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Heteroduplex Mobility Assay and Phylogenetic Analysis of V3 Region Sequences of Human Immunodeficiency Virus Type 1 Isolates from Gulu, Northern Uganda

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We analyzed peripheral blood mononuclear cells from 19 asymptomatic seropositive pregnant women from the district of Gulu in northern Uganda. A 700-bp fragment of the human immunodeficiency virus type 1 (HIV-1) *env* gene, including the V3-V5 region, was successfully amplified by PCR from 10 samples (52.6%) and was subsequently subjected to both a heteroduplex mobility assay for genetic screening and subtyping and DNA sequence analysis (approximately 300 bp) for nucleotide comparison and phylogenetic studies. The results show the presence of HIV-1 A and D subtypes (or clades) in this rural area, with the prevalence of the A subtype (8 of 10) being greater than that of the D (2 of 10) subtype, which is unlike what was previously reported for Uganda. By pairwise comparison analysis, the percentage of sequence divergence among samples within each subtype is low (the average intrasubtype divergence is 15.8%), but it is significantly higher between the two subtypes (the average intersubtype divergence is 23%). At the amino acid level, the two HIV-1 subtypes show distinct tetramers at the apex of the V3 loop and, in particular, GPGQ in clade A and GPGR in clade D. In addition, 10 of the 19 viral samples (52.6%) have been isolated in vitro. Nine of the samples have been classified as rapid/high, which reflects a high in vitro replication capacity for the HIV-1 field isolates from this country, even for those obtained from seropositive asymptomatic individuals. These observations, despite being made on the basis of a limited sample size, show a modest degree of genetic divergence among samples isolated in the last 4 years in this country by comparison with those based on the 1990 data on HIV-1 isolates from Kampala. The results reported here are, therefore, extremely relevant for Uganda, which is one of the selected World Health Organization field sites for future HIV-1 vaccine evaluation programs.

The human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus characterized by extensive genetic variability. One of the most variable regions of the complete viral genome is found in the structural *env* gene, in which the distribution of sequence variability is not uniform and five hypervariable regions have been identified (V1 to V5) (3, 14, 22, 47, 55, 60, 69, 75, 76, 78, 84). Nucleotide sequence databases of the *env* gene of viruses isolated throughout the world have allowed phylogenetic analyses to be performed. On the basis of these analyses, HIV-1 strains have been subtyped into six major clades (A to F) with an average intraclade sequence divergence of 11% and an interclade divergence of 26% (60). Additional HIV-1 subtypes, named G and H, have been recently identified, and clades A to H have now been designated as the M group (8, 60). A limited number of HIV-1 viruses recently identified in Cameroon have been included in a second cluster termed the O group, and they have a sequence divergence of >50% with M group viruses (31, 51, 79). Furthermore, a specific clade distribution has been identified for different geographical regions (83, 85).

The biological relevance of genetic variations in the *env* gene is due to the central role of envelope gp120 in the virus-host interaction, particularly in the binding of the virus to the surface CD4 molecule on human T lymphocytes (46, 49). Within the gp120 molecule, the third hypervariable (V3) loop is involved in syncytium formation as well as in membrane fusion (15, 16, 46, 49) and represents the domain for cell tropism (13, 38, 73, 82). Furthermore, the V3 loop is one of the HIV-1 major antigenic epitopes (29, 65) and is the principal neutralizing determinant (PND). It is indeed the target of type-specific anti-HIV-1 neutralizing antibodies, although primary virus isolates have been shown to be significantly resistant to in vitro neutralization by anti-gp120 monoclonal antibodies and cytotoxic T lymphocytes (43, 48, 56, 68, 77, 86). Both immune functions are strongly related to sequence variations around the crown of the V3 loop, which is relatively well conserved within each clade (30, 50, 54, 60, 70, 77, 83, 86). On the basis of these observations, the V3 loop is considered one of the best candidates for anti-HIV intervention and therapeutic strategies. In this regard, complete protection of chimpanzees against infection with laboratory-adapted HIV-1 strains has been obtained by eliciting anti-V3 antibodies, by immunizing them with homologous gp120 (7, 27), and by administering specific anti-V3 monoclonal antibodies (21). Recent studies,

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however, have shown that anti-V3 antibodies are generally type specific, reacting in peptide binding and in vitro neutralization assays to the autologous V3 domain at significantly higher titers and frequencies (32, 36, 41, 66). On the other hand, sera cross-reacting with a wide-range panel of V3 loop peptides (11, 12, 35, 41, 42, 80, 81) as well as sera with binding activity to the autologous V3 peptides lower than that to the heterologous V3 peptides have been reported (41).

All these considerations indicate that HIV-1 genetic and antigenic variability is a crucial challenge. This variability requires that a worldwide monitoring of the geographical distribution of HIV-1 genetic subtypes, which dynamically changes over time, be carried out in order to develop broadly effective anti-HIV-1 vaccines or passive immune therapies, which are urgently needed in Third World countries because of the high incidence and prevalence of HIV-1 found in such countries.

In this framework, we have focused our attention on Uganda, one of the countries with the highest incidence and prevalence of HIV-1 infection. Nationwide serosurveys conducted in the last few years have shown an overall HIV-1 seroprevalence of 12%, but this prevalence is not uniformly distributed throughout the country (72). Very recent surveys in the Masaka district in southwest Uganda showed a 12.25% seroprevalence rate for the 15-to-44-year-old age group and a 4.32% rate for the older age group (61). The highest rates occurred for women aged 20 to 24 years (21.4%) and for men aged 25 to 34 years (18.0%) (61). On the other hand, a longitudinal study of pregnant women in the Kampala urban area (January 1989 to March 1990) showed an overall seroprevalence rate of 28.1%, with the highest rate (45.7%) occurring for people at the age of 22 (37). Furthermore, in the Masaka district, a 1% incidence rate in the general adult population and a 10.3% 1-year progression to death rate among HIV-1 seropositive adults have been reported (57). These data appear more dramatic when they are considered together with the evidence of a mother-to-child HIV-1 transmission rate of above 30% (9).

We have analyzed, at a molecular level, the V3 regions of 10 HIV-1 isolates from asymptomatic seropositive pregnant women living in the Gulu district, a rural area in the north of Uganda. For this purpose, we used the heteroduplex mobility assay (HMA) methodology, a recently reported new approach to predicting the genetic variability among different sequences and to subtyping HIV-1 strains, in place of the more complex DNA sequencing analysis (5, 19). Heteroduplexes, which are formed by the annealing of divergent single-stranded DNA molecules, show an electrophoretic mobility reduction that is proportional to the divergence of the sequences (point mutations, deletions, and insertions) (5, 19). This method has previously been used to discriminate among 1.2-kb fragments spanning the V1-V5 region of the *env* gene that diverge by 2 to 30% (5, 19). With better electrophoretic resolution and consequently easier discrimination among clades, a shorter 700-bp fragment comprising the V3-V5 region was used in our molecular analysis. Eight of 10 samples were subtyped as clade A and 2 samples were typed as clade D, the two clades previously identified in Uganda (5, 6, 60, 83). These results have been confirmed by nucleotide sequence homology comparison and phylogenetic analysis.

