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Phylogenetic Relationship and Geographic Distribution of Multiple Human T-Cell Lymphotropic Virus Type II Subtypes

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The current *env*-based subtyping of human T-cell lymphotropic virus type II (HTLV-II) identifies only two heterogenetic groups, HTLV-IIa and HTLV-IIb. To better understand the genetic diversity and phylogeny of HTLV-II, we examined the most divergent genomic region of HTLV-II, the long terminal repeat, by using restriction fragment length polymorphism (RFLP) and sequence analysis. Long terminal repeat sequences were amplified from peripheral blood mononuclear cells by PCR and digested with seven restriction endonucleases that differentiated HTLV-II into five HTLV-IIa (IIa0 to IIa4) and six HTLV-IIb (IIb0 to IIb5) restriction types, with HTLV-IIa0 and HTLV-IIb0 being prototypes for the MoT and NRA isolates, respectively. We examined 169 HTLV-II-infected samples, including 123 from blood donors and intravenous drug users (IDU) from the Americas, 16 from IDU from Europe, and 30 from Amerindians. Of the 169 samples, 109 (64.5%) were categorized as HTLV-IIa and 60 (35.5%) were categorized as HTLV-IIb. The predominant restriction types seen among the U.S. blood donors and U.S. IDU were IIa0 (68.7%) and IIb4 (10.4%). Four Spanish and seven Italian samples were IIb4, while five Norwegian samples were IIa2. Twelve Guaymi and all ten Seminole samples were single restriction types (IIb1 and IIb5, respectively), whereas the two Navajo and six Pueblo samples had a mixture of restriction types IIa0, IIa4, and IIb5. Of the HTLV-IIb restriction types observed in the U.S. non-Indians, 42.8% appear to have originated from the North Amerindian (IIb5), while 57.2% were similar to the European IIb4 restriction type. Sequences of 15 selected HTLV-II samples were determined and phylogenetically compared with 7 previously published HTLV-II LTR sequences. The derived topologies revealed three HTLV-IIa phylogroups (A-I to A-III) and four HTLV-IIb phylogroups (B-I to B-IV). Furthermore, the HTLV-IIa phylogroups appear to have evolved from the HTLV-IIb phylogroups. In the HTLV-IIa cluster, a Navajo (A-I) and a Brazilian (A-II) sequence formed separate phylogroups, while the remaining IIa sequences formed a single phylogroup (A-III). The four HTLV-IIb phylogroups were represented predominantly by a New York IDU (B-I), European IDU (B-II), North Amerindian and NRA (B-III), and Central Guaymi Indian (B-IV) sequence(s). Comparison of the phylogenetic data with the RFLP results revealed that results of the two methods correlated completely, demonstrating the ability of the RFLP method to predict the phylogroup of HTLV-II-infected samples accurately and quickly.

Human T-cell lymphotropic viruses type I (HTLV-I) and HTLV-II are closely related oncoviruses in the *Retroviridae* family (40). Unlike HTLV-I, little is known about the natural history of infection and pathogenicity of HTLV-II. HTLV-I has been clinically associated with adult T-cell leukemia/lymphoma (57), HTLV-I-associated myelopathy/tropical spastic paraparesis (18), and polymyositis (39). HTLV-II has not yet been clearly associated with any disease, though isolated cases of atypical T-cell hairy-cell leukemia or large granular lymphocyte leukemia (21, 26, 35, 38, 45), and "tropical" ataxic neuropathy (47) have been reported in HTLV-II-infected patients. Like HTLV-I, HTLV-II is transmitted sexually, from mother to child (27, 31, 37), and by contaminated blood through transfusion or needle sharing among intravenous drug users (IDU) (27, 37).

Infection with HTLV-I is endemic in Melanesia, Japan, the Caribbean, and sub-Saharan Africa (27, 37). Seroprevalence studies have demonstrated that HTLV-II is prevalent among IDU populations in the United States (27, 37), Italy (3, 59), Spain (50), France (56), Norway (13), and the United Kingdom (27, 37, 53). Additional seroprevalence studies have shown endemic foci of HTLV-II in Amerindians (1, 9, 12, 14, 25, 30, 34, 36). These studies led to the assumption that HTLV-I is an Old World virus and HTLV-II is a New World virus (10, 36). However, recent observations of a high HTLV-II seroprevalence among Pygmies in Zaire and Cameroon (19) and of rare HTLV-II infection in several central and West African countries (2) have raised doubts as to the New World origin of HTLV-II.

