

Isolation and molecular characterization of a human T-cell lymphotropic virus type II (HTLV-II), subtype B, from a healthy Pygmy living in a remote area of Cameroon: An ancient origin for HTLV-II in Africa

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ABSTRACT We report characterization of a human T-cell lymphotropic virus type II (HTLV-II) isolated from an interleukin 2-dependent CD8 T-cell line derived from peripheral blood mononuclear cells of a healthy, HTLV-II-seropositive female Bakola Pygmy, aged 59, living in a remote equatorial forest area in south Cameroon. This HTLV-II isolate, designated PYGCAM-1, reacted in an indirect immunofluorescence assay with HTLV-II and HTLV-I polyclonal antibodies and with an HTLV-I/II gp46 monoclonal antibody but not with HTLV-I gag p19 or p24 monoclonal antibodies. The cell line produced HTLV-I/II p24 core antigen and retroviral particles. The entire *env* gene (1462 bp) and most of the long terminal repeat (715 bp) of the PYGCAM-1 provirus were amplified by the polymerase chain reaction using HTLV-II-specific primers. Comparison with the long terminal repeat and envelope sequences of prototype HTLV-II strains indicated that PYGCAM-1 belongs to the subtype B group, as it has only 0.5–2% nucleotide divergence from HTLV-II B strains. The finding of antibodies to HTLV-II in sera taken from the father of the woman in 1984 and from three unrelated members of the same population strongly suggests that PYGCAM-1 is a genuine HTLV-II that has been present in this isolated population for a long time. The low genetic divergence of this African isolate from American isolates raises questions about the genetic variability over time and the origin and dissemination of HTLV-II, hitherto considered to be predominantly a New World virus.

The origin and the routes of the worldwide dissemination of primate T-cell lymphotropic viruses (PTLVs), which include human and simian T retroviruses [HTLV-I (1), HTLV-II (2), STLV-I (3), and STLV-II (4)] and the recently isolated PTLV-L (5), PTLV-PP1664 (6), and STLVpp (7), are under active investigation. Epidemiological and phylogenetic analyses indicate that HTLV-I and STLV-I have been present in the Old World (Africa and Asia) for several millennia (8–11), whereas HTLV-II, which is highly endemic in certain native Amerindian tribes (12–17), has been considered to be a New World virus, brought from Asia into the Americas some 10,000–40,000 years ago during the migration of HTLV-II-infected populations that occurred over the Bering land bridge (12–17).

Recent serological and molecular evidence of sporadic cases of HTLV-II infection in western (18, 19) and central Africa (20–26), however, raises the possibility that HTLV-II or a related retrovirus has also been present in Africa for a long time. This hypothesis was first suggested after the detection of

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HTLV-II-like serological markers (not verified by sequencing) in Pygmies in remote areas of Zaire (22, 24) and Cameroon (25), who are considered to belong to the oldest ethnic groups in central Africa, having lived in those areas for at least 20,000 years (27, 28).

We report here the isolation, molecular characterization, and phylogenetic analysis of an HTLV-II from a healthy, 59-year-old Pygmy woman of the Bakola population, an ethnic group living in a remote area of the equatorial forest of south Cameroon in which HTLV-II-like serological markers were found 10 years ago in four individuals (25).**

MATERIALS AND METHODS

Population Studied. The Bakola Pygmies are an ethnic group of about 400 individuals, living in the area of Akok (Campo district) (2°30' N, 10° E), near Kribi in the Ocean Department of southern Cameroon. This group, traditionally hunter-gatherers, is scattered throughout the forest; they now practice rudimentary agriculture but also exchange game for staple foods and manufactured goods with neighboring farmers (27, 28). In February 1994, 41 Bakola Pygmies (19 men and 22 women; mean age, 39 years) were visited, given a medical examination, and then bled (5 ml) after informed consent had been obtained. They were either relatives of the four individuals found to have HTLV-II-like serological markers in 1984, three of whom are now dead and the fourth untraceable, or people living in the same area.