Finally, 10 samples were successfully isolated in vitro, and 9 of them showed a rapid/high (R/H) replication pattern at the secondary biological characterization, confirming the high in vitro replicative and syncytium induction capacities of field isolates from this country (23, 67, 83).

Uganda, together with Brazil, Rwanda, and Thailand, is one of the World Health Organization (WHO)-sponsored HIV vaccine sites and is currently monitored by the WHO Network for HIV Isolation and Characterization in order to assess the prevalence and distribution of the HIV-1 clades (83). The results reported here represent the first data from a district in the north of Uganda and give a more complete picture of the distribution and spreading of HIV-1 subtypes in Uganda.

MATERIALS AND METHODS

Sample collection. The study was performed as part of a multicenter international collaborative program involving the Ist. Naz. Tumori "Fond. G. Pascale," the Directorate General for Development and Cooperation of the Italian Ministry of Foreign Affairs, the Istituto Superiore di Sanità, the St. Mary's Hospital (Lacor), and the Ugandan Virus Research Institute. It has been endorsed by WHO and approved by the Ugandan AIDS Commission and its Ethics Committee, the Institutional Panel of St. Mary's Hospital (Lacor, Gulu District), and the Istituto Nazionale Tumori "Fond. G. Pascale." Verbal informed consent for participation in this study was obtained from the pregnant women.

Blood samples were drawn in March 1994 from 217 pregnant women attending the antenatal clinic at St. Mary's Lacor Hospital, Gulu, Uganda. The age of the subjects enrolled in the study ranged between 17 and 37 years, and the route of HIV-1 transmission was prevalently heterosexual. The initial serological screening was performed locally with two enzyme immunoassay systems (Cambridge Biotech Corporation and Wellcome Diagnostics) and was confirmed in Naples by indirect immunofluorescence and Western blotting (immunoblotting).

A typical full designation for a sample, according to nomenclature proposed by WHO (45), is HIV194UG001INT.01_di1PD, where INT stands for Ist. Naz. Tumori "Fond. G. Pascale." For the sake of simplicity, however, the samples in this paper are indicated with only the year of isolation, the country of origin, and the isolate number (e.g., 94UG001).

PCR. Peripheral blood mononuclear cells (PBMC) were purified from fresh HIV-1-positive blood samples by Leucoprep density gradient centrifugation (1). Cellular lysates from PBMC (approximately 10^6 cells) were prepared by proteinase K digestion at 56°C (10, 34) and subjected to a 40-cycle PCR analysis of the HIV-1 *gag* gene with the standard SK38-SK39 primer set, and these procedures were followed by a liquid hybridization with the radiolabeled SK19 internal probe in a procedure modified from that of Ou et al. (63). The *env* gene amplification was performed by nested PCR analysis. Cellular lysates (25 μl) were used as the DNA template for the first round of amplification in a reaction mix containing 1.25 mM $MgCl_2$ and the primers ED3 and ED14, corresponding to bases 5537 to 5566 and 7509 to 7538, respectively, of the HIV-1_{IIIB} BH10 isolate sequence (19). Primers ES7 (positions 6579 to 6598) and ES8 (positions 7225 to 7245) were used in a mix containing 1.8 mM $MgCl_2$ for either the second round of amplification on 5 μl of the first reaction or on 10 ng of the reference plasmids (19). The amplification reactions were carried out in a Perkin-Elmer Thermocycler 9600 for 36 cycles under the following conditions: 3 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 32 cycles at 94°C for 15 s, 55°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR amplification products (size, approximately 700 bp) were detected by electrophoresis on a 1% agarose gel.

Heteroduplex formation and mobility analysis. Heteroduplex molecules were obtained by mixing two divergent PCR-amplified DNA fragments denatured at 94°C for 10 min and renatured by rapid cooling on wet ice. The reaction was performed in 100 mM NaCl, 10 mM Tris-HCl (pH 7.8), and 2 mM EDTA in a final volume of 10 μl (19). The heteroduplex formation was resolved by electrophoresis analysis at 200 V for 2.5 h on a nondenaturing 5% polyacrylamide gel in TBE buffer (88 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) and was detected after being stained with ethidium bromide. The electrophoretic mobility of the heteroduplexes was inversely proportional to the sequence divergence of the two annealed strands. Standard curves for our experimental conditions were obtained by plotting heteroduplex mobility against the degree of divergence of the annealed PCR products from different known HIV-1 sequences (see Fig. 2A). The relative mobilities of the heteroduplexes were calculated as previously described by dividing the migration distance of the heteroduplex bands by the migration distance of the corresponding homoduplex bands (expressed in centimeters) (19). The mobility values were then plotted against the percentages of genetic distances between the fragments, which were calculated on the basis of sequence alignments that discounted gaps due to nucleotide insertions and deletions. The best-fit curve of the data was found with a linear function. Under our experimental conditions, with 0.7-kb DNA fragments and an electric field of 200 V for 2.5 h, the equation was

$$\%\ \text{divergence}\ (\alpha) = [35.9 - 35.44\ (\text{heteroduplexmobility})],$$

for a domain $\{\alpha \mid 0 \leq \alpha \leq 35\}$ (see Fig. 2B). The standard sequences used for the HMA, the countries of origin, and the corresponding GenBank accession numbers, respectively, were as follows: A1 (RW20), Rwanda, and U08794; A2 (UG37), Uganda, and U09127; A3 (SF170), Rwanda, and M66533-35; B1 (BR20), Brazil, and U08797; B2 (TH14), Thailand, and U08801; B3 (SF162), United States, and M65024; D1 (UG21), Uganda, and U08804; D2 (UG38), Uganda, and U08808; and D3 (UG46), Uganda, and U08809.

DNA sequencing. Direct sequencing reactions were performed on PCR products purified by a rapid method developed in our laboratory (10). Briefly, the amplified DNA fragments were digested with proteinase K for 30 min at 37°C, phenol-chloroform extracted, and polyethylene glycol precipitated for 10 min at 37°C. DNA samples were denatured at 95°C in the presence of dimethyl sulfoxide, immediately cooled in liquid nitrogen, and sequenced by following the Sequenase protocol (United States Biochemical, Cleveland, Ohio) as modified in the labeling step (3 min on ice). The internal annealing primers V3 B and C (322 bp around the C2-V3 region) (74) were used to prime sense and antisense sequence reactions, respectively. The sequences were then analyzed on 6% polyacrylamide wedge sequencing gels, and comparison analysis was performed by alignment with reference HIV-1 sequences from the Los Alamos database (60) by using the GENEPRO software (Riverside Scientific Enterprises, Seattle Wash.).

Phylogenetic analysis. Sequences obtained in this study (approximately 300 nucleotides) were aligned with those of corresponding fragments of 22 known HIV-1 isolates belonging to different subtypes, with the simian immunodeficiency virus cpzgab (SIV_{cpzgab}) isolate being used as the outgroup. Multiple sequence alignments were performed with the MegAlign option of the Lasergene software (DNASTAR Inc., Madison, Wis.) by the Clustal method (33). The final phylogenetic tree was produced with MegAlign by applying the neighbor-joining method to the distance and alignment data (71).

