In Vivo Genomic Variability of Human T-Cell Leukemia Virus Type I Depends More upon Geography than upon Pathologies

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To investigate the geography- and disease-associated genomic variation of human T-cell leukemia virus type I (HTLV-I), we studied *ex vivo* DNA from peripheral blood lymphocytes from nine patients by polymerase chain reaction and direct DNA sequencing. For each viral strain, 1,918 bp was sequenced, including parts of the long terminal repeat, the *env* gene, and the *px* II, *px* III, and *px* IV coding frames of the *px* region. The number of genomic variations observed in the U3 region of the long terminal repeat was higher than that seen in the *env* and *px* genes. Very few mutations were present in the *px* II and *px* III genes. In contrast, the *px* IV open reading frame exhibited numerous single point mutations. While no specific mutation could be linked to any pathology (adult T-cell leukemia/lymphoma or tropical spastic paraparesis/HTLV-I-associated myelopathy), variations among HTLV-I isolates from different geographic areas (Ivory Coast, Caribbean, and Japan) existed. The Ivory Coast HTLV-I appeared to represent a group by itself.

Human T-cell leukemia virus type I (HTLV-I) infection is known to be associated with two pathologies: adult T-cell leukemia/lymphoma (ATL) (21, 35) and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM) (13, 33). These diseases are widespread worldwide, with higher prevalence in the Caribbean (2, 13), southern Japan (52), and, to a lesser extent, equatorial Africa (9).

Studies of the long terminal repeat (LTR) and of the envelope (*env*) regions of HTLV-I isolates associated with either ATL or TSP/HAM (50, 8) have not yet demonstrated sequences specifically linked to either neurotropism or lymphotropism. This contrasts with the murine experimental model, in which specific sequences in the *env* gene (38, 48) and in the LTR (25, 26) have been involved in determining tissue tropism and pathogenesis of neurological disease. Only a few studies have been done on the degree of sequence conservation of the HTLV-I *px* gene (39), despite the fact that the transactivating Tax protein encoded by the *px* IV open reading frame plays an important role in the transactivation of the LTR via host cell factors (22, 30, 32, 49, 54) and in the dysregulation of cellular genes involved in cell growth (10, 18, 31, 42). Only one amino acid change could suppress the Tax activity (46), which in turn could influence the outcome of the HTLV-I infection. The existence of host genetic factors possibly controlling the pathogenic potential of HTLV-I has been hypothesized (51), but alone these could not be sufficient to explain disease development.

Classical epidemiology, using serological data and clinical records, enables us to appreciate the spread of HTLV-I infection in different parts of the world and to determine the spectrum of associated diseases (13, 14, 53). The study by Gray et al. (17) of the genomic variability of HTLV-I isolated from different geographic areas concluded that great homology exists between HTLV-I isolates of Japanese or Caribbean origin, whereas Malik et al. (28) stressed great homogeneity only within geographic areas. These few ob-

servations were based on the sequence analysis of HTLV-I in long-term cultures.

In this study, to investigate further whether particular HTLV-I sequences could be linked either to any geographic areas or to specific associated pathology, we analyzed *in vivo* DNA sequences, including the LTR, *env*, and *px* region, from peripheral blood lymphocytes obtained from patients with ATL or TSP/HAM from Africa, the Caribbean, and Japan by direct sequencing of amplified DNA.

MATERIALS AND METHODS

Samples. Peripheral blood lymphocytes were collected from nine HTLV-I-seropositive adult patients from different geographic areas and with different associated pathologies. Two patients originated from the Ivory Coast, three were from the Caribbean, and four were from Japan (Table 1). The two patients from the Ivory Coast (Sie and Akr isolates) were coinfecting by HTLV-I-human immunodeficiency virus type 2 (HIV-2) and HTLV-I-HIV-1, respectively. None of the patients studied here were related to each other.

TABLE 1. Origin of HTLV-I isolates

Geographic origin	Pathology	Sex ^a /age (yrs)	Name of isolate
Caribbean	TSP/HAM	F/52	Xav
	TSP/HAM	F/48	Bou
	B lymphoma	F/35	Gro
Ivory Coast	ATL	F/19	Sie
	TSP/HAM	M/45	Akr
Japan (Kagoshima)	ATL	F/40	Jap1
	ATL	M/53	Jap2
	TSP/HAM	F/57	Jap3
	TSP/HAM	M/46	Jap4

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^a F, female; M, male.