Serological Tests. The sera were screened for HTLV-I/II by an enzyme-linked immunosorbent assay (ELISA; Platelia HTLV-I new; Sanofi, Paris) and an immunofluorescence assay involving HTLV-I (MT2) or HTLV-II (C19) producing cell lines. All positive or borderline samples were tested in a Western blot assay (HTLV 2.3 Diagnostic Biotechnology, Singapore) (29).

Cell Culture and Virus Isolation. In March 1994, heparinized blood specimens were collected from the individuals identified as HTLV-I/II-seropositive in the pilot survey and rushed to the Centre Pasteur in Yaoundé, where peripheral blood mononuclear cells (PBMCs) were separated and stored in liquid nitrogen. The frozen cells were transported 2 weeks later to Paris and thawed, and 10 million cells were placed in a 50-ml culture flask containing 5 ml of RPMI 1640 medium

Abbreviations: PTLV, primate T-cell lymphotropic virus; HTLV, human T-cell lymphotropic virus; STLV, simian T-cell lymphotropic virus; PBMC, peripheral blood mononuclear cell; LTR, long terminal repeat.

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**The sequences reported in this paper have been deposited in the GenBank data base [accession nos. Z46883 (LTR) and Z46889 (*env* gene)].

Table 1. Oligonucleotide primers for DNA amplification

| Target sequence | Expected size, bp | Forward primer (5' → 3') | Reverse primer (5' → 3') |
|-----------------|-------------------|--|--------------------------------------|
| LTR | 784 | LTRII TTTGAGCGGCCGCGCCAGGGCGAGTCATCGACCC | 5PLTRE ACTTAGAATTCTCCCGGACGAGCCCCCAA |
| Pol-env-Px | 1606 | AGP1 TTTGAGCGCGCGTCTCTGCCAGTGGATCCCCTGG | WH2 ACTTAGAATTCTCGAGAAGCTAGCAGGTCTA |
| Pol-env-Px | 1575 | AGP2 TTTGAGCGGCCGAGCGAGGTGCATGCCAAGACC | WH2 ACTTAGAATTCTCGAGAAGCTAGCAGGTCTA |
| Env-Px | 1013 | WH1 TTTGAGCGGCCCTGCAACAACCTCCATTATCCT | WH3 ACTTAGAATTCGAGGAGCAGCGGGAGATCTTA |
| Env-Px | 595 | ET403 TTTGAGCGGCCGCGGCTGGGGACTAACTGGGATCTTGG | WH3 ACTTAGAATTCGAGGAGCAGCGGGAGATCTTA |

Primers internal to the amplified *env* and long terminal repeat (LTR) products were used as oligonucleotide probes. The *env* probe was HTLV-II PR (5'-GTCATATTGTTGGCCCCCTGTATCTCCGC-3') and the LTR probe was LTRII (5'-AAACAAGGCTCTGACGATTACCCCCCT-3').

with 20% heat-inactivated fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin. During the subsequent 3 days, the cells were stimulated with phytohemagglutinin at 2 mg per 10^6 cells and then cultivated in a humidified 5% CO₂ atmosphere in the same medium as described above but with the addition of 10% interleukin 2. Cells were also cocultivated with BJAB cells after 2 weeks of culture alone.

Indirect Immunofluorescence, Immunophenotyping, and Antigen Detection. Indirect immunofluorescence was performed on cultured cells; either mouse monoclonal antibodies directed against HTLV-I p19 or p24 (Cambridge Biotech) or HTLV-I/II gp46 7G5D8 (30) or human polyclonal serum from HTLV-I- or HTLV-II-infected individuals was used. Cytofluorographic analysis of the cultured cells was performed as described (31). Production of the p24 core antigen in the culture supernatant was detected in a capture ELISA that recognizes both HTLV-I and HTLV-II (Coulter).

