

Molecular Characterization and Phylogenetic Analyses of a New, Highly Divergent Simian T-Cell Lymphotropic Virus Type 1 (STLV-1*marc1*) in *Macaca arctoides*

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A serological survey of a captive colony of Asian monkeys indicated that six *Macaca arctoides* had antibodies to human T-cell leukemia/lymphotropic viruses (HTLV). Over a 4-year interval, sera from these animals continued to exhibit a peculiar Western blot (WB) pattern resembling an HTLV-2 pattern (p24^{gag} reactivity of equal or greater intensity than that of p19^{gag} and a strong reactivity to recombinant gp21) but also exhibiting, in five of six cases, a reactivity against MTA-1, an HTLV-1 gp46 peptide. PCR experiments on DNA extracted from peripheral blood mononuclear cells using HTLV-1- or HTLV-2-specific long terminal repeat, *gag*, *pol*, *env*, and *tax* primers yielded negative results. However, highly conserved primers successfully amplified three different gene segments of *env*, *tax*, and *env-tax*. The results of comparative sequence analysis demonstrated that STLV-1*marc1* was not closely related to any known STLV-1 strain, was the most divergent strain of the HTLV-1–STLV-1 group, and lacked the ATG initiation codons corresponding to the p12 and p13 proteins of HTLV-1. Phylogenetic analyses incorporating representative strains of all known HTLV-STLV clades consistently depicted STLV-1*marc1* within the HTLV-1–STLV-1 type 1 lineage, but it probably diverged early, since its position is clearly different from all known viral strains of this group and it had a bootstrap resampling value of 100%. Genetic distance estimates between STLV-1*marc1* and all other type 1 viruses were of the same order of magnitude as those between STLV-2*PanP* and all other type 2 viruses. In light of the recent demonstration of interspecies transmission of some STLV-1 strains, our results suggest the existence in Asia of HTLV-1 strains related to this new divergent STLV-1*marc1* strain, which may be derived from a common ancestor early in the evolution of the type 1 viruses and could be therefore considered a prototype of a new HTLV-STLV clade.

The origin and mode of dissemination of both human and simian T-cell leukemia/lymphotropic viruses (HTLV and STLV, respectively) are topics under active investigation. Analyses of type 1 viral strains from throughout the world yield a general pattern of dissemination that include the following: transmission of STLV type 1 (STLV-1) to humans, as exemplified by the high homology between viral strains from African chimpanzees with those from human patients in Zaire (21, 32); the likely transmission of STLV-1 between simian species (21, 26, 27, 30); persistence of the virus over long periods of time in remote areas, as seen, for example, for HTLV type 1 (HTLV-1) in Melanesia (1, 6, 29); and global distribution of the virus via large-scale human migration of populations infected by HTLV-1, e.g., the slave trade from Africa to the New World (8). In contrast, the high levels of HTLV-2 in several Amerindian groups (14), combined with the absence of STLV-2 in New World monkeys, support the hypothesis that the virus entered the Americas 10,000 to 40,000 years ago with human migrations from Asia across the Bering Strait (14, 15). Inherent to this hypothesis is the expected occurrence of HTLV-2 or STLV-2 in Asian primates. While sporadic cases of HTLV-2 infection may have occurred in some inhabitants of Mongolia (15), STLV-2 has yet to be isolated from any Asian

simian species. Finally, the recent discovery that some African pygmy tribes have high levels of HTLV-2 (7, 11, 12), and the isolation of a type 2-like virus from bonobo chimpanzees (*Pan paniscus*) from neighboring regions (10, 22, 31) complicate the current global overview of both the origin and geographical distribution of this human retroviral infection.