The study of the genetic heterogeneity of HTLV-II is important for several reasons. First, it provides a laboratory tool for epidemiologic studies to monitor viral transmission or to identify genetic markers of possible pathogenicity. Second, it provides insights into the origin of HTLV-II and the movement of ancient populations. While such studies have been successfully applied to HTLV-I (5, 6, 16, 28, 29, 48, 55), little is known about the genetic diversity of HTLV-II.

Recently, Hall et al. demonstrated two subtypes of HTLV-

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II, designated HTLV-IIa and HTLV-IIb, in eight IDU from New York (20). This differentiation was based on restriction mapping and a 4.3% sequence divergence of the gene encoding the envelope transmembrane protein (52). The HTLV-IIa sequences were more similar to the sequence of the prototypic MoT isolate, whereas the HTLV-IIb sequences were closer to the sequence of the NRA isolate. This *env*-based clustering was further supported by Dube et al., who examined 140-bp *pol* fragments from 17 Amerindians and North Americans and demonstrated that the HTLV-II sequences of the Amerindian samples were similar to the subtype IIb sequence, while the HTLV-II sequences of the North American samples were a mixture of subtype IIa and IIb sequences (8).

On the basis of analysis of the gp21 region of HTLV-II, other studies demonstrated the existence of both subtypes in the indigenous New World population and in the IDU population and hypothesized that HTLV-II in the IDU population may have originated from the endemically infected Amerindians (23). However, this hypothesis could not be adequately addressed by analyzing the gp21 region of HTLV-II, which shows a high degree of sequence homology and discriminates only between two subgroups. Thus, it is important to analyze other gene regions to understand the genetic heterogeneity, the global distribution of various HTLV-II subtypes, and the origin of HTLV-II.

In contrast to the low sequence diversity in the gp21 region of HTLV-II, analysis of previously published sequences of HTLV-II isolates, including NRA and G12 (Guaymi), have revealed the long terminal repeat (LTR) to be the most divergent region within the HTLV-II genome (8, 33, 43, 49, 58). The LTR is a noncoding region and may not be subjected to the same strict environmental selection forces of the structural genes of HTLV-II. Therefore, to expand our understanding of the genetic diversity in HTLV-II, we analyzed the LTR region by using two methods, restriction fragment length polymorphism (RFLP) and sequence analysis. A similar approach demonstrating the simplicity and rapidity of the RFLP technique has been successfully applied to the study of the genetic heterogeneity of HTLV-I (29). The current study evaluated the prevalence of 11 LTR restriction types in a large number of HTLV-II-infected individuals from different geographic regions. Selected HTLV-II samples with unique restriction patterns, and additional European and Amerindian samples, were sequenced and phylogenetically compared with previously published HTLV-II LTR sequences. The implications of these findings on the phylogeny of HTLV-II and the usefulness of the LTR-based RFLP method in studying the molecular epidemiology of HTLV-II are discussed.

MATERIALS AND METHODS

Study population. Peripheral blood mononuclear cells (PBMC) from 169 HTLV-II-infected individuals were collected and stored at −80°C (Table 1). These subjects included 84 blood donors and 1 child (81 from the United States and 3 from Brazil), 4 prostitutes and 1 child from Mexico, 32 IDUs and 1 child from the United States, 16 IDUs from Europe, and 30 Amerindians residing in Florida (10 Seminole), New Mexico (6 Pueblo and 2 Navajo), and Panama (12 Guaymi). HTLV-II infection in all subjects has been previously confirmed by PCR or by type-specific serologic tests (Western blot 2.3; Genelabs Diagnostics, Singapore).

