransfected into H9 cells with a *vif*-deficient proviral vector. Harvested viruses were used to challenge the indicator cell line C8166/HIV-CAT. Levels of CAT activity indicate levels of Vif-mediated infectivity. These data represent the means of four independent experiments. (B) Whole-cell lysates of transfected H9 cells were separated by SDS-PAGE and electroblotted onto a membrane filter. HIV-1 Vif was detected using a Vif-specific monoclonal antibody.

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