Polymerase Chain Reaction (PCR). The PCR was carried out in a DNA thermocycler (Cetus) using high molecular weight DNA extracted from PBMCs, before and after 1 month of culture. Each initial reaction mixture contained 1.5 μ g of DNA, 0.2 mmol of dNTP mix per liter, 10 μ l of a 10 \times reaction buffer, 0.2 mmol of each oligonucleotide primer per liter, and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer/Cetus) in a total volume of 100 μ l. After denaturation at 94°C for 5 min, the reaction mixtures containing DNA were cycled 35 times for 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C. An extension of 2 min per cycle was included. A seminested PCR was performed on DNA from uncultured PBMC DNA, as described above. For the first PCR run, we used the outer primer

sets AGP1 and WH2 (Table 1). Then, 2 μ l was transferred to 100 μ l of the second PCR run, which included the inner primer sets AGP2 and WH2 (Table 1), which amplified a fragment of 1509 bp [nt 5133–6641 of the MO prototype (32)], including 55 bases of the *pol* gene and the entire *env* gene (1462 bp). We also amplified 522 bp of the *env*/PX region (nt 6469–6991) using as the outer primer set WH1 and WH3 and as the inner set ETH403 and WH3 (Table 1). From the DNA of cultured cells (1 month) a 715-bp fragment of the LTR (nt 52–766) was also amplified using the primer set LTRII and 5PLTRE (Table 1).

Cloning, Sequencing, and Phylogenetic Analysis. The DNAs amplified by the primer sets AGP1 and WH2, ET403 and WH2, and LTRII and 5PLTR (which contains *Not* I and *Eco*RI restriction sites) were digested with *Not* I and *Eco*RI restriction enzymes and cloned into a Bluescript vector and their nucleotide sequences were determined by the dideoxynucleotide chain-termination method. Multiple alignment of the sequences was performed using the clustalV program (33) and analyzed with programs of the PHYLIP package version 3.52c (Josef Felsenstein, University of Washington, joegenetics.washington.edu) (34).

RESULTS

Eight of the 41 sera from the Bakola Pygmies had antibodies to HTLV-I/II in the ELISA; two had optical densities of >1 and <2 and six had optical densities of >3. In the confirmatory Western blot assay (Fig. 1), one had a typical HTLV-I pattern, with reactivity against p19, p24, rgp21, and MTA-1, and one had typical HTLV-II seroreactivity, against p24 >> p19, rgp21,

