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Complete Nucleotide Sequences of the Genomes of Two Brazilian Specimens of Human T Lymphotropic Virus Type 2 (HTLV-2)

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ABSTRACT

We report the complete nucleotide sequences of the genomes of two human T cell lymphotropic viruses type 2 (HTLV-2) isolated from a Kayapó Indian (K96 isolate) and from an inhabitant of an urban region in the south of Brazil (RP329 isolate). The general structure of the K96 and RP329 genomes did not differ from that of other HTLV-2 genomes described in the literature. The K96 genome consisted of 8955 bp and the RP329 genome consisted of 8964 bp. The general similarity between the nucleotide sequences of the K96 and RP329 genomes was 99.4%. Comparison between the nucleotide sequences of the K96 and RP329 genomes and the nucleotide sequences of isolates considered to be HTLV-2 prototypes of subtype 2a (Mo isolate), 2b (NRA isolate), and 2d (Efe2 isolate) showed a global similarity of 98.8, 95.6, and 93.3%, respectively, for the RP329 isolate, and of 99.1, 95.6, and 93.3%, respectively, for the K96 isolate. Phylogenetic analysis permitted the classification of the K96 and RP329 isolates as HTLV-2 subtype 2a. Detailed phylogenetic analyses of the LTR, *env*, and Tax regions showed that the Brazilian isolates tend to form a distinct phylogenetic subgroup within subtype 2a, previously called HTLV-2c, which differs from the subtype 2a isolates found in North America, Europe, and Africa. The K96 genome is the first HTLV-2 genome obtained from a Brazilian Indian that was completely sequenced, whereas the RP329 genome represents the first specimen derived from an inhabitant of a Brazilian urban region who was not coinfected with HIV-1.

INTRODUCTION

HUMAN T LYMPHOTROPIC VIRUSES TYPE 1 (HTLV-1) and type 2 (HTLV-2) are related human T cell lymphotropic retroviruses.[1,2] HTLV-1 is clearly associated with two well-characterized diseases: HTLV-1-associated myelopathy/tropical spastic paraparesis and adult T cell leukemia/lymphoma, whereas the association of HTLV-2 with a defined disease remains to be proved.[3–8]

HTLV-2 infects two types of populations with completely different geographic and epidemiological characteristics. The first type of infection, called "epidemic," occurs in large urban centers and mainly affects those population groups that present risk factors for infection through body fluids (intravenous drug users, sex workers, individuals undergoing polytransfusions). This epidemic type of HTLV-2 infection occurs in parallel to the HIV-1 epidemic, although in a less pronounced manner due to the different characteristics of HTLV-2 infectivity.[9]

The second type of infection, called "endemic," occurs in native and isolated population groups (Amerindians and native Africans). HTLV-2 is mainly transmitted among these populations by maternal breastfeeding and the sexual route.

The epidemic form of the infection is believed to originate from infected individuals belonging to populations where the infection is endemic. This hypothesis is supported by the fact that high prevalences of HTLV-2 infection have been demonstrated among culturally and geographically isolated groups.[10,11]

The epidemic form and its most effective transmission vector, i.e., the use of illicit intravenous drugs, does not respect national frontiers, and therefore is not informative with respect to epidemiological and anthropological aspects and population genetics. In contrast, the endemic form, which is assumed to have long existed among affected populations isolated for tens of thousands of years, represents an excellent population marker that may help in the clarification of migrations of ancestral populations.[12] Recent studies have shown that viral dynamics also

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differs among these populations, with the rhythm of viral evolution being faster for the epidemic infection than for the endemic infection.[9,13]

Various native populations endemically infected with HTLV-2 exist throughout the Americas, such as the Navajo, Pueblo, and Seminole Indians of North America, the Guaymi Indians of Central America, the Wayuu Indians from Colombia, the Toba from Argentina, and the Kayapós and Krahos from Brazil. In Africa, endemic HTLV-2 infection has been identified among the Pygmies from Zaire and Cameroon.[11,14–21]

Three HTLV-2 subtypes have been recognized thus far: HTLV-2a, HTLV-2b, and HTLV-2d. Subtypes 2a and 2b circulate in the large urban centers of North America and Europe (epidemic areas) and among native North American populations (endemic areas). Subtypes 2b and 2d have been found among native populations from Africa, whereas only subtype 2b has been observed among indigenous populations from Central America, Colombia, Venezuela, and Argentina. The situation in Brazil is peculiar since only subtype 2a has been found both in the large urban centers and among native populations.[11,22] However, it was found that the Brazilian isolates are characterized by an extended TAX protein similar to HTLV-2b, but clustering into the HTLV-2a subtype in phylogenetic analysis. Previously, the proposal was made to call the Brazilian isolates subtype HTLV-2c.[23] Thus far, eight complete sequences of the HTLV-2 genome are available, including two subtype 2a sequences,[24,25] five subtype 2b sequences,[26–30] and one subtype 2d sequence.[22]

The complete genome of a Brazilian HTLV-2 isolate derived from an intravenous drug user coinfected with HIV-1 has been recently described.[23] In the present study, we determined the complete nucleotide sequence of the genomes of two Brazilian HTLV-2 isolates derived from a Kayapó Indian and from a blood donor not coinfected with HIV-1.