Isolation and secondary characterization of virus isolates. Isolations of virus from PBMC were performed by the cocultivation procedure recommended by the WHO Network for HIV Isolation and Characterization (67), with 10^6 PBMC from infected individuals being used. Briefly, PBMC from HIV-1-infected individuals were prepared at the St. Mary's Hospital laboratory from buffy coats by Leucoprep density gradient centrifugation and were then frozen in liquid nitrogen (1). PBMC from two different HIV-1-seronegative donors were prepared from buffy coats and were stimulated for 72 h with 10 μg of phytohemagglutinin per ml in RPMI 1640 medium supplemented with 15% fetal calf serum. After the stimulation, 1.2×10^7 donor PBMC and thawed PBMC from HIV-1-infected individuals were cocultivated in RPMI 1640 medium supplemented with 15% fetal calf serum and 20 U of recombinant interleukin-2 per ml. The cultures were monitored daily during the whole cultivation period (28 days), and if necessary, medium and/or freshly stimulated donor PBMC were added. The cell supernatants were monitored weekly for viral growth by a p24 antigen assay (DuPont), and after the first sign of p24 antigen positivity, the cultures were expanded for 3 more days and then were frozen in liquid nitrogen for subsequent analysis.

For the biological characterization, 10^6 phytohemagglutinin-stimulated PBMC from HIV-seronegative donors were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 μg of polybrene per ml, and 10 U of recombinant interleukin-2 per ml and were infected with cell supernatant aliquots containing 1 ng of primary HIV-1 isolate p24 antigen (52). After 7 to 10 days, at the time of the p24 antigen peak, a cocultivation with target cell lines (CEM, U937, MT-2, and Jurkat-Tat) in a 1:3 ratio was set up, and then the cell cultures were incubated for 4 weeks, with the supernatants being tested for p24 antigen twice a week (4, 24, 25). Additionally, MT-2 cell cultures were scored for syncytium formation. Three consecutive positive tests were required to score virus isolates as replication positive in a given cell line, and the biological phenotypes were classified as slow/low and R/H (24, 25, 67).

RESULTS

Serologic screening of selected members of the cohort and detection of the HIV-1 *env* gene by PCR amplification. A total of 217 pregnant women were enrolled in the study and screened for HIV-1 seroprevalence. Twenty-nine seropositive individuals (13.36%) were identified by two enzyme immunoassay systems (Cambridge Biotech Corporation and Wellcome Diagnostics), and the results were confirmed in Naples by indirect immunofluorescence and Western blot analyses. All the seropositive women were asymptomatic, with no evident clinical symptoms related to AIDS or to a general status of immune depression, such as general weight loss (slim disease), tuberculosis, and/or lymphadenopathy. No information, however, on the seroconversion time was available.

PBMC from 19 of the 29 positive samples were lysed so that the HIV-1 *gag* sequence (nucleotides 1541 to 1638) and the V3-V5 region of the HIV-1 *env* gene could be amplified by PCR. All the samples were positive for the conserved *gag* gene (data not shown). Of the 19 samples, 10 were also positive for the 0.7-kb *env* DNA fragment, as shown in Fig. 1. The additional low-intensity upper band that is visible in most of the gel lanes and that has a mobility of 1,700 bp might be either the product of amplifications driven by one of the internal and one of the external primers of the two subsequent reactions or the result of heteroduplexes formed between DNA PCR products of heterogeneous virus variants that were both present within the same sample (Fig. 1) (19).

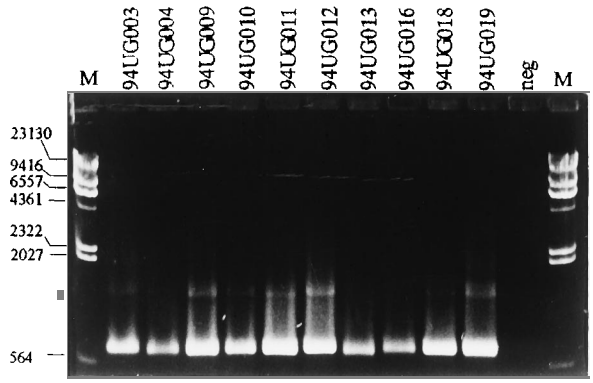
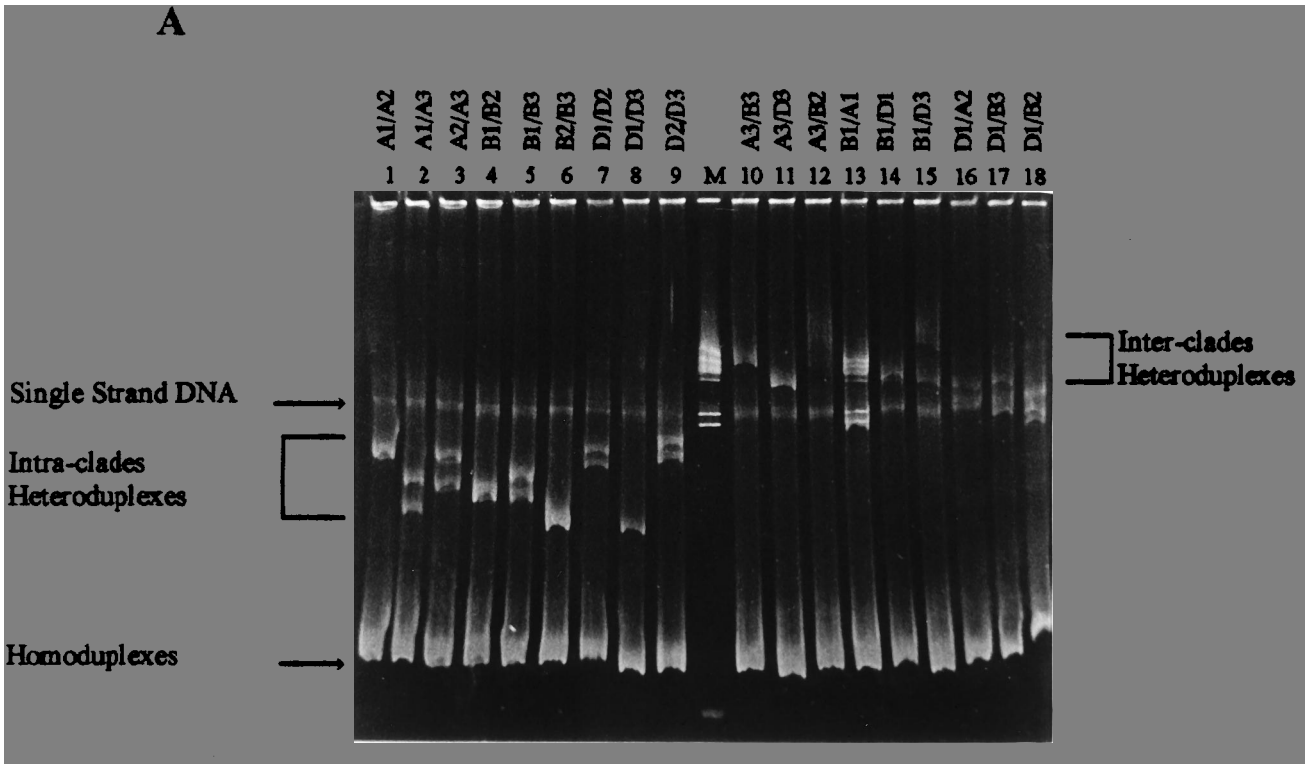


FIG. 1. Analysis of DNA fragments obtained by a second round of nested PCR. The 0.7-kb V3-V5 region of the HIV-1 *env* gene was purified as previously described (10) and electrophoretically separated on a 1% agarose gel in 1× TBE buffer. The samples are indicated above each lane. The negative control lane (neg) denotes a reaction without the addition of the target DNA. The additional upper band in each lane could be explained as possible heteroduplexes or even as an amplification product driven by one of the internal and external primers used in the two subsequent reactions. The molecular weight marker lanes (M) are λ DNA digested with *Hin*dIII, and the sizes of the bands are indicated at the left (in nucleotides).