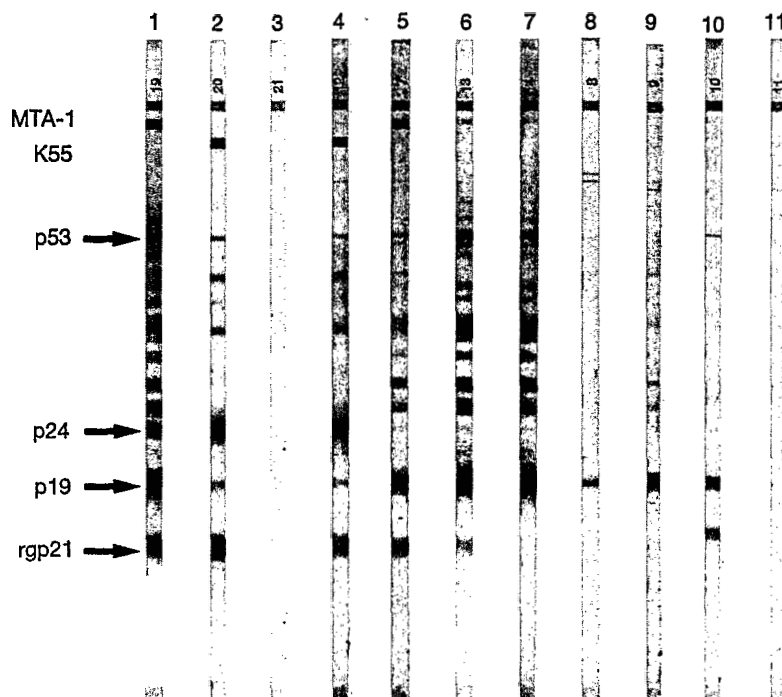


FIG. 1. Western blot patterns of the sera of Bakola Pygmies from southwestern Cameroon, using the Western blot HTLV-I/II 2.3 from Diagnostic Biotechnology. Lanes: 1, HTLV-I-positive control; 2, HTLV-II-positive control; 3, HTLV-I/II-negative control; 4, serum of the index case: HTLV-II-infected Pygmy woman, aged 59 years; 5, serum of the HTLV-I-infected individual; 6–10, sera of five of the six Pygmies showing indeterminate Western blot pattern; 11, serum of an HTLV-I/II-negative Pygmy.

and K55. The latter pattern was indistinguishable from that of the HTLV-II-positive controls, which included Tobas Amerindians (35). Six further subjects, who had mainly *gag*-encoded proteins, were considered to be seroindeterminate (Fig. 1). The serum found to be HTLV-II-positive in the Western blot assay was that of a woman estimated to be 59 years old, who had never been transfused and had had no intimate contact with foreigners from Europe or the Americas. Higher HTLV antibody titers were found on C19 (1/1280) than on MT2 (1/320) cells, confirming the presence of an HTLV-II infection.

After PBMCs from this woman were cultured for 2 weeks, a few reacted with both HTLV-I and HTLV-II polyclonal antisera and the patient's serum. The number of fluorescent cells increased progressively, reaching 10–15% after 40 days of culture and 30–40% after 5 months. Twenty-five to 30% of the cells were also faintly stained, after 5 months of culture, with the 7G5D8 monoclonal antibody to HTLV-I/II gp46, but none was stained by the HTLV-I-specific p19 and p24 antibodies.

When PBMCs were cocultivated with BJAB cells after 14 days in culture, syncytia and giant cells (36, 37) were seen after 3 days; their numbers increased rapidly, leading to cell death. The syncytia were stained in the indirect immunofluorescence assay by both HTLV-I and HTLV-II polyclonal antisera. A high level of free p24 antigen was detectable in the supernatant of cell cultures after 2 weeks and remained so in further culture. After 5 months in culture, a few retroviral type C-like particles were detected in PBMCs under the electron microscope. The

HTLV-II-infected cells were of T-cell lineage (95% CD2, 94% CD3, 26% CD4, 81% CD5, 89% CD7, 87% CD8, 1% CD19, and 1% CD20) with activation markers (94% HLA DR and 70% CD25).

A fragment consisting of 1509 bp (encompassing all of the *env* gene) was amplified, cloned, and sequenced from the DNA of uncultured PBMCs by seminested PCR with the AGP2 and WH2 inner primer set. Comparison of this sequence with HTLV-II prototype strains demonstrated a very close homology with the subtype B group. Thus, in the total *env* gene (1462 bp), HTLV-II PYGCAM-1 exhibited only 0.6% (9 substitutions) nucleotide divergence from G12 (15) and 0.75% (11 substitutions) divergence from NRA (38) but exhibited 4% divergence from the HTLV-II MO subtype A isolate (Fig. 2). Analysis of the 715-bp fragment of the LTR confirmed that PYGCAM-1 belongs to subtype B, with a divergence of nine bases (1.3%) from the G12 prototype: two C deletions at position 145–146 (as compared with the MO isolate), five substitutions (T → C at 293, G → C at 312, G → T at 313, A → G at 340, and C → T at 405), and two insertions (one A at 217 and one G at 667); and a divergence of only four bases (0.6%) from the NRA prototype: one deletion of a C at position 145, one insertion of an A at 217 and two substitutions, a G → A at 343 and an A → G at 651.