Table 1. Sequence and Position of the Primers Used for the Sequencing of the HTLV-2 Genome

Primers	*Position*	*5′–3′ Sequence*
A1	27–47	agc cac cca ggg cga gtc at
A2	1099–1118	tag gtg tcg gaa ctg ggg cg
A2a	1112–1133	agt agt agg gca gat agg tgt c
A1b	419–438	ggc ctc ggc acc tcc tga ac
B1	907–926	gca gcc tag gcc ctc cga tt
B2	1958–1977	tct tgg cat agg ggg cag gg
B1a	1489–1507	atg gca ggg ccc cta aga a
B2a	1515–1536	acc ttg ctg ggc ggg gtt att a
C1	1878–1897	ccc cca cac agc cct gct tt
C2	2972–2991	gtt gtt cga aga ggg tgg gg
C2a	2437–2455	gcg tcc ctt cca atg atg g
D1	2380–2399	ttc cga agg tcc ccc gtt at
D2	3463–3482	agg tag ggc ggg gtt gag gc
D1a	2805–2824	gac agc ctt acc cca cct ac
E1	2972–2991	ccc cac cct cttcgaacaac
E2	3743–3761	gtg gtg aat gag gat gcc g
F1	3463–3482	gcc tga acc ccg ccc tac ct
F2	4341–4360	gcg cca aaa gag act aga gc
G1	3994–4012	aaa cac att ccg cac aaa a
G2	5018–5037	ggg att gca atg gac ctt tc
H1	4521–4540	tgc ctc cac gtc tgg gta ga
H2	5410–5428	atg tgg gaa taa gta taa
H1a	4899–4918	aac ccg atg gca aat cca cc
H2a	5478–5497	tag cga gca agg gtc att gt
H1b	4989–5008	taa act ccc cgg cct tac ca
I1	5252–5271	tca cga ttg gta tct cct cc
I2	5992–6011	tgt agg cga ggt tgg tag ca
J1	5912–5931	aac aaa cct cct ccc gaa cc
J2	6695–6714	ggt ata gag gac tgt gga tg
L1	6283–6302	cag tat gca gcc caa aat ag
L2	6967–6986	ttc tgc agg agc gtg agg ag
L1a	6476–6492	caa tgg gca cga gaa gc
M1	6773–6792	ttc ctc taa ccc ccg ctc ac
M2	7246–7265	cac gta gac ggg gta tcc at
N1	7143–7162	ggt ctc cta acg gca atc tc
N2	7681–7700	tgt cat ggg tgg gga aag ct
N2a	7643–7662	tac ggt ttt tcc cca ggt gg
O1	7590–7609	ctc ccc tcc ctc gcc ttc cc
O2	8252–8271	gat tgt ttg tgt gag acg gt

MATERIAL AND METHODS

DNA

DNA of the Kayapó Indian (K96) sample was extracted from the buffy coat using the phenol-chloroform method, followed by ethanol precipitation.[31]

DNA of the HTLV-2 seropositive blood donor (RP329) was extracted from cells present in 5 ml whole blood using the Super Quick Gene DNA Isolation kit (Analytical Genetic Testing Center - AGTC, Denver, CO).

Oligonucleotides

Primers and probes were synthesized by the phosphoramidite method using the Gene Assembler Special apparatus (Amersham-Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The primers and probes used are shown in Table 1.

DNA sequencing

PCR for sequencing of the HTLV-2 genome. DNA samples identified as RP329 (blood donor) and K96 (Kayapó) were submitted to polymerase chair reaction (PCR) for amplification of the different regions of the HTLV-2 genome, using the primers described in Table 1. PCR was carried out with 5 μl total DNA at a concentration of 0.1 μg/μl, 10 μl 10× buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.5, 15 mM $MgCl_2$), 53 μl ultrapure sterile water (Milli-Q), 0.2 mM of each deoxyribonucleotide—dNTPs (dATP, dGTP, dCTP, dTTP, Perkin Elmer Cetus, Norwalk, CT), 10 μl of 2.5 μM of each set of primers (see Table 1), and 2.0 U *Taq* DNA polymerase (Amersham-Pharmacia, UK), in a final volume of 100 μl. The reaction was carried out at 94°C for 5 min, followed by 35 cycles under the following conditions: 94°C for 60 sec, 50–60°C for 60 sec, and 72°C for 90 sec. At the end of the 35 cycles, the amplification product was maintained at 72°C for 10 min and then stored at 4°C.