Genetic subtyping of HIV-1 samples by HMA. In order to determine the genetic variability (distance) of and to subtype the HIV-1 samples identified in Gulu, HMA was performed on heteroduplexes obtained by reannealing the 0.7-kb *env* PCR product of each sample with the corresponding PCR product of the HIV-1 reference strains (19, 60).

To establish the relationship between electrophoretic mobility and sequence divergence under our experimental conditions, HMA was first performed on heteroduplexes formed by pairwise combination of V3-V5 fragments from the HIV-1 reference sequences that belonged to clades A, B, and D and that were commonly used in our study (Fig. 2A). The results clearly show that electrophoretic mobility for heteroduplexes formed by fragments of HIV-1 strains belonging to the same clade (Fig. 2A, lanes 1 to 3 for clade A, 4 to 6 for clade B, and 7 to 9 for clade D) is greater than that for heteroduplexes formed by fragments from strains belonging to different clades (Fig. 2A, lanes 11 to 19). The heteroduplex mobilities were plotted against the expected nucleotide sequence divergences, and the best-fit curve was determined with a linear equation (Fig. 2B; see also Materials and Methods). The correlation coefficient for the linear regression of DNA mobility against sequence variation was equal to 0.919, with a significance level (*P* value) of <0.0001, confirming the significant relation between the two variables and therefore the good predictability and validity of the HMA as an HIV-1 genetic screening methodology (Fig. 2B) (5, 18, 19).

The analysis of heteroduplexes of the PCR products of the Gulu samples and the reference sequences showed unequivocal electrophoretic patterns. Figure 3 shows the typical HMA pattern of the 94UG013 sample. In each lane, three types of bands are visible: (i) the double-stranded homoduplex band at the bottom of the gel, corresponding to the fragment molecular size (0.7 kb); (ii) the single-stranded band in the middle of the gel, resulting from the fraction of DNA molecules which have not reannealed during the reaction process; and (iii) the heteroduplex bands at different positions in the gel lanes. The



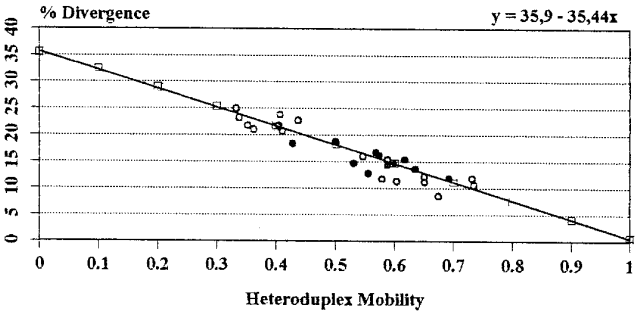


FIG. 2. The heteroduplex mobilities of the 0.7-kb V3-V5 region of the *env* gene of HIV-1 strains representing different clades were analyzed on a nondenaturing 5% polyacrylamide gel in 1× TBE buffer and were graphed against sequence divergences. (A) DNA fragments from clades A, B, and D were mixed in order to have intraclade combinations and 9 of the 36 possible interclade pairwise combinations. Heteroduplexes formed by fragments from intraclade strains clearly show greater electrophoretic mobilities than those formed by fragments from interclade strains; the bands at the bottoms of the lanes are the homoduplexes running just above the 0.5-kb fragment of λ DNA digested with *Hin*dIII. The standard sequences used for the HMA are A1 (RW-20), A2 (UG-37), A3 (SF-170), B1 (BR-20), B2 (TH-14), B3 (SF-162), D1 (UG-21), D2 (UG-38), and D3 (UG-46). M, molecular weight marker. (B) Graphic representation of the correlation between HMA heteroduplex mobilities and DNA sequence divergences. Mobility values were calculated by dividing the migration (expressed in millimeters) of the heteroduplex band (or the midpoint between two bands) by the migration of the corresponding homoduplex. The best-fit curve was derived by the equation % DNA divergence = [35.9 − 35.44 (heteroduplex mobility)], where the range of the percentage of DNA divergence covered is between 1 and 35. The positions of the points obtained under our experimental conditions for the Gulu samples (●) and the HIV-1 standards (○) significantly correspond to those (□) for the equation-derived curve.

94UG013 sample, as with most of the Gulu samples, can be clearly subtyped because of a clear-cut electrophoretic pattern, with heteroduplex bands reannealed to the least divergent reference sequences (D strain) running much faster than those of heteroduplexes formed with the other reference strains (Fig. 3). Furthermore, the sample showed similar sequence divergences from the three D references (13 to 15% by interpolation on the standard curve), since the heteroduplex bands have comparable electrophoretic mobilities (Fig. 3, lanes 4 to 6). Finally, the simple heteroduplex electrophoretic pattern strongly suggests the molecular homogeneity of HIV-1 sequences in the sample (Fig. 3); the reannealing of two double-stranded DNA molecules (one from the sample and one from the reference) can, indeed, originate only two heteroduplex molecules.

All 10 amplified samples were analyzed under the same experimental conditions, and similar clear-cut results were obtained, with 8 samples being identified as subtype A and 2 samples as subtype D. The intraclade sequence variation between each Gulu sample and the HIV-1 reference strains ranged from 14 to 20%, as calculated by interpolation of HMA mobility values on the standard curve (Fig. 2B).