As part of an ongoing study on the phylogeny of HTLV and STLV, we conducted a serological analysis on Asian monkeys housed at the Strasbourg Primatology Center (N. Herrenschmidt and E. André). Among the 130 serum samples tested for the presence of STLV-1 or -2 infection, 14 of the 25 samples positively detected by an HTLV-1 or -2 enzyme-linked immunosorbent assay (Diagnostic Biotechnology, Singapore, Singapore) and a particle agglutination test (HTLV ATLA, Fujirebio, Tokyo, Japan) exhibited strong Western blot (WB) reactivities (HTLV2-3; Diagnostic Biotechnology). Of these, eight *Macaca mulatta* had a typical HTLV-1 WB profile with a reactivity against p19 stronger than that against p24 and a reactivity against both a recombinant gp21 (rgp21) and the specific HTLV-1 gp46 peptide MTA-1 (data not shown). However, six *Macaca arctoides*, all older than 10 years and originating from Southeast Asia, exhibited a peculiar WB profile with an HTLV-2-like reactivity (p24^{gag} reactivity of equal or greater intensity than that of p19^{gag} and a strong reactivity against rgp21) but also exhibiting, in five of six cases, a reactivity against MTA-1, an HTLV-1 gp46 peptide (Fig. 1). Such reactivities resemble those present in the HTLV-2-like virus isolated from *P. paniscus* (2, 10, 22, 31). These six HTLV- or STLV-seropositive *M. arctoides*, unrelated males and females

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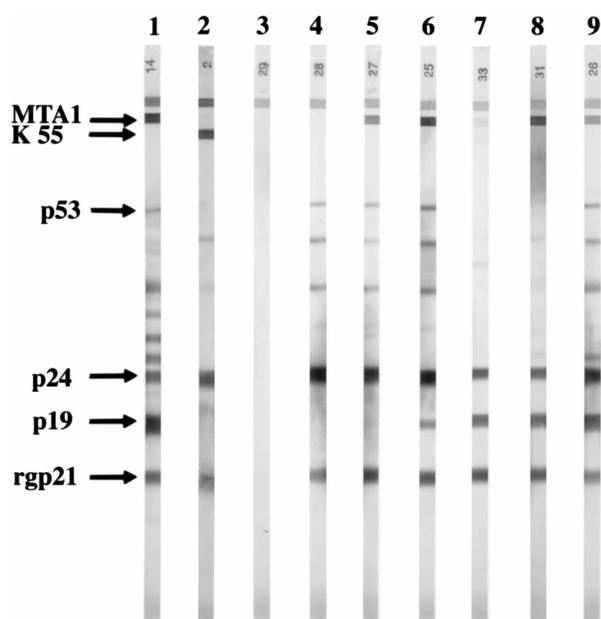


FIG. 1. WB analysis. The WB used was from Diagnostic Biotechnology (HTLV2-3). Lane 1, HTLV-1-positive control; lane 2, HTLV-2-positive control; lane 3, HTLV-1- and HTLV-2-negative control; lane 4, 1 plasma sample from *Macaca arctoides*, with an HTLV-2-like seroreactivity (strong reactivity against p24 and rgp21 and lack of reactivity against p19) but without K55. Lanes 5 to 9, HTLV-2-like seroreactivity (reactivity of p24 \geq to that against p19 and rgp21), but with MTA-1. MTA-1 is a peptide that is a fragment of the gp46 of HTLV-1 (amino acids 162 to 209), while K55 is a peptide that is a fragment of the gp46 of HTLV-2 (amino acids 162 to 205).

with no offspring and previously housed at the Sukhumi Primate Center (in the former USSR) 15 years ago, were very probably never intravenously or orally injected with human or simian materials. Further, this particular HTLV or STLV WB profile remained unchanged for a 4-year interval and was marked by constant low antibody titers detected by serial dilution of plasma or sera in an immunofluorescence assay using MT2 HTLV-1-producing cells (titers ranging from 1/80 to 1/160) and C19 HTLV-2-producing cells (titers ranging from 1/40 to 1/160).