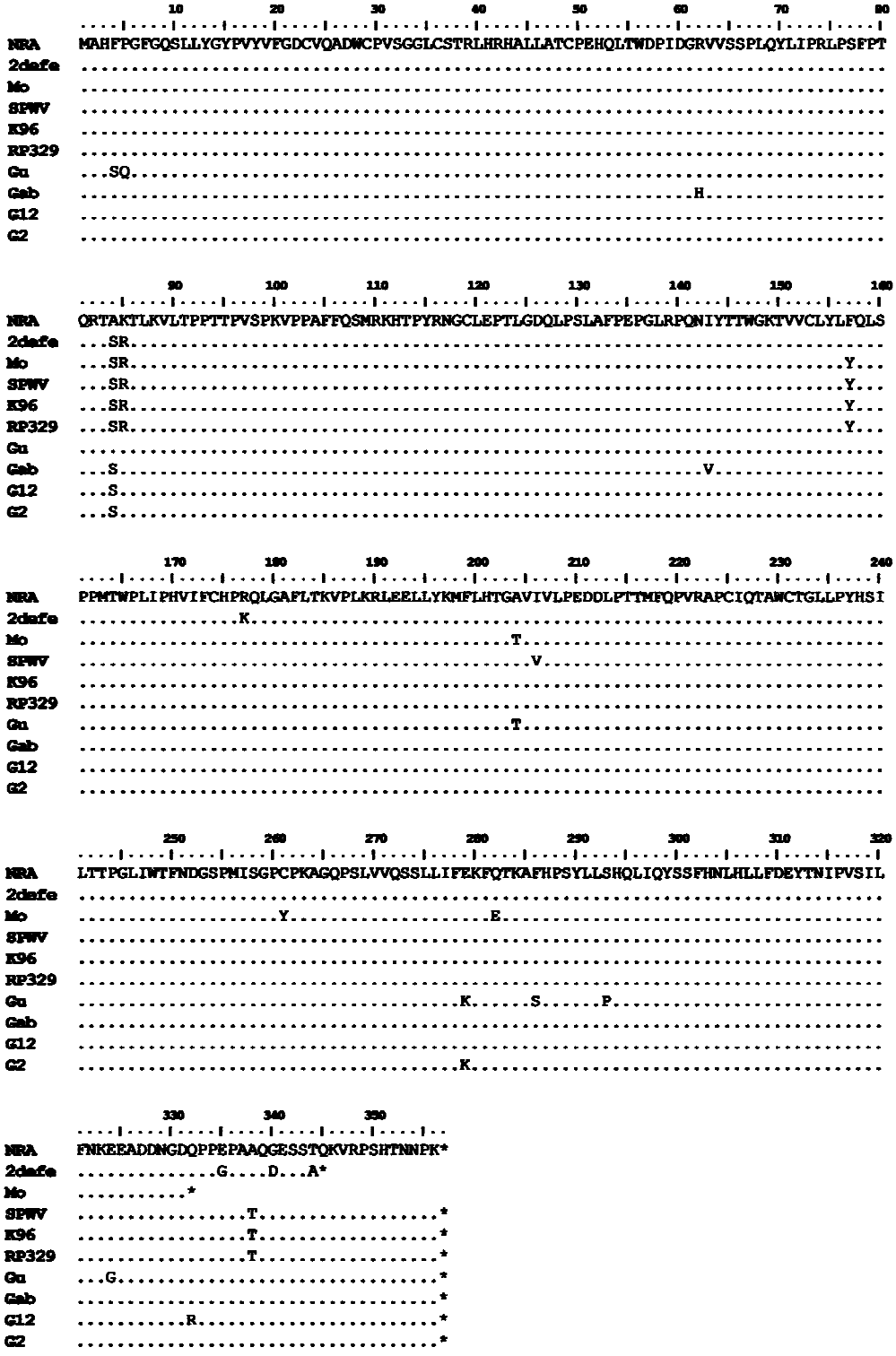

FIG. 1. Alignment of the putative TAX protein of the K96 and RP329 isolates with the TAX protein of the HTLV-2 prototypes (Mo, NRA, and Efe2) and 5 other strains whose complete genome has been described in the literature. The HTLV-2b subtypes have an extended TAX protein in comparison with the HTLV-2a prototype (Mo). The Brazilian isolates (SPWV, K96, and RP329) also have an extended TAX. The stop codon is indicated by an asterisk (*).