FIG. 1. Positions of HTLV-I primers (bottom) and number of nucleotide changes (top) observed in the amplified regions compared with the ATK-1 sequence (45). Primer positions are numbered as in the EMBL data base. Arrows indicate regions amplified by PCR. The sequences of the primers were: LTR1, 5'-ACCATGAGCCCCAAATATCCCCC3'; LTR2, 5'-AATTTCTCTCCTGAGAGTGCTATAG3'; Flo4, 5'-CTCCCTTCTAGTCGACGCTCCAGG3'; Flo5, 5'-GCCACCGGTACCGCTCGGCGGGAG3'; pxA1, 5'-ATAGCCCGTCCACCAATTCTCC3'; pxA2, 5'-CTGTGGTGAGGGAAATTTTATAGA3'; pxB1, 5'-GGAGGCTCCGTTGTCTGCATGTA3'; pxB2, 5'-CGTCAGGGCCTAGCCGAGCCGG3'. During the PCR, 30 cycles were performed in a DNA thermocycler (Perkin-Elmer) under the following conditions: denaturation at 94°C for 2 min, annealing at 55°C for 1 min, and extending at 70°C for 2 min.

DNA amplification. DNA was extracted from Ficoll-Paque-separated peripheral blood lymphocytes. DNA (1 µg) was subjected to polymerase chain reaction (PCR) with four primer pairs in *env*, the LTR, and the *px* genes as described in the legend to Fig. 1. For each pair, one of two primers was chemically 5' phosphorylated to obtain, after PCR, a double-stranded product with one phosphorylated strand. The PCR was performed as described elsewhere (40, 43). The amplified product was analyzed according to its molecular weight after electrophoresis in a 4% agarose gel. It was also transferred to a nylon filter and hybridized with labeled oligonucleotide probes to confirm the specificity of the PCR.

Amplified DNA treatment. The double-stranded amplified product was treated with 1 U of lambda exonuclease (Bethesda Research Laboratories) for 40 min at 37°C (20). The phosphorylated strand of the amplified DNA was preferentially digested by the exonuclease. The single-stranded DNA remaining was used for direct sequencing as described below.

Direct sequencing of single-stranded DNA. The dideoxynucleotide-chain termination sequencing method (44) was performed with *Taq* DNA polymerase (Cetus Corp.). The labeling and termination reactions were done at high temperatures (42 and 70°C, respectively). Depending on the se-

TABLE 2. Nucleotide changes in the HTLV-I *env* gene (positions 5772 to 6060)

Study	Geographic origin	Pathology	Name of isolate	Nucleotide at the following position ^a :				
				5775 ^b	5795	5837	5903	5949 ^c
Seiki et al. (45)	Japan	ATL	ATK-1 ^d	C	C	C	C	T
Tsujimoto et al. (50)	Japan	TSP/HAM	H5					
Malik et al. (28)	Caribbean	ATL	HS-35	T	T	T	A	C
Present study	Caribbean	TSP/HAM	Xav				A	
		TSP/HAM	Bou				A	
		B lymphoma	Gro				A	
							A	
	Ivory Coast	ATL	Sie	T	T	T	A	C
		TSP/HAM	Akr	T	T	T	A	C
	Japan	ATL	Jap1					
		ATL	Jap2					
		TSP/HAM	Jap3				A	
		TSP/HAM	Jap4				A	

^a Nucleotide positions are numbered according to the HTLV-I (ATK-1) sequence in the EMBL data base.

^b Pro → Ser.

^c Ser → Pro.

^d ATK-1 is used as a reference (45).