To genetically characterize the STLV present in these *Macaca arctoides*, PCR was performed on DNA extracted from uncultured peripheral blood mononuclear cells (PBMCs) of three of these animals using several specific HTLV-1 or HTLV-2 primers located within the long terminal repeat, *gag*, *pol*, and *env* regions. Each PCR mixture contained 1.5 μ g of PBMC DNA, 0.2 mM deoxynucleoside triphosphates (Boehringer, Mannheim, Germany), 10 μ l of a 10 \times reaction buffer (Perkin-Elmer Cetus, Norwalk, Conn.), 2.5 mM MgCl₂, 10 μ M (each) oligonucleotide primer, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) in a total volume of 100 μ l. For all PCR experiments, amplification mixes were made in a special room physically separated from the laboratory. Following a denaturation step of 5 min at 94°C, the reaction mixtures containing DNA were subjected to 40 cycles of PCR, with 1 cycle consisting of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. There was an extension step of 2 s per cycle, with an extension step of 10 min on the last cycle (23). Despite using *env1* and *env2* primers known to amplify STLV-1 from 12 different species of nonhuman primates (21) as well as strains from four recognized HTLV-1 clades, no signal was detected with any of these primers, either by UV visualization or hy-

bridization with specific [γ -³²P]dATP-radiolabelled viral probes. The failure to amplify proviral DNA by PCR using such conventional HTLV-1 or -2 primers indicated a high divergence in the proviral genome of such STLV compared to prototypical STLV or HTLV. In contrast, using highly conserved primers from the *tax* region (KKpX1, [5'CCCACTTC CCAGGGTTTGGACAGAG, sense] and KKpX2 [5'CTGTA GAGCTGAGCCGATAACGCG3', antisense]), a clear PCR-positive signal of 203 bp was obtained from DNA purified from the PBMCs of three *Macaca arctoides* (monkeys 16, B43, and 757). A second, overlapping fragment of the *tax* region (406 bp) was obtained by PCR using primer set Mac2 (positions 7453 to 7483, 5'ACCAGGTCACCTGGGACCCCATCGATG GACG3', sense) and Mac4 (positions 7761 to 7786, 5'ATGG GGGGGAAAGCTGGTAGAGTA3', antisense), 1.25 mM MgCl₂, and 60°C annealing temperature. The PCR products were purified on agarose (GeneClean; Bio 101, La Jolla, Calif.) and cloned (PCR script SK+; Stratagene, La Jolla, Calif.). Twenty-five randomly chosen colonies were analyzed for the presence of the recombinant insert by digestion with *EcoRI* and *NotI* restriction enzymes which are absent in this part of HTLV and STLV genomes (25, 28, 33). Positive clones were purified (Midiprep Qiagen) and sequenced (Sequenase version 2.0; U.S. Biochemical Corp.). Comparative sequence analysis of this very conserved *tax* region demonstrated that the new STLV-1*marc1* (present in *M. arctoides* 16) was different from any known STLV or HTLV, was more closely related to HTLV-1 (13.4% nucleotide divergence to ATK prototype) than to HTLV-2 (21% nucleotide divergence to MO prototype) and was the most divergent strain of the HTLV-1-STLV-1 group (Fig. 2). The *tax* fragment of 203 bp was also sequenced for two other *M. arctoides* (B43 and 757). Analysis of these sequences showed a great nucleotide similarity with the sequence of monkey 16 (97.5 and 100%, respectively), confirming that these three monkeys were infected with closely related viruses.

To compare the STLV-1*marc1* strain with other known HTLV and STLV strains (most of which are sequences for *env*), we designed highly conserved primers for the gp21 region: Mac5 (positions 6127 to 6148, 5'TGGCAGTCTGGCTT GTCTCCGC3', sense) and Mac3 (positions 6455 to 6477, 5'AGATCCCAATTTAGGCCCCAGCC3', antisense). PCR experiments were conducted using the same conditions as described above but with a 51°C annealing temperature and a hot start. To obtain adequate amounts of product, three PCR products of the same DNA (*M. arctoides* 16) were pooled, coprecipitated, and cloned as described above. Comparative sequence analysis confirmed that the new STLV-1*marc1* was different from the STLVs and HTLVs already known and revealed a greater divergence of this novel strain in the *env* gene (with a 19.6% nucleotide divergence with ATK) than in the *tax* gene (13.4%). In this gp21^{env} fragment, STLV-1*marc1* diverged from the HTLV-II MO prototype by only 24.1%. Note also that 40% (25 of 59) of the *env* nucleotide mutations observed within the STLV-1*marc1* isolate were shared with those found in the HTLV-2 genome in the same region (data not shown). Taken together, these data confirm the greatest degree of similarity observed in the N-terminus end of the HTLV or STLV *tax* protein which contains some of the active sites for function. By contrast, the *env* variability appears higher, and upon translation into amino acids, the gp21^{env} sequence of STLV-1*marc1* exhibited specific mutations (positions 331 and 333) (17, 21) to Asian HTLV-1/STLV-1.