Sample purification. The PCR amplification products (100 μl) were purified using the Wizard PCR Preps DNA Purification kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions.

Automatic sequencing. The purified PCR products were submitted to direct sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). Two microliters of Big Dye, 2 μl of the purified PCR product, and 1 μl of the primer (2.5 μM) were added to 96-well microplates and the reaction was carried out at 95°C for 2 min, followed by 25 cycles at 95°C for 10 sec, 50°C for 20 sec, and 72°C for 4 min. After this interval, the samples were stored at 4°C.

The samples were then submitted to 4% polyacrylamide gel electrophoresis with 6 M urea, using the ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA), at 1.0 kV, 35.0 mA, 50 W, and 51°C, for 6–8 hr. The fluorograms obtained were analyzed using the ABI PRISM 377 DNA Sequencing Analysis program version 3.3 (Applied Biosystems, Foster City, CA). The consensus sequences were obtained based on comparison with the Mo prototype (GenBank Accession No. M10060), using the Sequencher program version 3.1 (Gene Codes Corporation, Ann Arbor, MI).

Alignment

The consensus sequences obtained for the RP329 and K96 isolates were manually aligned with the sequences of the prototypes representative of the HTLV-2a (Mo—GenBank Accession No. M10060), HTLV-2b (NRA—Accession No. L20734), and HTLV-2d subtypes (Efe2—Accession No. Y14365), using the XEsee software (Eyeball Sequence Editor, version 3.0) and automatically aligned using the ClustalX program, version 1.6 (www.igbmc.u-strasb.fr/BioInfo/ClustalX/Top.html).

Putative amino acid sequences

The amino acid sequences were deduced from the nucleotide sequences using the XEsee and MEGA programs (Molecular Evolutionary Genetics Analysis, version 2.1—www.megasoftware.net/).[32]

Construction of the phylogenetic trees

Phylogenetic analyses were conducted using the MEGA computer program. We estimated the evolutionary distance between nucleotide sequences using the Kimura 2 parameters method.[33] The phylogenetic tree was constructed using the neighbor-joining (NJ) method and by performing 1025 replicates (bootstrap) with the unrooted option.[34] Sites with alignment gaps in each pair of sequences under comparison were omitted (the pairwise deletion option in MEGA). The transition/transversion rate for each analysis was calculated automatically by MEGA and was checked using the software TREE-PUZZLE version 5.0 (www.tree-puzzle.de). To examine the reliability of the tree topology we constructed a maximum likelihood parsimony tree using the MPL option of the MEGA with the option Close-Neighbor-Interchange (CNI) with search level 3 and random addition trees.

RESULTS

Nucleotide sequences

The proviral HTLV-2 genome of the K96 isolate consisted of 8955 nucleotides and the genome of the RP329 isolate consisted of 8964 nucleotides. The genomic organization of the K96 and RP329 isolates was the same as that described for other HTLV-2 isolates.

We compared the nucleotide sequences of the K96 and RP329 isolates with the complete proviral HTLV-2 sequences published thus far, including two subtype 2a sequences (Mo and SPWV), five subtype 2b sequences (NRA, G12, G2, Gu, and Gab), and one subtype 2d sequence (Efe2). The proviral genomes of the K96 and RP329 isolates showed a higher general similarity to the subtype 2a isolates (Mo and SPWV), with the similarity being 99.1 and 99.2% for the K96 isolate and 98.8 and 99.2% for the RP329 isolate, respectively. Similarity was 95.6% between the two isolates and the subtype 2b prototype (NRA) and 93.3% between the two isolates and the subtype 2d prototype (Efe2). The largest differences between the K96 and RP329 isolates and the Mo prototype were observed in the LTR, gag, and nontransducible regions (NTR). The similarity between the K96 isolate and Mo for these regions was 97, 98.8, and 97.5%, respectively, whereas the similarity to the RP329 isolate was 96.2, 97.7, and 98.1%, respectively. For the remaining regions, the similarity between the two isolates and Mo was higher than 99%. Despite the wide general variability, the functional LTR elements, including the promotor region, cap site, polyadenylationsignal and site, and 21-bp repeat segments, were found to be conserved.