TABLE 3. Nucleotide changes in the HTLV-I LTR (positions 89 to 650)

Study	Geographic origin	Pathology	Name of isolate	Nucleotide at the following position ^a :											
				89	105	113	122	125	144/145	150	163	168	193	202	231
Seiki et al. (45)	Japan	ATL	ATK-1 ^b	A	A	G	C	A	X ^c	G	G	G	G	T	A
Tsujimoto et al. (50)	Japan	TSP/HAM	H5							A					G
Malik et al. (28)	Caribbean	ATL	HS-35	T	G		A			A	A	A	A		G
Josephs et al. (23)	United States	TSP	CR-1	C						A		A			G
Present study	Caribbean	TSP/HAM	Xav	C					C	A		A			G
		TSP/HAM	Bou	C					C	A		A			G
		B lymphoma	Gro	C						A		A		A	G
	Ivory Coast	ATL	Sie	C	G	A	A			A	A	A	A		G
		TSP/HAM	Akr	C	G	A				A	A	A	A		G
	Japan	ATL	Jap1	ND ^d	ND					A					G
		ATL	Jap2	ND	ND					A					G
		TSP/HAM	Jap3	ND	ND	ND				A		A			G
		TSP/HAM	Jap4	ND				G		A		A			G

^a Nucleotide positions are numbered according to the HTLV-I (ATK-1) sequence in the EMBL data base.

^b ATK-1 is used as a reference.

^c X, deletion.

^d ND, not done.

quence analyzed, electrophoresis was run at 65 W and 1.8 kV on a 4 or 6% polyacrylamide gel for 4 to 6 h.

Validity of the method. To check the reliability of the direct sequencing of amplified DNA, part of the *env* gene and of the LTR of HTLV-I from the CNS-1 line were sequenced, leading to sequences identical to those published by Tsujimoto et al. (50). Furthermore, the HTLV-I sequences obtained from two independent PCRs were compared. The results were identical, thus confirming the reliability of the method.

RESULTS

Sequence analysis of *env* gene. About 290 bases (5772 to 6060) spanning one-third of the *env* gene and coding for gp46 were sequenced and compared with the envelope sequences of the reference ATK-1 isolate of Seiki et al. (45) (Table 2). A strong sequence conservation was observed in this part of the gene, with only five nucleotide substitutions found among the nine HTLV-I isolates studied. Two mutations led to two changes in the amino acids of the Env glycoprotein. The substitution in position 5903 was common to all the HTLV-I isolates tested, except the two Japanese ATL isolates (Jap1 and Jap2), which were completely identical to the ATK-1 sequences.

The two Ivory Coast HTLV-I isolates (Sie and Akr) shared four common mutations in positions 5775, 5795, 5837, and 5949, mutations not observed in the Caribbean and Japan HTLV-I isolates. No specific mutations observed in the *env* gene could be associated with ATL or TSP/HAM pathology.

Sequence analysis of the LTR. The LTR gene was sequenced from base 89 to base 650, corresponding to part of the U3 region, to the complete R region, and to the first 40 bp of the U5 region. The observed sequence changes are summarized in Table 3. Sixty-nine percent of the nucleotide variation observed in the LTR was localized in the U3 region; the R and U5 regions appeared to be more conserved. The well-conserved structures, such as the three imperfect 21-bp repeats, the poly(A) signal, and the TATA box of the U3 region, showed no mutation.

The substitution rate in the LTR region was higher than

that in the *env* gene, with a strong preference for A→G and G→C substitutions. Three insertions and two deletions were detected. Some mutations (150, 231, 232, and 338) were common to all our HTLV-I isolates, raising questions about the suitability of ATK-1 as the reference prototype (see Discussion).

The three Caribbean HTLV-I isolates (Xav, Bou, and Gro) exhibited five mutations that were also observed in the two Japanese TSP/HAM HTLV-I isolates (Jap3 and Jap4). Six mutations were specific for the Ivory Coast HTLV-I isolates (Akr and Sie). As expected, HTLV-I from Japanese patients with ATL (Jap1 and Jap2) showed a greater homology with the published ATK-1 sequences. The sequence comparison made between individual HTLV-I isolates (Table 4) enabled us to assess the genomic variation according to geographic areas.

The Caribbean HTLV-I isolates (Xav, Bou, and Gro) exhibited 4 to 16 bases that were different from the Japanese and African HTLV-I isolates. The Ivory Coast HTLV-I isolates (Akr and Sie) showed the same number of nucleotide changes as observed with the Japanese or Caribbean HTLV-I isolates. As discussed below, no sequence change could be linked to ATL or TSP/HAM.