Subsequently, a third 952-bp fragment (positions 6424 to 7354 of ATK) encompassing open reading frames I and II of the pX region and comprising the recently described p12 and p13 protein initiation codons in HTLV-1 strains (4, 20) was

A

	Asian STLV-I and HTLV-I type C								HTLV-I A		PTLV-L	
	PSHU-1	MMU-173-78	MEL5	MFA-C194	MSHR-1	MTO-TE4	MMU-39-83	MNE-PTM3	ATK	HS35	MO	PH-969
STLV _{marc1}	82,68	81,37	80,06	80,72	79,74	80,06	81,04	80,06	80,39	81,04	75,82	71,8
PSHU-1		98,04	88,24	89,22	88,89	87,60	98,04	87,25	87,25	87,90	72,22	70,16
PHA-969		69,18	70,82	72,46	70,82	71,80	68,52	71,80	69,83	69,83	78,03	

B

	Asian STLV-I and HTLV-I type C					HTLV-I A and B				HTLV-II A			PTLV-L
	STLV _{marc1}	MNE-PTM3	MTO-TE4	MEL5	PANP	EL	ATK	HS35	TSP1	NRA	G12	MO	
PHA969	78,5	78	77,6	80,1	76,7	79	78,75	78,5	79	79,2	79,3	78,75	
STLV _{marc1}		85,7	85,2	85	77,8	86,6	86,6	86,4	86,9	79,7	79,7	79	
MNE-PTM3			90,8	92,2	76,9	92,6	92,4	92,2	92,2	79	79,2	78,75	
MTO-TE4				93	77,6	93,5	93,8	93,5	93,5	79,4	79,6	79	
PANP				78		77,2	77,4	77,6	77,4	87,5	87,8	86,9	

FIG. 2. (A) DNA nucleotide sequence similarity in the 306-bp *env* region (bases 6149 to 6454 of ATK [28]). (B) DNA nucleotide sequence similarity in the 406-bp *tax* region (bases 7355 to 7760 of ATK [28]). HTLV-1 type A, B, and C correspond respectively to the cosmopolitan, Central African, and Melanesian subtypes (23). All values shown are percentages. PTLV-L, primate T-lymphotropic virus type L.

amplified by nested PCR with primer sets and probes specific for the new STLV-1*marc1* sequence (data not shown). The resultant proviral sequence of the pX homologous region of the HTLV-1 open reading frames I and II contained neither the p12 nor the p13 initiation codons, as previously described for the Asian STLV-1 TE4 and Melanesian HTLV-1 mel5 strains (6, 17). Since p12 exhibited functional homologies with oncoproteins and interacts with both beta and gamma chains of the interleukin 2 receptor, p12 could be involved in the transformation pathway (4). Therefore, it is possible that the absence of p12 ATG codon in some HTLV-1 and STLV-1 strains may indicate a less-pathogenic virus.

Data used for phylogenetic analyses consisted of STLV-1*marc1* combined with representative sequences of all known clades of STLV and HTLV types 1 and 2 available in GenBank. The sequence of STLV-2*PanP* was obtained with permission from G. Franchini (2). Two data sets were compiled: 28 sequences of gp21^{env} gene (306 bp) and 16 sequences of the region spanning the interval from *env* (position 6149 in ATK) through the *tax* gene (position 7760) of 1,612 bp. In preparation for phylogenetic analyses, sequences were aligned by the method of Needleman and Wunch (24) using the GCG program Pileup (5), and the empirical transition/transversion ratio for each gene segment was estimated with CMATRIX3 (A. Burke, unpublished program). Phylogenetic analyses consisted of three major algorithms each with different optimality criteria: minimum evolution estimated by neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). Concordance among the resultant topologies was viewed as a strong indicator of the true phylogeny. Genetic distances were estimated among all pairs of sequences to be used in the NJ analyses for each gene. Computer programs used for phylogenetic analyses were PHYLIP version 3.5 (3) for the NJ and ML analyses. MP analyses were performed by using PAUP*

(PAUP test version used with permission from David Swoford).