Putative amino acid sequences

The putative amino acid sequences of the different genic regions of the K96 and RP329 isolates were compared to the amino acid sequences obtained for the other completely sequenced HTLV-2 isolates. The highest amino acid similarity was observed for the subtype 2a isolates (Mo and SPWV). With respect to the specimens of this subtype, the largest divergence was observed for the GAG protein from RP329, which showed

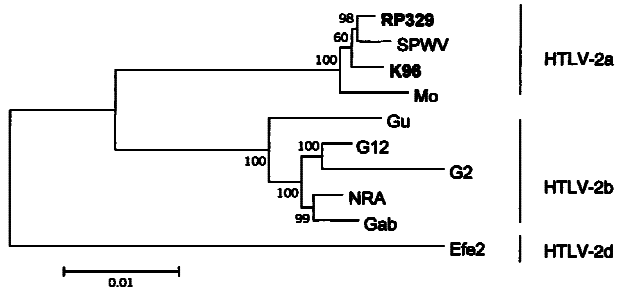

FIG. 2. Dendogram showing the phylogenetic relationship between the entire genomes of the K96 and RP329 isolates and eight completely sequenced HTLV-2 subtypes and prototypes available in the literature. The tree was constructed using the neighbor-joining (NJ) method with the Kimura two-parameter model included in MEGA software, and by performing 1025 replicates (bootstrap). The transition/transversion rate used was 4.88. Only bootstrap values higher than 50% are shown.

a divergence of 3.6% compared to the Mo isolate and of 2.7% compared to the SPWV isolate. For the other proteins, the similarity between the different subtype 2a isolates was higher than 98%.

Special attention should be paid to the TAX protein. The Tax region of the Mo isolate comprises 996 bp, which results in a protein of 332 amino acids, whereas the Tax region of the subtype 2b isolates comprises 1071 bp that encodes a 356 amino acid protein. The additional 25 amino acids are due to a nucleotide change at position 8215 (T → C), which leads to the abolition of a stop codon in the Mo isolate and its substitution with a glutamine. The K96 and RP329 isolates, due to the similarity of the subtype 2b isolates, present an extended TAX protein, a characteristic previously described by Eiraku *et al.*[23] and confirmed in the present study. Based on this peculiarity, the similarity between the TAX proteins of the K96 and RP329 isolates and the Mo isolate was only 91.9% but was 98.8% between the two isolates and the prototype 2b (NRA). The putative TAX protein alignment of the isolates K96 and RP329 in comparison with the HTLV-2 prototypes and others with the entire genome published is shown in Fig. 1.