Sequence analysis of *px* gene. Three coding frames of the *px* gene (*px* II, *px* III, and *px* IV) were sequenced, covering 1,069 bp. We did two different PCRs of the region of interest, each giving an amplified product of about 700 bp (Fig. 1). The majority of the sequence changes observed (22 changes involving 29 mutations) were detected in the *px* IV coding frame, precisely in the region not overlapping with the *px* III coding frame. Among these nucleotide changes (Table 5), three were common to seven HTLV-I isolates (Xav, Bou, Gro, Sie, Akr, Jap3, and Jap4) but were never observed in the Japanese ATL isolates (Jap1 and Jap2). Two substitutions (7949 and 8243) were only found in the Ivory Coast HTLV-I isolates (Sie and Akr). Single mutations represented 75% of the total mutations observed in the *px* amplified region. Among the 29 above-mentioned mutations in the *px* region, 5 led to an amino acid change in the p21 and p27 proteins and 11 were responsible for an amino acid modification of the p40 protein (Table 6). The two substitutions

TABLE 3—Continued

Nucleotide at the following position ^a :																						
232	233	235	262	263	268	308/309	310	323	328	335	338	381	387	476	498/499	503	559	595	596	603	631	634
A	G	A	A	A	T	X	C	T	C	C	G	G	C	A	X	A	C	T	C	A	C	T
G											A											
G								C		A	A					G						
G	X				C				G		A											
G	X				C	T			G		A		T			G		C	T	G		C
G	X				C				G		A		T			G		C		G		C
G	X		G		C				G		A			X		G				G		C
G								C		A	A					G						
G								C		A	A					G	T					
G		G		C							A	A										
G	X				C				G		A	A				G				G		C
G	X				C		T		G		A					G				G	T	C

(7949 and 8243) observed in the Ivory Coast HTLV-I isolates (Akr and Sie) induced no amino acid modifications; thus, the biological activity of their Tax proteins should not be altered. Sie virus had a higher nucleotide divergence when compared with the reference isolate ATK-1, but only 2 of the 12 substitutions observed in its *px IV* coding frame led to an amino acid change. Two amino acid changes were observed in the Bou and Xav HTLV-I isolates and four amino acid changes were seen in the Akr HTLV-I isolate. The largest number of amino acid changes was found in the Gro virus, with the stop codon of the p21 and p27 proteins substituted by a Trp codon, leading to a truncated protein. The patient from whom the Gro isolate was obtained had a B-cell lymphoma and was HTLV-I seropositive but had no sign of ATL or of TSP/HAM. The lymphomatous cells could not be studied for the presence of HTLV-I provirus.

As seen in Table 7, the two Japanese ATL HTLV-I isolates (Jap1 and Jap2) were 100% homologous with the reference isolate (ATK-1), whereas the Ivory Coast and Caribbean HTLV-I isolates were found to be more distant (98.4 to 99.5% homology).

DISCUSSION

The direct sequencing of amplified DNA without cloning enabled the dominant HTLV-I species from each patient to be rapidly sequenced. This procedure was well adapted to

the aim of our study, which was to search for geographic variations in *in vivo* HTLV-I sequences.

In this context, three regions of the HTLV-I genome, i.e., parts of the LTR and the *env* and *px* genes involving more than 20,000 bp, were sequenced in isolates from nine patients from the Caribbean, the Ivory Coast, and Japan. The results obtained after sequencing were compared with the sequence of the reference strain ATK-1 (45). However, some of the original ATK-1 data may not represent the consensus LTR sequence, since the mutations observed in positions 150, 231, 232, and 338 found in all isolates could either represent errors in the original ATK-1 sequencing or suggest that ATK-1 is a variant vis-à-vis the consensus prototype, still to be agreed on. The fidelity of the *Taq* DNA polymerase isolated from *Thermus aquaticus* in the gene amplification reaction has previously been properly investigated (1, 43) and thus did not seem to be an obstacle here. In direct sequencing, the random misincorporations of the *Taq* polymerase are averaged out, making this method less disturbing than it would be in the sequencing of cloning products.