PCR. Cryopreserved PBMC were lysed at a concentration of 6×10^6 cells per ml in PCR lysis buffer supplemented with 60 μg of proteinase K per ml at 56°C for 1 h (22). The proteinase K was then inactivated at 94°C for 10 min, and the PCR lysates were stored at −20°C. Twenty-five-microliter aliquots of PCR lysates were used in first-round amplification of the LTR and gp21 sequences under conditions described previously (22). The DNA primers BSQF6 (5′ CAG GGC GAG TCA TCG ACC CAA AAG 3′) and BSDR3 (5′ GAA GAC AAT GCT CCT AGG GCG GGC 3′) were used to amplify a 712-bp LTR sequence, while the primers GP21FA (5′ TGC TAC CAA CCT CGC CTA CAG 3′) and GP21RA (5′ GTG GAT GGG TCA ATG GTA GGG 3′) were used to amplify a 689-bp gp21 DNA fragment. Five microliters of the first-round amplification product was used in a nested PCR assay to amplify a 672-bp LTR product and a 630-bp gp21 product, using primer pairs BSQF2 (5′ ACC GTC TCA CAC AAA CAA TCC C 3′)-BSDR4 (5′ GCG GGC CTG CCT ATA GCG ATG 3′) and GP21F1 (5′CTG CAA CAA CTC CAT TAT CCT 3′)-GP21R1 (5′ CTG CAG AAG CTA GCA GGT CTA 3′), respectively. To confirm their identify, the amplified products were Southern blot hybridized to the ^{32}P-labeled oligoprobes BSQR2 (5′ ACG CTT TTA TAG ACT CGG CAT C 3′) and GP21P2 (5′ CCT CCT CCG GCG ACA AGA CG 3′) for the LTR and gp21 sequences, respectively (22). Negative controls included reactions using uninfected PBMC or Hut78 (uninfected T-cell line) DNA lysates and water.

Restriction endonuclease analyses. The DNASIS software program was used to generate restriction enzyme maps from the published HTLV-II LTR sequences of MoT (49), NRA (33), G12.1 (43), HTLV-IIa E.D. and HTLV-IIb J.G. from New York, N.Y. (52), and Gu and Va from Italy (58), as well as five other HTLV-IIa and HTLV-IIb sequences identified in this study as described below.

The restriction enzyme digestion consisted of 8 μl of the nested LTR or gp21 PCR products, 3 to 14 U of restriction enzyme, and 1 μl of specific enzyme buffer supplied by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). The enzyme reaction was allowed to proceed for 2 h at 37°C. The entire restriction digest reaction was electrophoresed in a 1.8% agarose gel and visualized after staining with ethidium bromide. Positive controls were included with each restriction enzyme digest to confirm the quality of the restriction enzymes and to provide the expected restriction fragment patterns.

Cloning and sequencing of LTR PCR fragments. Fifteen samples were selected for sequencing to confirm their distinct RFLP patterns and to permit their phylogenetic analysis. Six HTLV-IIa samples were from California (LA8A), Georgia (ATL18), New Mexico (NAV.DS, PUEB.RB), Brazil (BRAZ.A21), and Norway (NOR2N), and nine HTLV-IIb samples were from Florida (SEM1050, SEM1051), New Mexico (PUEB.AG), New York (NY185), Pennsylvania (PENN7A), Italy (ITA47A, ITA50A), and Spain (SPAN129, SPAN130). The PCR-amplified LTR sequences from these HTLV-II-infected individuals were cloned into the pT7Blue vector (Novagen, Madison, Wis.), and plasmid DNA from recombinant clones was extracted by using the Qiagen midiprep system (Qiagen, Studio City, Calif.). The LTR inserts from two to three clones of each sample were sequenced in both directions, using Sequenase according to the manufacturer's instructions (United States Biochemical, Cleveland, Ohio). In the rare cases when sequence differences were seen among multiple clones, a consensus sequence was generated by using the Wisconsin Genetics Computer Group sequence analysis software (7) on a VAX computer system.

Phylogenetic analysis. Twenty-two HTLV-II LTR sequences and one HTLV-I (strain ATK used as an outgroup) LTR sequence were aligned by using the Clustal version V multiple alignment program (included in the Genetic Data Environment package [15]), which provides the interface to the phylogenetic programs on a Sun SPARC workstation. The gaps and regions flanking the gaps were trimmed until a reliable alignment could be established to eliminate the source of random and systematic errors (see Fig. 4). As a result of this trimming, some conserved regions of the HTLV-II sequences were removed in order to maintain the proper alignment with the HTLV-I_{ATK} LTR sequence. The final length of the edited sequences was 553 bp and comprised a total of 56 variable sites. Phylogenetic analysis was done by using the maximum-likelihood method with the fastDNAML program, version 1.0.8, which utilizes randomized data input and global rearrangement (32). Additionally, the neighbor-joining method included in the Phylip 3.5c package (11) was used, with and without bootstrapping. The Treetool program was used to display the phylogenetic trees (32). The stability of the topology of the tree was tested by pruning. Pruning consists of removing one species from the alignment and rerunning the phylogenetic analysis. This process is repeated with each sequence in the alignment.