Nucleotide sequence and phylogenetic analysis of the V3 region. In order to complete the qualitative analysis begun by HMA analysis of the V3 regions, approximately 300 bp (the *env* gene C2-V3 region) were sequenced directly from the PCR products. The nucleotide sequences were subsequently compared pairwise, and the homology rates were obtained by alignment analysis, with gaps due to nucleotide insertions and deletions being discounted (GENEPRO). The results show, when both clades are considered together, an average intraclade divergence of 15.9% (13 to 21%) and an average interclade divergence of 23.6% (22 to 31%), confirming the presence of two distinct clusters of HIV-1 V3 sequences, as suggested by the HMA analysis (Table 1). The single intraclade distance percentage between the two D samples

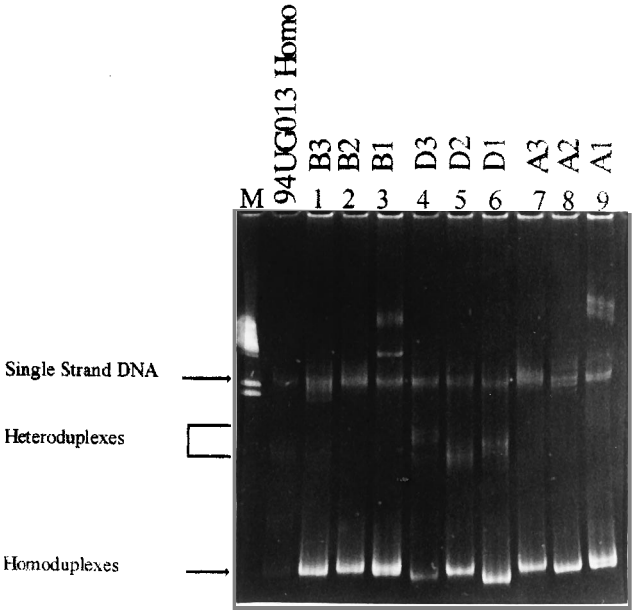


FIG. 3. The PCR fragment from HIV-1 isolate 94UG013 was mixed and reannealed with standard HIV-1 strains (as indicated above the lanes). Faster-running heteroduplexes indicate the higher homology of the reannealed strands and allow the 94UG013 sample to be subtyped as D. In the 94UG013 Homo lane, the sample is loaded by itself in order to visualize all the intrinsic bands so that they can be subtracted from the other lanes. The bands running between the single-stranded DNA and homoduplex bands can be explained by the heterogeneous virus molecular species in the sample itself forming heteroduplexes. M, molecular weight marker.

(94UG010 and 94UG013) is very close to the general mean (15%) (Table 1). This sequence clustering is much more evident when the alignment is performed with the 114 nucleotides corresponding to the V3 loop region; for the A strains, the intraclade arithmetical mean divergence dramatically drops to 7.6% (2 to 11%), while the interclade divergence remains at 22.5%. Similarly, the divergence among the D strains drops to 10% (Table 2). The same analysis was performed to compare the sequences of our samples with published HIV-1 sequences representative of three different clades (A, B, and D) (60), and the results again show a very clear pattern of homology rates (Table 3). The two groups of Gulu sequences show a lower divergence from those of reference intraclade strains (16.47% for clade A and 16.9% for clade D) than from those of interclade strains (28.1% for clade A and 26.1% for clade D). Furthermore, the pairwise comparison of the sequences of the Gulu samples with the two consensus sequences identified in 1990 in Uganda (groups A and B) (2, 41), indicates that in this country, HIV-1 isolates can be largely grouped into two clades and that no significant change in the degree of divergence has been observed for the HIV-1 sequences identified in the last 4 years.

In order to perform phylogenetic studies, Gulu HIV-1 *env* linear 300-nucleotide sequences were aligned among themselves and with data bank sequences, in particular with 22 HIV-1 sequences from different clades and with the chimpanzee SIV_{cpzgab} isolate sequence (60). Phylogenetic trees were inferred by the neighbor-joining method, and confidence values for the individual branches were determined by the MegAlign option of the Lasergene software (DNASTAR Inc.) (Fig. 4). The two groups of Gulu sequences significantly cluster in two branches together with those of the reference strains belonging to clades A and D.

All of the nucleotide sequence information, therefore, fully confirms the data obtained with the HMA and shows the good agreement between the rates of sequence divergence and the HMA patterns for HIV-1 samples within each clade.

V3 peptide analysis and comparison. In order to verify the impact of nucleotide variations on peptide composition, the V3 loop amino acid sequences were deduced by computer analysis. HIV-1 samples from clade A show an average 50.9% degree of stability (27 of 53 amino acids [aa]) in the V3 region and a greater homology (68.5%) in the conserved core of the V3 loop sequences (24 of 35 aa); the clade D samples, on the other hand, show a constant higher degree of stability (>73%) in the whole region (38 of 52 aa) and the V3 loop (27 of 34 aa) (Fig. 5). The two groups of HIV-1 strains present distinct V3 loop patterns; the crown consensus sequence is GPGQ(A/T)F for clade A viruses and GPGRA(L/Y) for clade D viruses (Fig. 5). In addition, the V3 loop consensus sequence of the Gulu A subtype is 94.3% homologous (33 of 35 aa) to the global consensus sequence for clade A obtained from 124 worldwide samples, and the V3 loop consensus sequence of the Gulu D subtype is 94.1% homologous (32 of 34 aa) to the global clade D sequence based on 73 worldwide samples (Fig. 5). The two clade D samples (94UG010 and 94UG013) also have a specific amino acid deletion in the V3 loop (for a total length of 34 instead of 35 aa), which is a feature of clade D (Fig. 5) (55). All samples from Gulu have two conserved cysteines (C) at the V3 loop extremities involved in the secondary stem-and-loop structure formation and the highly conserved N-linked glycosylation sites (Fig. 5). Finally, it is very important to note that

TABLE 1. Nucleotide diversity among DNA sequences from the Gulu samples[a]

Sequence	Diversity (%) from:									
	94UG003	94UG004	94UG009	94UG011	94UG012	94UG016	94UG018	94UG019	94UG010	94UG013
94UG003	0									
94UG004	**16**	0								
94UG009	**15**	**14**	0							
94UG011	**18**	**18**	**12**	0						
94UG012	**13**	**17**	**16**	**13**	0					
94UG016	**18**	**14**	**15**	**21**	**17**	0				
94UG018	**16**	**16**	**14**	**12**	**13**	**17**	0			
94UG019	**19**	**20**	**17**	**17**	**14**	**15**	**19**	0		
94UG010	26	28	27	28	22	26	24	31	0	
94UG013	27	28	28	29	25	27	23	27	**15**	0

[a] The numbers indicate the percentages of nucleotide diversity for the DNA sequences. The analysis was performed on the approximately 300 nucleotides of the C2-V3 region. Positions that included gaps were excluded from the alignment. Boldface indicates intraclade diversity, and lightface indicates interclade diversity.

TABLE 2. Nucleotide diversity among DNA sequences from the Gulu samples[a]

Sequence	Diversity (%) from:									
	94UG003	94UG004	94UG009	94UG011	94UG012	94UG016	94UG018	94UG019	94UG010	94UG013
94UG003	0									
94UG004	**10**	0								
94UG009	**11**	**8**	0							
94UG011	**11**	**10**	**8**	0						
94UG012	**9**	**8**	**3**	**5**	0					
94UG016	**10**	**9**	**7**	**8**	**6**	0				
94UG018	**9**	**9**	**7**	**7**	**4**	**8**	0			
94UG019	**10**	**9**	**5**	**7**	**2**	**7**	**6**	0		
94UG010	24	24	23	23	22	21	22	22	0	
94UG013	25	22	22	24	22	21	20	23	**10**	0

[a] The numbers indicate the percentages of nucleotide diversity for the DNA sequences. The analysis was performed on the 114 nucleotides of the V3 loop. Positions that included gaps were excluded from the alignment. Boldface indicates intraclade diversity, and lightface indicates interclade diversity.

the sequences of V3 crown octapeptides of the Gulu samples, even the divergent ones, correspond to the worldwide reported sequences of clades A and D, respectively (Table 4), except for that of the 94UG011 sample, whose octapeptide sequence has been more frequently associated with clade F (60).