Among the nucleotide changes observed, some, localized in the LTR, appeared to be associated with the geographic origin of the virus. The Ivory Coast HTLV-I isolates (Sie and Ak) appeared to form a separate group distant from the Caribbean and Japanese HTLV-I isolates. A Zairian HTLV-I isolate (HTLV-Ib) differing by 17 restriction en-

TABLE 4. Comparison of HTLV-I sequence: number of base changes in the LTR in each HTLV-I studied

[illegible]

TABLE 5. Nucleotide changes in the *px* III and *px* IV coding frames of the HTLV-I *px* gene^a

Coding frame	Study	Geographic origin	Pathology	Name of isolate	Nucleotide at the following position ^b :							
					7580 ^c	7583 ^c	7644 ^c	7675 ^c	7746	7829 ^c	7833 ^c	7886
<i>px</i> III	Seiki et al. (45)	Japan	ATL	ATK-1 ^d	C	G	A	A	C	C	A	
	Tsujimoto et al. (50)	Japan	TSP/HAM	H5								
	Ratner et al. (39)	Zaire	ATL	MC-1								
	Malik et al. (28)	Caribbean	ATL	HS-35								
	Present study	Caribbean	TSP/HAM	Xav								
			TSP/HAM	Bou								
			B lymphoma	Gro		A	G				G	
			ATL	Sie	T					T		
<i>px</i> IV	Present study	Caribbean	TSP/HAM	Akr				G	T			
	Seiki et al. (45)	Japan	ATL	ATK-1			A	A	C	C	A	G
	Tsujimoto et al. (50)	Japan	TSP/HAM	H5								
	Ratner et al. (39)	Zaire	ATL	MC-1								
	Malik et al. (28)	Caribbean	ATL	HS-35						T		
	Present study	Caribbean	TSP/HAM	Xav								
			TSP/HAM	Bou								
			B lymphoma	Gro			G				G	
			ATL	Sie						T		A
		Ivory Coast	TSP/HAM	Akr				G	T			
	Present study	Japan	ATL	Jap1								
			ATL	Jap2								
			TSP/HAM	Jap3								
			TSP/HAM	Jap4			ND	ND	ND	ND	ND	

^a No mutations were observed in the *px* II coding frame.^b Nucleotide positions are numbered according to the HTLV-I (ATK-1) sequence in the EMBL data base.^c Indicates the nucleotide changes leading to an amino acid modification.^d ATK-1 is used as a reference.^e ND, not done.

zyme sites from any other HTLV-I isolate (39) has been sequenced in the *px* region. This showed one mutation, possibly specific for the Ivory Coast group. Since the *px* IV coding frame harbored a limited number of sequences linked to a geographic origin, this region does not appear to be suitable for the comparison of HTLV-I isolates. Surprisingly, the HTLV-I nucleotide changes observed in the African patients were also reported in the HS-35 Caribbean HTLV-I isolate of Malik et al. (28). Of interest, this patient's ancestors were African (23a).

Furthermore, it was interesting that the three Caribbean HTLV-I isolates (Xav, Gro, and Bou) studied here were closely related to the two Japanese TSP/HAM HTLV-I isolates (Jap3 and Jap4), whereas the two Japanese ATL HTLV-I isolates (Jap1 and Jap2) differed but showed a very strong homology with the reference ATK-1 sequences (45). The possibility of PCR contamination between Caribbean and Japanese TSP/HAM HTLV-I isolates could be excluded, since single mutations characterized each HTLV-I isolate.

To verify this relationship between Caribbean and Japanese TSP/HAM HTLV-I isolates, two additional Japanese TSP/HAM HTLV-I isolates were sequenced in their LTRs (data not shown). One of these was closely related to the Caribbean-Japanese TSP/HAM HTLV-I profile mentioned above, whereas the other showed a strong homology with the Japanese ATL HTLV-I isolate investigated in this study. These preliminary results suggest that two HTLV-I subpopulations independent of the associated pathology have

evolved in Japan. Whether the Japanese HTLV-I subpopulation sharing sequences with the Caribbean HTLV-I isolates reflects specific population migration remains an open question.

Among the three sequenced regions of HTLV-I, the LTR appeared to be the best adapted for searching for geographic markers. In contrast, the *px* region, with numerous single point mutations (75%), more than in any other genes studied, does not appear to vary according to geography. Also, the 288 bases sequenced in the *env* gene, corresponding to a part of gp46 in which sequences exhibited strong conservation (28), showed no differences in the present study between Caribbean and Japanese HTLV-I isolates, although the Ivory Coast isolates had specific changes. Similar observations have been reported for the whole *env* gene (17). This gene might be a highly conserved cross-species as suggested by Cianciolo et al. (7). In contrast to HTLV-I, ex vivo HIV sequences exhibit great sequence variability (16). This difference probably reflects the different strategies and host-virus relationships of oncoretroviruses and lentiretroviruses in humans. The genomic diversity of HIV has been explained in part by in vivo genomic recombination (47). In our study, no recombination between HTLV-I and HIV sequences was observed in the samples from the two doubly infected patients. The two viruses could, however, cooperate in vivo, with HTLV-I Tax activating the HIV LTR (3), thus affecting the prognosis of AIDS (24, 34).