Separate analyses of *env* and *env-tax* gave similar phylogenetic patterns despite differences in viral strains within each data set (Fig. 3). Phylogenetic reconstruction indicated strong divergence among the three major lineages of HTLV and STLV: type 1 viruses, type 2 viruses and a STLV strain isolated from a baboon from Eritrea (PHA-969) (13). As previously described (17, 21, 26, 27, 30, 32), phylogenetic associations among viral strains within each lineage did not correspond to host species phylogeny. For example, HTLV-1 strains grouped with nonhuman primate strains in phylogenetic trees derived using the *env* gene (Fig. 3A).

Results of phylogenetic analyses of the two gene segments consistently depict STLV-1*marc1* probably as an early divergence within the HTLV-1 lineage. Analysis of 28 *env* sequences place STLV-1*marc1* within viral type 1 strains (Fig. 3A), with a very high bootstrap resampling value of 100% and a significant result with ML ($P < 0.01$) (data not shown). A nearly identical tree was obtained by MP analysis that varies only in the relative placement of EL and also had a 100% bootstrap value for inclusion of STLV-1*marc1* within the type 1 lineage. The average estimate of genetic distance between STLV-1*marc1* and other type 1 viruses of 23.72% is nearly twice that estimated for all other pairs of type 1 viruses and reflects the deep divergence of this new viral STLV strain within this lineage (Fig. 3). Similarly, analysis of the *env-tax* region conclusively identifies STLV-1*marc1* as a type 1 virus (Fig. 3B), yet it is distinctly separate from the remaining strains within the lineage with a bootstrap value of 100% (NJ) and ML significance of $P < 0.01$ (data not shown).

Comparison of all phylogenetic trees from both gene segments are consistent with respect to other viral strain associations as well (Fig. 3). Common to both gene analyses are viral