Therefore, the results of the amino acid sequence analysis of the HIV-1 isolates from Gulu show a high degree of intraclade homology and very limited variation in the V3 loop region sequence compared with global consensus sequences (Table 4).

Virus isolation and biological characterization. In order to isolate the HIV-1 strains prevalent in Gulu, PBMC from all 19 samples were cocultured with PBMC from two seronegative donors (1). A 52.6% isolation efficiency was obtained (10 of 19 samples), even though a low number of PBMC ($\approx 10^6$) from infected individuals was used (67). Most of the virus isolates demonstrated high in vitro replicative capacity by p24 antigen detection in the cell culture supernatant during the second week of cocultivation; the 94UG016 sample was positive during the first week, and the 94UG004 sample was positive during the third week (Table 5). In order to reduce the in vitro selection of virus variants, isolates were harvested 3 days after the detection of p24 antigen, when its levels were generally >100 pg/ml (300 to 29,800 pg/ml) (Table 5).

The primary virus isolates were subsequently tested for replicative capacity with T-lymphoid (CEM, Jurkat-Tat, and MT-2) and monocytoid (U937) cell lines. Nine of 10 isolates showed active replication in all tested cell lines, inducing cellular syncytia in MT-2 cells, and have been classified R/H; 94UG015, on the other hand, was able to replicate only with the support of the Tat protein constitutively expressed in the Jurkat-Tat cell line. It, therefore, has been classified as slow/low group 2 (Table 5) (25, 67).

These data support previous observations on the prevalently high in vitro replication rate of Ugandan HIV-1 field isolates (67).

DISCUSSION

A new methodological approach (HMA) to characterize HIV-1 strains present in a cohort of pregnant women living in the district of Gulu in northern Uganda was used. The HIV-1 seroprevalence rate for 217 subjects was 13.36% (29 of 217), compared with a 15.03% rate reported for a 20-to-40-year-old group of women from the Masaka district in southwestern Uganda (61) and a 12% rate for the general Ugandan population (72). PBMC samples from 19 HIV-1-seropositive individuals were purified, and the infecting HIV-1 virus was biologically and genetically characterized. Nine of the 10 isolates obtained by in vitro cocultivation showed an R/H replication pattern. Most of the field isolates from asymptomatic HIV-1-infected individuals at other WHO field sites (Brazil, Rwanda,

TABLE 3. Nucleotide diversity among DNA sequences from the Gulu and HIV-1 reference samples[a]

Sequence	Diversity (%) from:									
	94UG003	94UG004	94UG009	94UG011	94UG012	94UG016	94UG018	94UG019	94UG010	94UG013
Group A	27	33	33	20	24	27	21	21	**13**	**11**
Group B	**13**	**15**	**11**	**14**	**12**	**18**	**13**	**17**	26	25
A SF1703	**12**	**14**	**10**	**10**	**13**	**16**	**10**	**9**	23	26
A U455	**23**	**21**	**20**	**19**	**19**	**21**	**18**	**19**	28	29
A Z321	**19**	**17**	**16**	**13**	**16**	**18**	**16**	**17**	28	27
A UG06	**22**	**18**	**20**	**19**	**21**	**21**	**19**	**20**	24	25
B MN	27	25	23	24	24	27	24	25	21	23
B SF2	26	25	24	24	23	27	23	26	20	23
D JY1	29	27	25	25	23	29	28	26	**14**	**16**
D ELI	33	31	27	26	26	32	29	31	**18**	**21**
D Z2Z6	34	30	28	26	27	33	30	29	**18**	**20**
D UG23	32	28	29	28	26	31	31	30	**18**	**20**

[a] The numbers indicate the percentages of nucleotide diversity for the DNA sequences. The analysis was performed on the approximately 300 nucleotides of the C2-V3 region. Positions that included gaps were excluded from the alignment. The reference HIV-1 sequences are representative of different clades and include the group A and B consensus sequences from Kampala, Uganda, that were previously described (2, 60). Boldface indicates intraclade diversity, and lightface indicates interclade diversity.

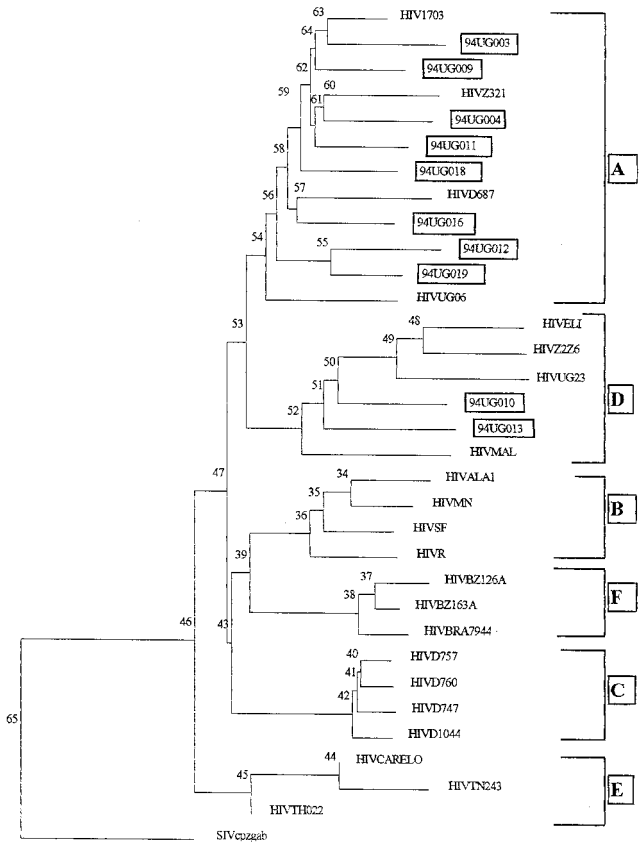


FIG. 4. Phylogenetic tree based on 300 unambiguously aligned positions of the HIV-1 *env* gene V3 region from the Gulu samples and 22 HIV-1 reference strains representing different clades. The tree was rooted with a corresponding region of the chimpanzee SIV_{cpzgab} isolate being used as an outgroup. The analysis was performed as described in Materials and Methods.

and Thailand) showed, on the contrary, a slow/low pattern (67). The clinical information on the samples analyzed in this study is too limited to allow us to offer an explanation for such observations, in particular to discriminate between preferential transmission of R/H HIV-1 strains and a seroconversion time of >2 years (67).

PCR analysis of fresh uncultured PBMC showed that all samples were positive for HIV-1 *gag* sequences after liquid hybridization. The V3-V5 region of the *env* gene was amplified by nested PCR, and it was detected by fluorescence in only 10 of 19 samples, which were further processed by HMA analysis and direct DNA sequencing (Fig. 1). The low quantity of infected cells in the samples, either because of the early stage of the disease or because of the low recovery efficiency with frozen cells, could generally account for the low percentage of success of the *env* PCR. Nevertheless, inefficient priming due to a high *env* gene sequence divergence could be also taken into consideration as well.