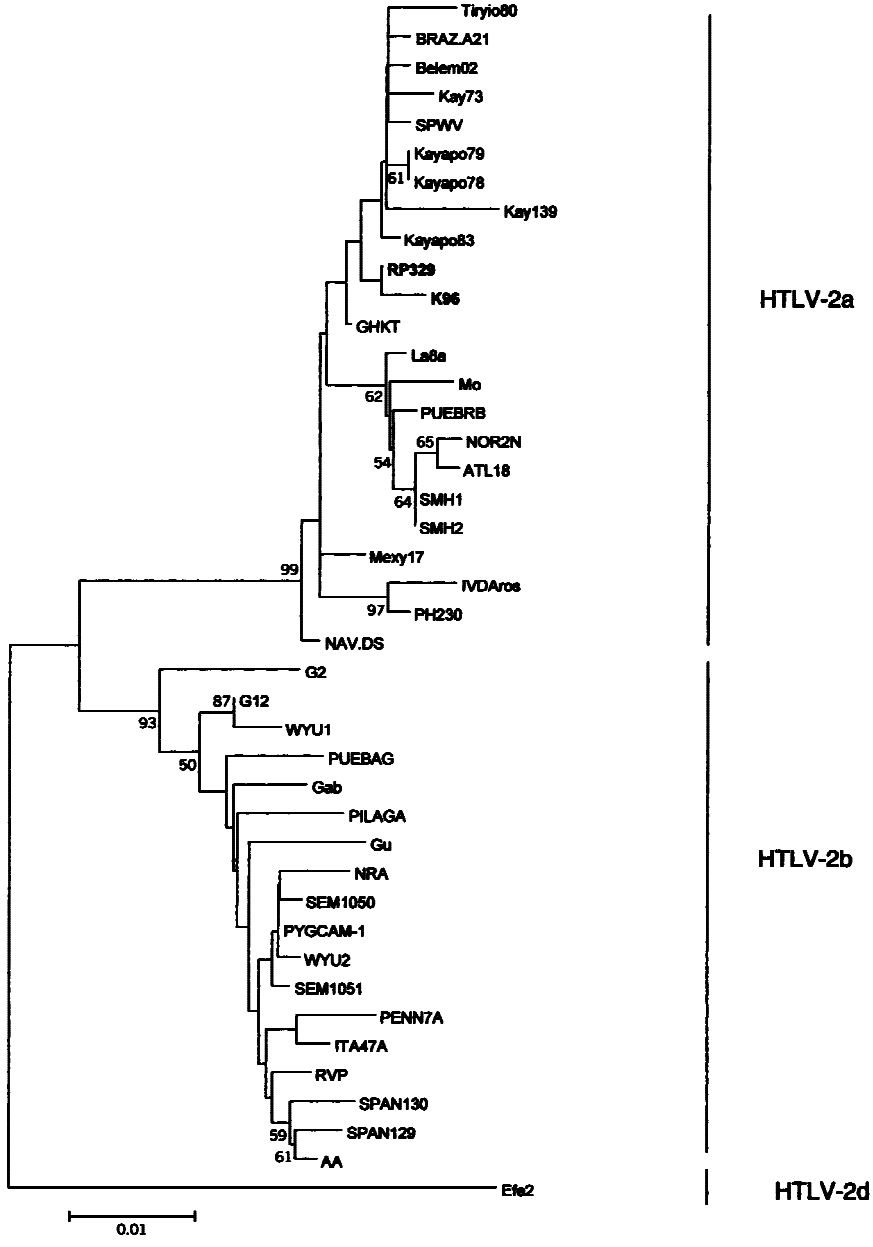

FIG. 3. Dendrograms showing the phylogenetic relationship between 581 bp of the LTR region various regions of the K96 and RP329 isolates and other sequences 40 HTLV-2 LTR sequences available in the literature. The tree was constructed using the neighbor-joining (NJ) method with the Kimura two-parameter model included in the MEGA software, and by performing 1025 replicates (bootstrap). The transition/transversion rate used was 2.75. Only bootstrap values higher than 50% are shown.

Phylogenetic analysis

Complete genome. The phylogenetic tree showing the relationship of the K96 and RP329 isolates with the prototypes and eight other HTLV-2 subtypes completely sequenced is shown in Fig. 2. The Brazilian isolates (RP329, K96, SPWV) segregated together with a bootstrap of 60%. The RP329 isolate segregated with the SPWV isolate with a bootstrap value of 98%.

LTR region. Phylogenetic analysis was conducted on 581 bp of the LTR region of the K96 and RP329 isolates and 40 other HTLV-2 sequences (21 subtype 2a isolates) available in the literature. The K96 and RP329 isolates could be grouped with the subtype 2a isolates (Fig. 3). The Brazilian isolates (K96, RP329, Kayapó 83, 79, 78, Kay 139, 73, Belém 02, SPWV, BRAZ.A21, and Tiriyo80) segregated together, although the bootstrap value of 20% did not permit the assumption that they formed an independent cluster. The tree topology was confirmed by the maximum parsimony method.

env *region.* Phylogenetic analysis was conducted on 535 bp of the *env* region, encoding the gp21 protein of the K96 and RP329 isolates and 30 HTLV-2 sequences (18 subtype 2a isolates) available in the literature. The result obtained by the neighbor-joining method employing 1025 bootstraps is shown in Fig. 4. The K96 and RP329 isolates could be grouped with