A dendrogram comparing the nucleotide sequences of 589 bp of the *env* gene (bp 6052–6640) showed that HTLV-II PYGCAM-1 was in the subtype B cluster (Fig. 3A), being closely related to G12 isolate. Phylogenetic trees were con-

| | |
|--------|---|
| | 6031 |
| MO | <u>CTGCAACAACCTCCATTATCCT</u> CCCCCTTTTTCCTCGCTCCCGTACCTCCTCCGGGACAAGACGCCCGTGCCTTC |
| NRA |C..... |
| G12 |C..... |
| PygCam |C..... |
| | 6112 |
| MO | AATAGCAGTGTGGCTTGTCTCCGCCCTAGCGGCCGAACAGGATCGCTGGTGGAGTAACAGGCTCCCTATCTCTGGCTTC |
| NRA |T.....G.....C.....A..... |
| G12 |T.....G.....C.....A..... |
| PygCam |T.....G.....C.....A..... |
| | 6193 |
| MO | CAGTAAAAGCCTTCTCCTCGAGGTGACAAAGACATCTCCACCTTACCAGGCCATAGTCAAAAATCATCAAAACATCCT |
| NRA |T.....T..... |
| G12 |T.....T..... |
| PygCam |T.....T..... |
| | 6274 |
| MO | CCGGGTGACACAGTATGCAGCCAAAATAGACGAGGATTAGACCTCCTATTCTGGGAACAAGGGGTTTGTGCAAGGCCAT |
| NRA |A.....G.....A..... |
| G12 |A.....G.....A..... |
| PygCam |A.....G.....C.....A..... |
| | 6355 |
| MO | ACAGGAGCAATGTTGCTTCTCAACATCAGTAACACTCATGTATCCGCTCCTCCAGGAACGGCCCCCTCTGAAAAACGTGT |
| NRA |T.....A.....G..... |
| G12 |T..... |
| PygCam |T.....A..... |
| | 6436 |
| MO | CATCACCGGCTGGGACTAAACTGGGATCTTGGACTGTCCCAATGGGCACGAGAAGCCCTCCAGACAGGCATAACCATTCT |
| NRA |T.....T.....G..... |
| G12 |T.....T.....A.....G..... |
| PygCam |T.....T.....A.....G..... |
| | 6507 |
| MO | CGCTCTACTCCTCCTCGTCATATTTGTTGGCCCTGTATCCTCCGCCAAATCCAGGCCCTCCACAGCGGTTACAAAACCG |
| NRA | .A.C.....T.....C.....A.....G..... |
| G12 | ...C.....T.....C.....A.....G..... |
| PygCam | ...C.....T.....C.....A.....G..... |
| | 6598 |
| MO | ACATAACCAGFATCCCTTATCAACCCAGAAACCATGCTATAATAGACTGCTAGCTTCTGCAG |
| NRA |G.....G.....A.....G..... |
| G12 |G.....G..... |
| PygCam |G.....G.....G.....C..... |

FIG. 2. Nucleotide sequence of the gene region encoding the gp21 of the PYGCAM-1 isolate. Primers employed in PCR amplification are underlined. The sequences selected as prototypes are MO (HTLV-II A) (32) and G12 and NRA (HTLV-II B) (15, 38).