The *env* DNA fragments were analyzed by HMA. This method, which used the 1.2-kb V1-V5 *env* fragment, has recently been described as a fast and accurate method to subtype HIV-1 strains and to predict the percentage of sequence divergence (5, 19). In this study, the HIV-1 *env* genetic divergence was correlated with the electrophoretic mobility of heteroduplexes formed by 0.7-kb fragments (V3-V5 region) (Fig. 2). Under our experimental conditions, a linear relationship between the two variables was observed, as determined by the formula % divergence (α) = [35.9 − 35.44 (mobility)], which fits for the domain $\{\alpha \mid 0 \leq \alpha \leq 35\}$. The data obtained in our study show a correlation coefficient of 0.919 for the two variables, with a significance level (*P* value) of <0.0001.

Each sample was subtyped on the basis of the higher mobility of the heteroduplexes formed with at least three HIV-1 reference strains for each clade, and the percentage of sequence variation, calculated with the formula given above, fell between 14 and 20% (Fig. 2B). The simple HMA pattern of each sample suggested that the number of variants present in the same individual was limited, perhaps because of an early stage of infection or a strong positive selection for a predominant variant.

The HIV-1 isolates from Gulu were determined by HMA to belong to clades A and D, the most common in Africa. In this northern area of Uganda, the prevalence of clade A (8 of 10) is greater than that of clade D (2 of 10). This finding contradicts data previously reported for the southeastern and southwestern regions of Uganda that showed either a strong prevalence of clade D (5, 6, 60, 83) or equivalent frequencies of clades A and D (2). The high prevalence of clade A is likely due to accidental trafficking (viral migration), with a predominant introduction of clade A subtype HIV-1 into the Gulu area over the period of the AIDS epidemic (59). However, the transmission route of the virus could also have an effect on the prevalence of clade A. In Thailand, for instance, the newly introduced clade E HIV-1 is overcoming the clade B virus that was predominant at the beginning of the epidemic (26, 53, 62, 64, 83). Clade E virus spreads through sexual contact, whereas clade B virus is more frequently transmitted by means of shared intravenous needles (26, 53, 62).

DNA nucleotide analysis was performed directly on PCR amplification products, without any subcloning step, in order to identify the most prevalent V3 region nucleotide and amino acid sequences in each sample. Pairwise alignment analysis was performed on 300-nucleotide fragments, and the A samples identified in Gulu showed a high intraclade clustering (average homology of 84.2%), which appears more evident when the analysis is performed on the 114-nucleotide V3 loop (92.4%). This pattern is also valid with the two D sequences, since the homology for these 114 nucleotides increased from 85 to 90%. The percentage of interclade homology, however, for either fragment was significantly lower (average homology of 77%) (Tables 1 and 2). Similar results were observed when the samples identified in Gulu were compared with HIV-1 reference strains of different clades. Such comparisons showed a strong sequence relationship of the Gulu strains to strains of clades A and D (Table 3). The consensus sequences of the Ugandan HIV-1 A and B groups reported by Albert et al. (2) have been included in this analysis. The DNA sequence comparison analysis showed that the group A consensus sequence has a significantly higher homology with those of the Gulu D samples and that the group B consensus sequence had a higher homology with those of the Gulu A samples (Table 3). It is striking that the divergence between the old and the current Ugandan HIV-1 sequences (14.1%) is similar to the one existing for the current sequences (15.8%), suggesting that there has been no major divergence in Ugandan HIV-1 strains isolated in the last 4 years (1990 to 1994) (Table 3).

The phylogenetic analysis comparing the 10 Gulu sequences with those of 22 known HIV-1 reference strains of the six designated clades found that, as was indicated by HMA and the DNA sequence analysis, eight samples clustered in the A branch and that 94UG010 and 94UG013 clustered in the D branch (Fig. 4). This confirms the accuracy of HMA for genetic

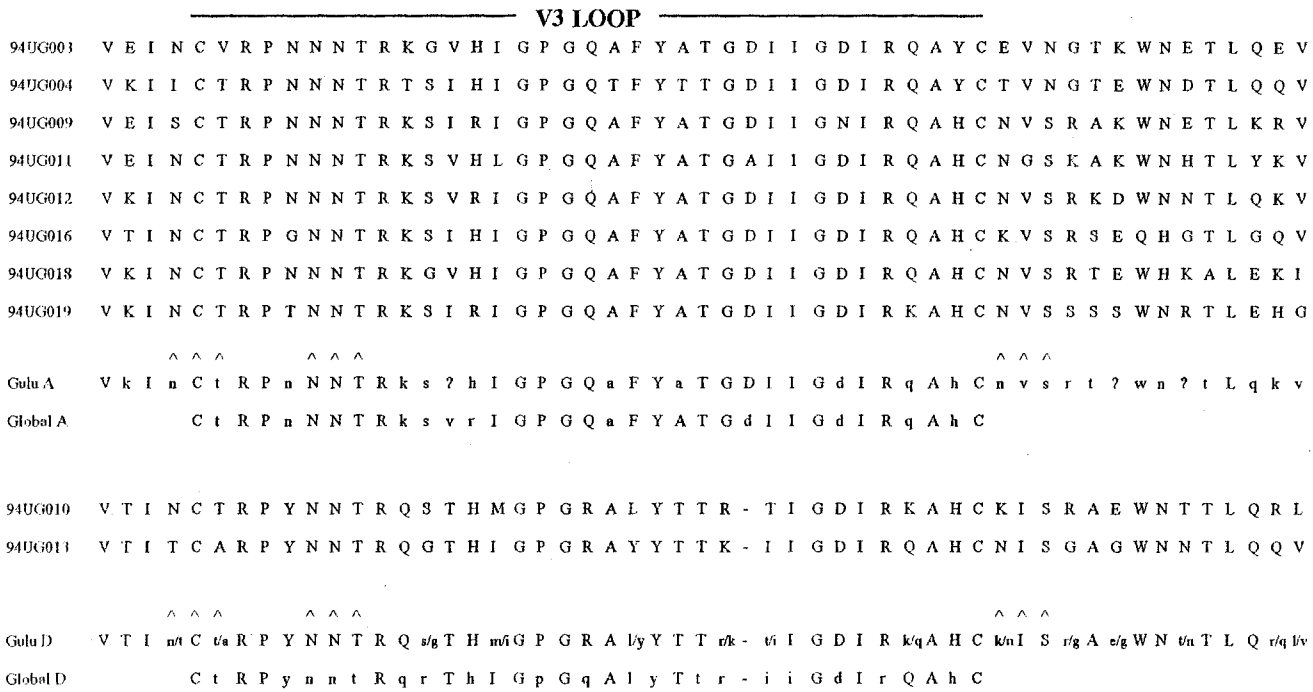


FIG. 5. Alignment of the derived amino acid sequences of the HIV-1 V3 region. The amino acid sequences corresponding to the V3 region (53 aa) of the HIV-1 isolates from Gulu were aligned on the basis of subtyping, and for each group, a consensus sequence was derived. Lowercase letters indicate that the residue was found in at least three of five sequences, and a question mark indicates that different residues were found in that position in fewer than two of five sequences. In the consensus sequences for the D samples (94UG010 and -013), the discordant residues found in the corresponding positions were both reported. The A and D global consensus sequences were derived from 124 and 73 sequences, respectively, and lowercase letters were used to denote residues with frequencies lower than 80% (60). ^, potential N-glycosylation sites.

screening by establishing the relationship of two unrelated DNA samples.