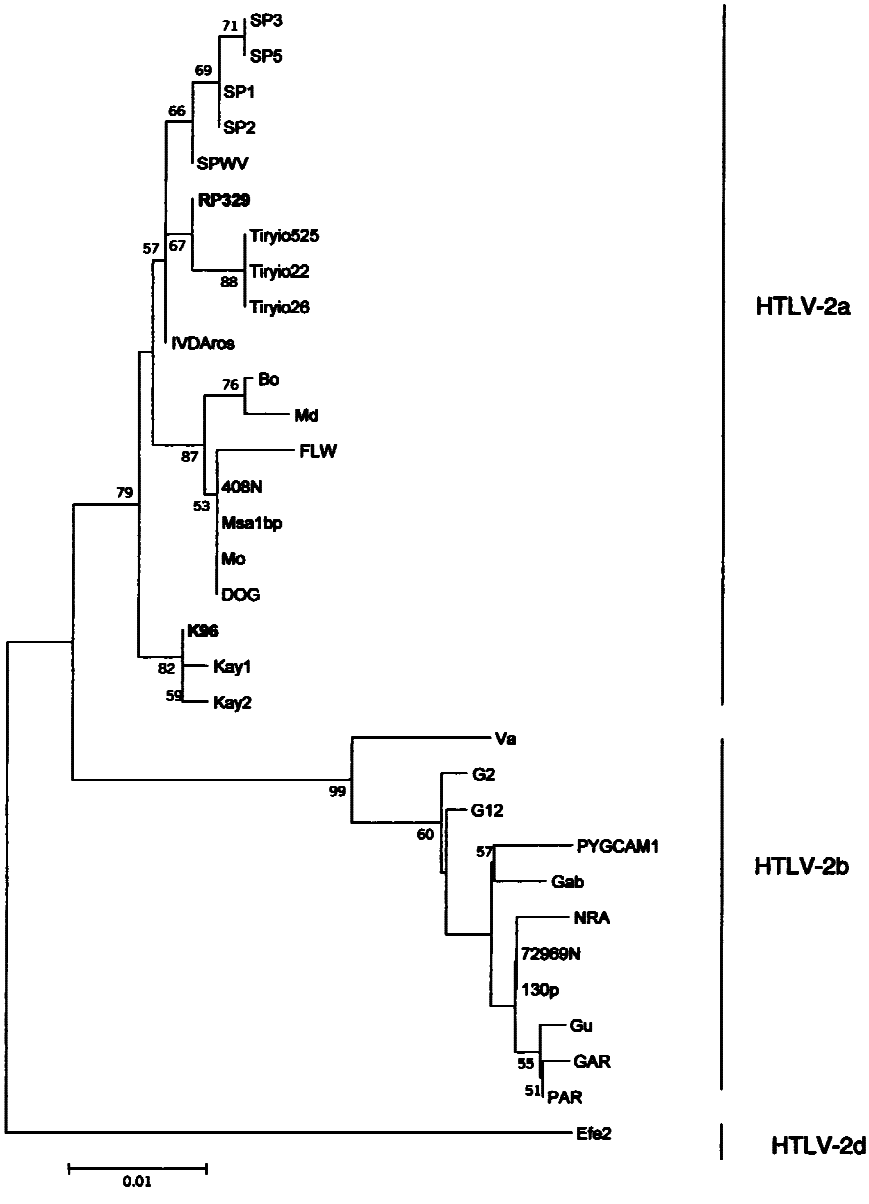

FIG. 4. Dendrogram showing the phylogenetic relationship between 535 bp of the *env* region of the K96 and RP329 isolates and 30 HTLV-2 sequences available in the literature. The tree was constructed using the neighbor-joining (NJ) method with the Kimura two-parameter model included in MEGA software, and by performing 1025 replicates (bootstrap). The transition/transversion rate used was 8.25. Only bootstrap values higher than 50% are shown.

the other subtype 2a isolates. The Brazilian samples obtained from drug users from urban regions (SP3, SP5, SP1, SP2, SPWV) formed a separate cluster, with a bootstrap value of 66%. Similarly, the RP329 isolate obtained from a non-drug user from an urban region could be grouped with three isolates obtained from Tiryió Indians (Tiryió 22, 26, and 525), forming a cluster with a bootstrap value of 67%. The K96 isolate formed a third cluster with the other samples obtained from Kayapó Indians (Kay1 and Kay2), with a bootstrap value of 82%. A fourth cluster was formed by isolates obtained from intravenous drug users from the Americas and Europe (Bo, Md, 408n, FLW, Mo, Dog, msa1bp), with a bootstrap value of 87%.

Tax region. A 667-bp segment of the Tax region of the K96 and RP329 isolates was compared with 24 HTLV-2 sequences available in the literature. Although the putative amino acid sequences of the Tax region showed a higher similarity between the K96 and RP329 isolates and subtype 2b isolates (Fig. 5), the nucleotide sequence similarity was higher between subtype 2a isolates (Fig. 5). Within subtype 2a, the HTLV-2 isolates obtained from Brazil (KAY2, RP329, k96K96, SP1, SP2, and SPWV) segregated independently in 44% of cases (1025 bootstraps), with the isolates obtained from intravenous drug users from urban regions (SP1, SP2, and SPWV) forming a distinct cluster (a bootstrap value of 87%) from the Indian isolates (K96 and KAY2) and the isolate obtained from a non-drug user from an urban region (RP329) (a bootstrap value of 60%). This segregation was confirmed by the maximum parsimony method.

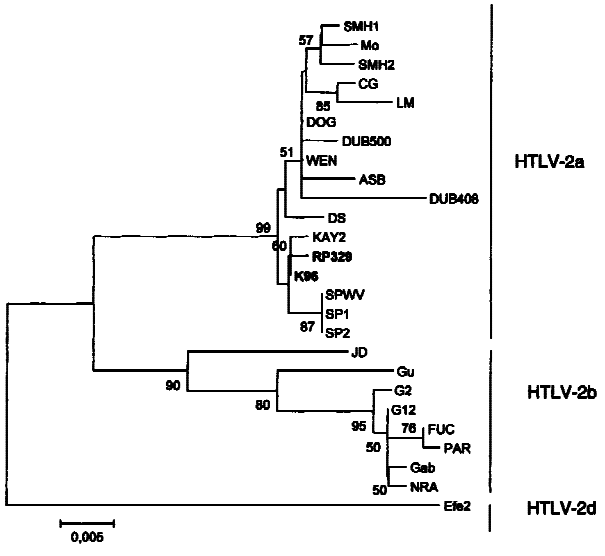

FIG. 5. Dendrogram showing the phylogenetic relationship between 667 bp of the Tax region of the K96 and RP329 isolates and 24 HTLV-2 sequences available in the literature. The tree was constructed using the neighbor-joining (NJ) method with the Kimura two-parameter model included in MEGA software, and by performing 1025 replicates (bootstrap). The transition/transversion rate used was 4.75. Only bootstrap values higher than 50% are shown.