A

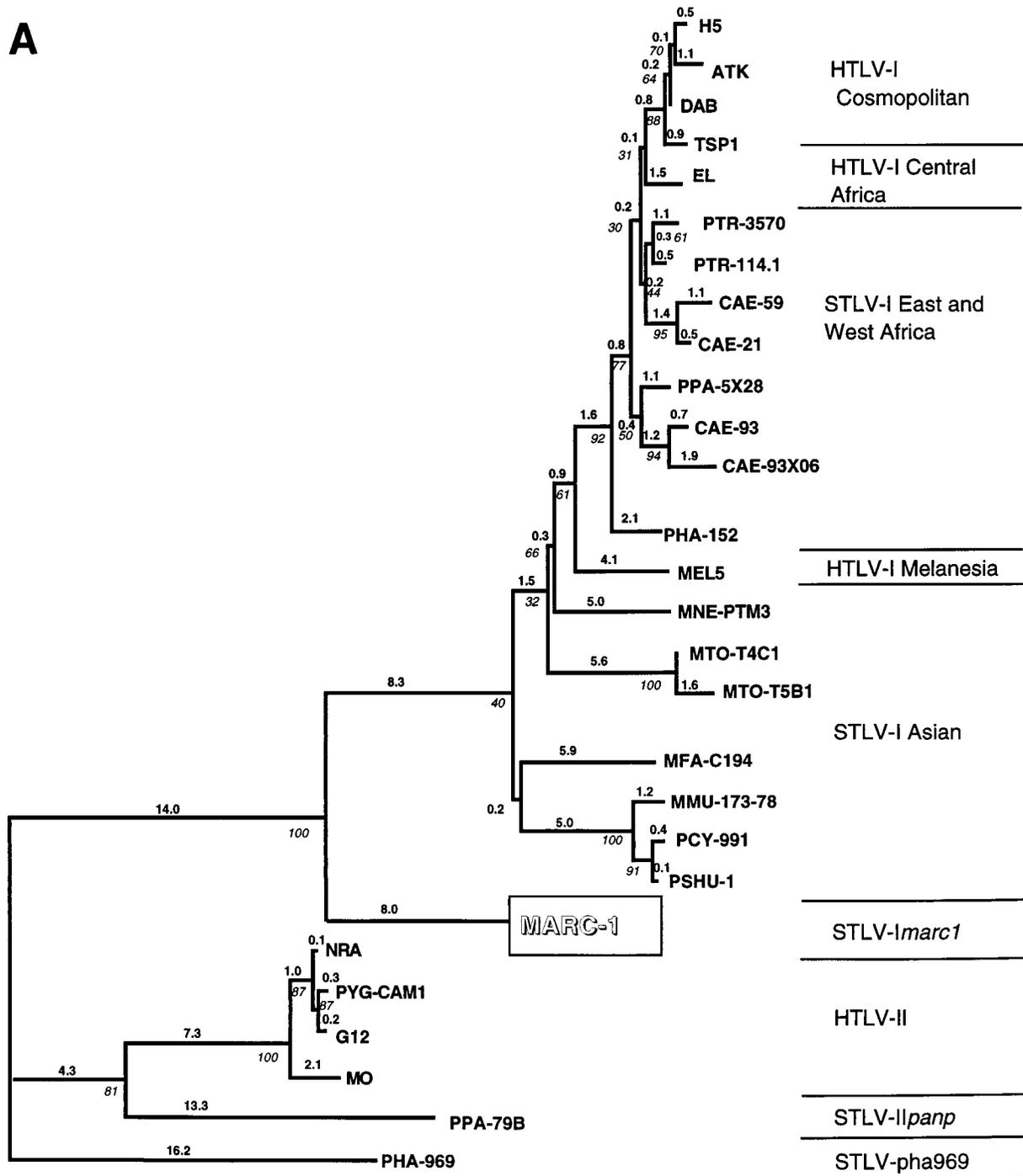


FIG. 3. (A) Phylogeny based on 306 bp of the *env* gene (bases 6149 to 6454 of ATK sequence [28]) on 28 HTLV and STLV isolates of African and Asian origin, representative strains of the different HTLV-1 clades, and the new STLV-*Imarc1* isolate. NJ tree constructed using genetic distances estimated by Kimura two-parameter model of substitution (19) and corrected with a transition/transversion bias ratio of 5:1. (B) Phylogenetic tree constructed by the NJ method with a fragment of 1,612 bp (bases 6149 to 7760 of ATK sequence [28]) encompassing most of the gp21 *env* gene and a large portion of the pX region on all available sequences of this genomic region, that is, 16 isolates including 3 STLV-1 isolates and the new STLV-*Imarc1* isolate. The NJ tree was based on Jukes-Cantor model of substitution (18). For both NJ trees, numbers on branches represent estimated number of substitutions per site $\times 100$. Number in italics represent bootstrap proportions in support of the adjacent node based on 100 resampling iterations, and values greater than 70% were considered strong support for the adjacent node (16).

type 1 isolates ATK, EL, Mel5, MNE-PTM3, MTO-TE4, and TSP1 and viral type 2 isolates of STLV-2*PanP*, MO, NRA, and G12. With each separate analysis, ATK, EL, TSP1 and HS35 were placed as more recently derived viruses and Mel5, MTO-

TE4, and MNE-PTM3 were placed as more ancestral viruses within the type 1 clade. Within the type 2 lineage, the phylogenetic pattern does not change, corroborating the placement of STLV-2*PanP* as an early divergence within the lineage (2, 9)