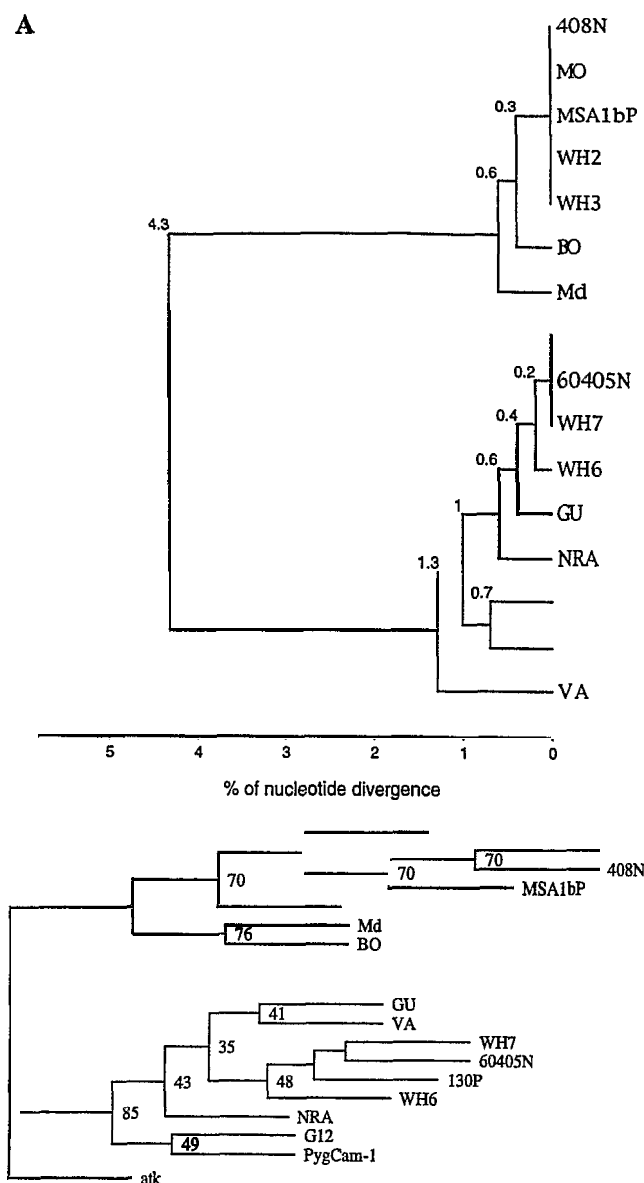


FIG. 3. (A) Dendrogram comparing the nucleotide sequences of 589 bp of the *env* region (bp 6052–6640) coding for the gp21. Two phylogenetic trees were obtained, one by the neighbor-joining method (34) (B) and one by maximum parsimony (data not shown). The dendrogram and the phylogenetic trees correspond to 16 different HTLV-II isolates including the PYGCAM-1 isolate generated in this study and 15 other available published sequences comprising HTLV-II subtype A (MO) (32), subtype B [NRA (38), G12 (15)], and HTLV-II isolates representative of the different geographical origins: Amerindians (408N, 60405N, 130P, MSA1bp) (17), i.v. drug abusers from the USA (WH2, WH3, WH6, WH7) (36) or from Italy (GU, VA, BO, Md) (kindly communicated by U. Bertazonni). The HTLV-I ATK isolate was used as outgroup to root the phylogenetic tree.

structed by two different methods on the same *env* region coding for the gp21 (Fig. 3B) and on the LTR (data not shown). In both cases, the topology of the two trees was very similar, demonstrating the existence of two molecular clusters of HTLV-II genotypes (with high bootstrap values, 85–100%) with the PYGCAM-1 always included within the subtype B cluster.

DISCUSSION

Culture of PBMCs from a healthy 59-year-old Bakola Pygmy woman with HTLV-II serological markers resulted in the

establishment of a continuous CD8⁺ cell line containing an HTLV-II provirus that produces HTLV-II antigens and retroviral particles. Molecular analysis of this HTLV-II, PYGCAM-1, demonstrated close homology with the HTLV-II subtype B prototypes (G12 and NRA) originally isolated from a Guaymi Amerindian in Panama (15) and from a United States patient with T hairy cell leukemia (38), respectively. We propose that this isolate, a genuine African HTLV-II, has been present in this remote population for a very long time, for the reasons outlined below.