A strong nucleotide variability was observed in the U3

TABLE 5—Continued

Nucleotide at the following position ^b :																				
7904	7919	7949	7981 ^c	8000	8013 ^c	8027	8100 ^c	8117	8145 ^c	8180	8230 ^c	8234	8243	8294	8298 ^c	8319 ^c	8335 ^c	8336	8339	8366
C	C	A	C	A	A	G	T	G	T	C	G	A	G	G	G	C	G	C	A	A
		G		G							A		T							C
	T	G		G							A	G	A							T
	T		T	G							A			A						C
	T		T								A									C
	T		T		C				C		A						A	G		C
	T	G		G		T	C	A			A	G	A							C
	T	G		G						T	A		A		A	G				C
G																				ND ^c
	T		T								A								G	C
	T		T								A									C

region of the HTLV-I LTR, which contains specific sequences for optimal *tax* transcriptional activity (11). Among these sequences, three 21-bp repeats were described (5, 41) constituting the Tax-responsive element named TRE1. A core octanucleotide, TGACGTCN, present in the center of each repeat and also reported in the cyclic AMP-responsive element (36) must be conserved to maintain a high level of transactivation (15, 22). No sequence variation was detected in the TRE1 of the nine HTLV-I isolates studied, indicating that these functional structures were very well conserved. Other sequences described as a second Tax-responsive element (TRE2) by Marriot et al. (29) or as an Ets-responsive element by Bosselut et al. (4), situated between the two last 21-bp repeats, have also been implicated in Tax responsiveness. Only three nucleotide changes in TRE2 were observed in our study: one deletion at position 233 found in the three Caribbean isolates and in the two Japanese TSP/HAM isolates and two substitutions at positions 231 and 232 present in all HTLV-I isolates. This may refer to the consensus sequence of the LTR, with no apparent biological consequence. As some U3 sequences appear to be implicated in the activating mechanisms of the LTR without an obligatory Tax regulation (37, 12) but with binding and involvement of cellular factors preexisting in the cells (15) and specific to the cell types (27), it is highly probable that the mutations localized in these regions could influence LTR efficacy.

About 37% of the mutations observed in the *px* IV coding frame of the nine HTLV-I isolates led to amino acid modifications in the Tax protein which could also lead to biological consequences. Only one amino acid substitution could suppress functional Tax activity (46), particularly if situated within the first amino acid at the NH₂ terminus or at the

carboxyl terminus. These amino acid-encoded functions are necessary for both transactivation and specificity of the Tax protein (6), but it is difficult to link the amino acid changes to a given biological property, since a modification in their chemical nature does not appear to be necessary to suppress Tax activity (6).

For the p27 protein, which is implicated as a regulator in the posttranscription of the Gag, Pol, and Env proteins (19), the substitution of the stop codon by Trp as observed for the Gro isolate should lead to the formation of a truncated protein with modified activity. The stop substitution was observed for an HTLV-I isolate obtained from a patient with no known HTLV-I-associated pathology, as we have no evidence for infection of the lymphomatous cells of the patient by HTLV-I.

The two distinct pathologies (ATL and TSP/HAM) associated with HTLV-I infection could not be linked to any specific mutations in the sequences analyzed in this study. This parallels the results of Daenke et al. (8) concerning the LTR and *env* gene of isolates from patients with different pathologies who originated from one geographic area. However, the technique used here allowed us to study only the dominant HTLV-I species present in the peripheral lymphocytes from each patient. The analysis of specific clones in these patients, if isolated from different tissues, might have indicated the existence of neurotropic or lymphotropic HTLV-I sequences selected for or adapted to specific tissues.

The study of genetic variability of HTLV-I confirmed the high conservation of the DNA of virus originating from three endemic areas. However, three subtype populations were distinguished and may have evolved independently from a common ancestor.

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