DISCUSSION

We describe here for the first time the complete nucleotide sequence of the genomes of two Brazilian HTLV-2 isolates belonging to the endemic type of the infection, i.e., the K96 isolate obtained from a female Kayapó Indian and the RP329 isolate obtained from a 39-year-old woman living in the urban region of the interior of the State of São Paulo who was probably infected more than 10 years ago through blood transfusion.

The general genomic organization of the K96 and RP329 isolates did not differ from the genomic organization of other HTLV-2 isolates described in the literature. The general similarity between the Brazilian isolates described here and the HTLV-2 subtype 2a prototype (Mo) was higher than 98.8%, whereas the similarity with the subtype 2b (NRA) and 2d (Efe2) prototypes was 95.6 and 93.3%, respectively.

In addition, we carried out phylogenetic analyses of the viral LTR, *env*, and Tax regions and the entire genomes of the two isolates and sequences of viral isolates obtained from various parts of the world. All analyses showed that the K96 and RP329 isolates could be clearly grouped together with the HTLV-2 subtype 2a, in agreement with previous studies.[23,25,35,36]

Analysis of the LTR region showed that the Brazilian isolates segregated as a single group, which, however, could not be characterized as an separate cluster compared to the other subtype 2a isolates. A large heterogeneity was observed in the group formed by the Brazilian isolates, with no clear separation between the isolates representing an epidemic infection and those representing an endemic infection.

Analysis of the *env* region revealed at least three distinct clusters for the subtype 2a isolates, with the K96 and RP329 isolates segregating as clearly distinct groups. One of the clusters consisted of the K96, Kay1, and Kay2 isolates corresponding to viruses obtained from Kayapó Indians. The second cluster was formed by intravenous drug users from North America and Europe. The third group included isolates from intravenous drug users from Brazil (SP1, SP2, SP3, SP5, SPWV) and Argentina (IVDAros), from a Brazilian blood donor (RP329), and from Tiryió Indians (Tiryió 22, 26, 525). A clear separation within this group existed regarding isolates from Brazilian intravenous drug users coinfected with HIV-1, which formed a cluster separate from the blood donor isolate (RP329) and the isolates from Tiryió Indians (Tiryió 22, 26, 525).

With respect to the Tax region, the HTLV-2a isolates could be separated into two distinct groups. The first group included drug users and Indians from North America and the second group contained Brazilian isolates. In the latter group, the Kayapó Indian isolates (Kay2 and K96) and RP329 segregated independently (a bootstrap value of 60%) from the isolates obtained from intravenous drug users.

It is interesting to note that the segregation between isolates of endemically and epidemically infected patients as seen in the coding *env* and Tax regions cannot be seen in the noncoding LTR region. A possible explanation for this fact could be the existence of different selective pressures on the coding and noncoding regions in the two types of infection.

The phylogenetic analysis of the complete genomes of 10 isolates of HTLV-2 available showed that isolates K96 and

RP329 segregated with the other subtypes of HTLV-2a (Mo and SPWV). Isolates RP329 and SPWV (epidemic transmission) segregated together (bootstrap = 98%), showing a tendency to separation of the K96 isolate (endemic transmission) (Fig. 2).

Taken together, the present data indicate a large heterogeneity among Brazilian HTLV-2 isolates, which does not permit their classification into a phylogenetically distinct group as proposed by Eiraku *et al.*[23] A tendency exists toward phylogenetic separation between isolates obtained from Kayapó Indians and those obtained from an urban population, notably intravenous drug users coinfected with HIV-1, which may be explained by the different evolutive rates of these isolates.[37] Part of the difficulty in obtaining a phylogenetically correct classification of the Brazilian isolates is due to the lack of molecular information about these viruses. The literature reports only partial nucleotide sequences of the LTR, *env*, and Tax regions, which account for no more than 25% of the total nucleotide sequence of the HTLV-2 genome of Brazilian isolates. The determination of the true place of the Brazilian isolates within the phylogeny of HTLV-2 requires additional data, including other complete subtype 2a genome sequences.

Sequence data

The Genbank Accession numbers of the sequences reported in this paper are Af326583 And Af326584.

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