(i) Serum from the father of this woman as well as from three unrelated Bakola Pygmies living in the same area had shown similar seroreactivity (p24 >> P19, rgp21, and K55), indicating the presence of HTLV-II, 10 years previously (25). Isolation of an HTLV-II subtype B from the woman suggests that the four individuals were infected by the same virus. Furthermore, the presence of HTLV-II antibodies in the father of the index case supports the hypothesis that HTLV-II has been endemic in this population for at least two generations. Close contacts between the Bakola Pygmy group and neighboring populations have been rare until recently. (ii) A retrospective seroepidemiological survey of sera collected between 1967 and 1970 from 214 Cameroonian Pygmies (24) showed HTLV-II-like seroreactivity in five. HTLV-II appears to be virtually absent in other ethnic groups in Cameroon (unpublished data). (iii) While most of the Pygmy groups tested were seronegative for HTLV-II antibodies, the only other African population described up to now in which HTLV-II is endemic is the Bambuti Pygmies in the Ituri region of northeast Zaire, where 14/102 individuals in 1970 (24) and 4/12 in 1991 (22) were found to be HTLV-II-like seropositive. We failed to detect any HTLV-II-infected individuals in representative samples of Biaka Pygmies from the Lobaye region of the Central African Republic and of Twa Pygmies from the Lake Tomba area of Zaire (39). The Bambuti and Bakola Pygmies, both of which are infected with HTLV-II, are located at the western and eastern ends, respectively, of the present area of Pygmy habitation in central Africa and are of different paleoanthropological origins, with a divergence, based on linguistic and cultural features, of 10,000 years (27, 28). Taken together, these results strongly suggest that some isolated Pygmy populations have for a very long time been an African reservoir for HTLV-II infection. Since any close contact between these central African Pygmies and Amerindian populations is very unlikely to have occurred in the past, African HTLV-II may have diverged from a common ancestor long before HTLV-II was introduced into the Americas by perhaps two waves of migration over the Bering land bridge, each carrying one of the two molecular subtypes of HTLV-II (24) proposed by Hall *et al.* (16, 36, 37). Subtype A (prototype MO) is present mainly in US i.v. drug abusers (14, 36, 37) but also in the Pueblo Indians (17), whereas subtype B strains are found mainly in certain Amerindian groups scattered throughout North, Central, and South America, (refs. 12, 14, 16, 40, and 41; M.B. and A.G., unpublished data). Subtype B is thus referred to as the Paleo-Indian strain. The nucleotide divergence between the A and B subtypes is about 4.8% (15, 16, 37, 38).

We were surprised to observe a genuine African HTLV-II so close genetically to the Paleo-Indian subtype B; however, genetic variability between strains of the same HTLV-II subtype seems to be extremely low (16, 41). Thus, a nucleotide divergence of only 0–0.4% (over 1000 bp of the *env* gene) was observed between HTLV-II of different Amerindian groups who have probably had no contact for several thousand years (14–17, 41). The possibility that HTLV-II subtype B is a genuine African HTLV-II is reinforced by our recent finding of a subtype B variant in a Gabonese family (ref. 42; A.G., unpublished data). An HTLV-II B provirus has also been detected in two individuals in another area of Gabon and in Zaire (26). Only two other subtype A African isolates have

been described to date, one in Ghana (19) and the other in Cameroon (unpublished data), both in prostitutes, suggesting an imported infection.

Preliminary data from Mongolia (43) showing the presence of a typical HTLV-II subtype A (16, 32, 36, 37) strongly support the hypothesis that genetic drift is much lower for HTLV-II than for HTLV-I, for which, assuming a similar evolutionary rate for HTLV-I/II in the different geographical locations, an estimated 0.5–1% nucleotide divergence (in the *env* and *pol* genes) during 1000 years of evolution has been proposed (8, 11, 44, 45).

In the context of recent evidence for interspecies transmission in central and west Africa of PTLV-1 (HTLV-I/STLV-I) strains (46), we suggest that some STLV-II closely related to HTLV-II subtype B might exist or have existed in Africa. The recent finding of new PTLVs in several pygmy chimpanzees of Zairian origin [PTLV-PP1664 (6) and STLV-PP (7)] and in wild-caught baboons in Eritrea, Ethiopia (PTLV-L) (5), supports the hypothesis of an African origin for some PTLVs (10).

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