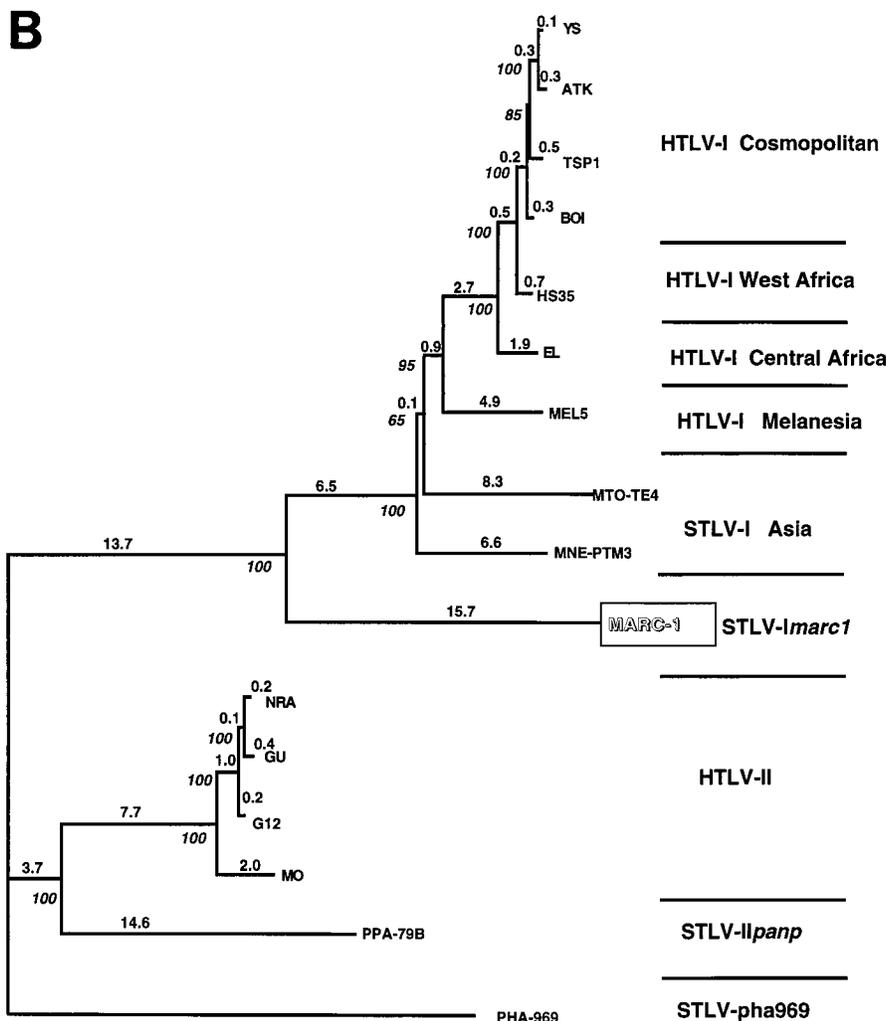


FIG. 3—Continued.

comparable to the position of STLV-1*marc1* within type 1 viruses.

In conclusion, despite the fact that STLV-1*marc1* shares certain serological traits with HTLV-2 and STLV-2*PanP* viruses, phylogenetic analyses of two gene segments consistently place STLV-1*marc1* within the type 1 lineage. However, its position, probably as an early divergence within the clade, suggests that STLV-1*marc1* arose from a common ancestor early within the evolution of type 1 viruses and could be considered a prototype of a new STLV-HTLV phylogenetic clade. Furthermore, when compared with the type 2 lineage, the position of STLV-1*marc1* is analogous to the position of STLV-2*PanP*, and genetic distance estimates between STLV-1*marc1* and all other type 1 viruses are roughly of the same order of magnitude as those of as STLV-2*PanP* relative to all other type 2 viruses. In addition, neither STLV-1*marc1* nor STLV-2*PanP* is closely related to the virus isolated from an hamadryas baboon from Eritrea (PHA-969). Although STLV-1*marc1* is the first Asian nonhuman primate strain to illicit an unusual serological response resembling that against an HTLV-2 infection, it is unlikely that STLV-1*marc1* represents an intermediate form between type 1 and type 2 viruses. However, STLV-1*marc1* provides further evidence that Asian nonhuman primates were the ancestral reservoir for HTLV-1 and

STLV-1. Finally, in light of the recent demonstration of the transmission of STLV-1 to humans in Africa and Asia (17, 23, 33), our results suggest the possible existence in Asia of HTLV-1 strains related to this new highly divergent STLV-1*marc1*.

Nucleotide sequence accession numbers. The GenBank accession numbers for the three new sequences of STLV-1*marc1* are as follows: *env*, U76624; *tax*, U76625; and *env/tax*, U76625.

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