Analysis of the derived amino acid sequences showed that different V3 loops have limited divergence within the two clades in the context of the entire V3 region and that the Gulu isolates show strong stability for amino acid residues in specific positions (Fig. 5). This is in accordance with what was previously described in an analysis of 302 C2-V3 amino acid sequences from the Los Alamos database (44). For all clades, with the exception of D, it is indeed evident that there is a lower rate of amino acid change within the V3 loop than in the V3 loop flanking regions (44). These findings suggest that there is a strong selection for specific V3 loop sequences involved in such strategic virus functions as tropism and cellular transmission (44).

Each of the two HIV-1 clades identified in Gulu has a specific tetrameric sequence at the apex of the V3 loop; in particular, the GPGQ sequence is associated with subtype A isolates and GPGR is associated with subtype D isolates. GPGQ is the most predominant tetramer in African subtype A and E isolates and appears to be globally the most common cross-clade sequence; GPGR, however, seems to be very much B specific, with a minor association with the subtype D (60). The limited number of cases analyzed in this study, however, does not allow for any final conclusion on the statistical significance of this observation. The pattern is more complex for the octameric apex of the loop, which is considered the target for the anti-V3 neutralizing antibodies (28). In particular, subtype A isolates show octamers HIGPGQAF and RIGPGQAF, which are equally the most represented (94UG009, -012, and -019 and 94UG003, -016, and -018, respectively), HIGPGQTF (94UG004), and HLGPGQAF (94UG011). Subtype D isolates show the HMGPGRAL (94UG010) and HIGPGRAY (94UG013) octamers. All these sequences are also found with a similar distribution in the samples described by Albert et al. (2), which is a further observation in favor of a very limited HIV-1 genetic variation in Uganda since 1990. In addition, of all the octamers in all of the 707 known isolates from all over

TABLE 4. Summary of the octameric regions at the apex of the V3 loop for the Gulu HIV-1 samples

Sample(s) (clade)	V3 loop octamer	Times octamer found in all 707 isolates	Times octamer found in clade[a]:						
			A	B	C	D	E	F	U
94UG009, -012, and -019 (A)	RIGPGQAF	36	**25**		8		1		2
94UG003, -016, and -018 (A)	HIGPGQAF	32	**27**	1				4	
94UG011 (A)	HLGPGQAF	28	2					**26**	
94UG013 (D)	HIGPGRAY	9				**9**			
94UG004 (A)	HIGPGQTF	5	**5**						
94UG010 (D)	HMGPGRAL	2				**2**			

[a] Boldface indicates the most common forms in each subtype. U, unclassified.

TABLE 5. Summary of the secondary (biological) characterization of the HIV-1 isolates from Gulu

Isolate	Time (wk) of first p24 positivity[a]	Amt (pg/ml) of p24 at harvest[b]	Characteristic by cell line[c]				Phenotype[d]	C2-V3 sequence[e]
			CEM	U937	Jurkat-Tat	MT-2		
94UG001	2	300	Pos	Pos	Pos	SI	R/H	−
94UG002								−
94UG003	2	20,500	Pos	Pos	Pos	SI	R/H	+
94UG004	3	1,500	Pos	Pos	Pos	SI	R/H	+
94UG005								−
94UG006								−
94UG007	2	4,600	Pos	Pos	Pos	SI	R/H	−
94UG008								−
94UG009	2	1,300	Pos	Pos	Pos	SI	R/H	+
94UG010	2	13,600	Pos	Pos	Pos	SI	R/H	+
94UG011	2	5,000	Pos	Pos	Pos	SI	R/H	+
94UG012								+
94UG013								+
94UG014								−
94UG015	2	4,400	Neg	Neg	Pos	NSI	Slow/low 2	−
94UG016	1	29,000	Pos	Pos	Pos	SI	R/H	+
94UG017								−
94UG018								+
94UG019	2	29,800	Pos	Pos	Pos	SI	R/H	+

[a] Positivity was determined by a p24 antigen enzyme immunoassay at the indicated week after initial cocultivation.
[b] The HIV-1-positive cultures were harvested and frozen in liquid nitrogen 3 days after evidence of p24 antigen positivity.
[c] Pos, positive HIV-1 replication; Neg, negative HIV-1 replication; SI, syncytium inducing; NSI, non-syncytium inducing.
[d] The phenotype classification is based on standard parameters (67). Virus isolates able to replicate in at least one cell line in addition to Jurkat-Tat and to induce syncytia in MT-2 cells were classified as R/H. 94UG015, which was able to replicate only in PBMC and Jurkat-Tat cells, was classified as slow/low 2.
[e] +, presence of sequence; −, absence of sequence.

the world, these are found mostly in clades A and D, indicating an increasingly consistent sequence-clade association. The only exception is the 94UG011 sample, whose octamer has been found to be associated mostly with clade F (Table 4) (60).

Positively charged amino acid residues are not observed in V3 loop positions 11 and 25 of the viral sequences identified in PBMC. Such a result is in apparent discord with the R/H in vitro phenotype of the viral samples (20). This observation, however, needs further investigation; in particular, it must be verified whether the in vitro isolates show the above-mentioned positively charged amino acids, are the less-represented variants in vivo, and are not identified by sequencing of the uncloned viral population. Nevertheless, the net charge of the entire V3 loop (C-C region) is between +3 and +5 in 6 of 10 samples; this parameter has previously been correlated with the ability of a virus to induce cellular syncytia in vitro (15, 67).

The results reported in this study show a limited complexity in the genotypic classification of HIV-1 isolates from a small sample from the Gulu district of Uganda, which is a remote rural region of the country. The demonstration in Uganda of the same clades (A and D) in two temporally and geographically distant situations and the existence of limited and recurring V3 loop sequences in association with a specific HIV-1 clade could be a strong signpost for the design of effective vaccine and/or passive immunization strategies. In other African countries, however, a very highly heterogeneous HIV-1 pattern, with a wide spectrum of cocirculating clades in addition to the appearance of novel clades, has been reported (17, 31, 39, 40, 58, 79).

The V3 loop has been shown to elicit both anti-HIV-1 humoral and cell-mediated immune responses; therefore, in a geographical area with a predominant HIV clade, a vaccine based on specific sets of peptides could possibly be used to cover the complete spectrum of locally present sequences and to induce significant levels of cross-reactive anti-V3 antibodies. On the other hand, the protective role of such cross-reacting antibodies, as described for HIV-1-infected Ugandan individuals (2, 41), needs to be verified.

In conclusion, the collection of all DNA sequence information in very recent studies, including the present one, should give a representative picture of the global HIV-1 clade distribution so that an effective and broadly protective anti-HIV-1 vaccine can be designed.

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