Nucleotide sequence accession numbers. The GenBank accession numbers of the HTLV-II LTR fragments sequenced in this study (see Fig. 4) are as follows: U10252, strain ATL18; U10253, strain BRAZ.A21; U10254, strain ITA47A; U10255, strain ITA50A; U10256, strain LA8A; U10257, strain NAV.DS; U10258, strain NOR2N; U10259, strain NY185; U10260, strain PENN7A; U10261, strain PUEB.AG; U10262, strain PUEB.RB; U10263, strain SEM1050; U10264, strain SEM1051; U10265, strain SPAN129; and U10266, strain SPAN130.

RESULTS

RFLP analysis. From the restriction maps generated by DNASIS, the following restriction endonucleases were chosen on the basis of their ability to differentiate between the sequences within the HTLV-IIa and HTLV-IIb groups: *Dra*II, *Ava*II, *Bgl*I, *Ban*II, *Sau*I, *Sac*II, and *Xho*I. The relative sequence locations of these restriction sites for the 11 restriction types are depicted in Fig. 1. The nomenclature of the specific HTLV-IIa0 to HTLV-IIb5 restriction types was arbitrarily assigned to these sequences. Five of the eleven restriction types

TABLE 1. Summary of RFLP analysis categorized by geographic location and risk behavior

Location and risk	No.[a]											
	Subtype a						Subtype b					
	n	a0	a1	a2	a3	a4	b0	b1	b2	b3	b4	b5
North America												
Blood donors												
Washington	3	2									1	
California	30	21			5						1	3
New Mexico	4	2			2							
Kansas	3	3										
Illinois	9	7			2							
Oklahoma	1	1										
Texas	1	1										
New York	3	2									1	
Pennsylvania	3											3
Tennessee	1										1	
Louisiana	8	6									1	1
Child	1	1										
Georgia	15	10		2	1						1	1
IDUs												
California	1				1							
Oklahoma	1	1										
New York	3	1									2	
Louisiana	27	21			1						4	1
Child	1				1							
Indian												
Navajo	2					1						1
Pueblo	6	3				1						2
Seminole	10											10
Subtotal (%)	133	82 (61.7)	0	2 (1.5)	13 (9.8)	2 (1.5)	0	0	0	0	12 (9.0)	22 (16.5)
Central and South America												
Mexico												
Prostitutes	4	1										3
Child	1	1										
Panama (Guaymi Indian)	12							12				
Brazil (blood donors)	3	3										
Subtotal (%)	20	5 (25.0)	0	0	0	0	0	12 (9.0)	0	0	0	3 (15.0)
Europe												
IDUs												
Italy	7										7	
Spain	4										4	
Norway	5			5								
Subtotal (%)	16	0	0	5 (31.3)	0	0	0	0	0	0	11 (68.8)	0
Total (%)	169	87 (51.5)	0	7 (4.1)	13 (7.7)	2 (1.2)	0	12 (7.1)	0	0	23 (13.6)	25 (14.8)

[a] Restriction types IIa0, IIa1, IIa2, IIa3, and IIa4 represent MoT, E.D. from New York, N.Y., ATL18 from Atlanta, Ga., LA8A from Los Angeles, Calif., and NAV.DS from a Navajo Indian from New Mexico, respectively. Restriction types IIb0, IIb1, IIb2, IIb3, IIb4, and IIb5 represent NRA, G12 from a Guaymi Indian from Panama, J.G. from New York, N.Y., GU and VA from Italy, ITA47A from an Italian, and SEM1051 from a Seminole Indian from Florida, respectively.

identified within the U.S. samples were based on unique restriction sites previously not found in the known HTLV-II LTR sequences. Within the IIa group, the presence of a *Xho*I site was termed restriction type HTLV-IIa2, the presence of an additional *Ban*II site was termed restriction type HTLV-IIa3, and the presence of a *Sac*II site and an additional *Ban*II site was termed restriction type HTLV-IIa4. Within the IIb group, the presence of an additional *Ban*II site and the loss of an *Ava*II site was termed restriction type HTLV-IIb4, and the presence of only one *Ban*II site and one *Ava*II site was termed restriction type HTLV-IIb5.

HTLV-IIa and HTLV-IIb typing. The restriction map of the LTR revealed the presence of a *Dra*II restriction endonuclease site in only the HTLV-IIb sequences. To determine whether this enzyme could be used to segregate the HTLV-IIa and HTLV-IIb groups, the LTR and gp21 sequences were amplified from 147 of 169 HTLV-II samples and digested with *Dra*II and *Xho*I, respectively. *Xho*I digestion of the gp21 region has

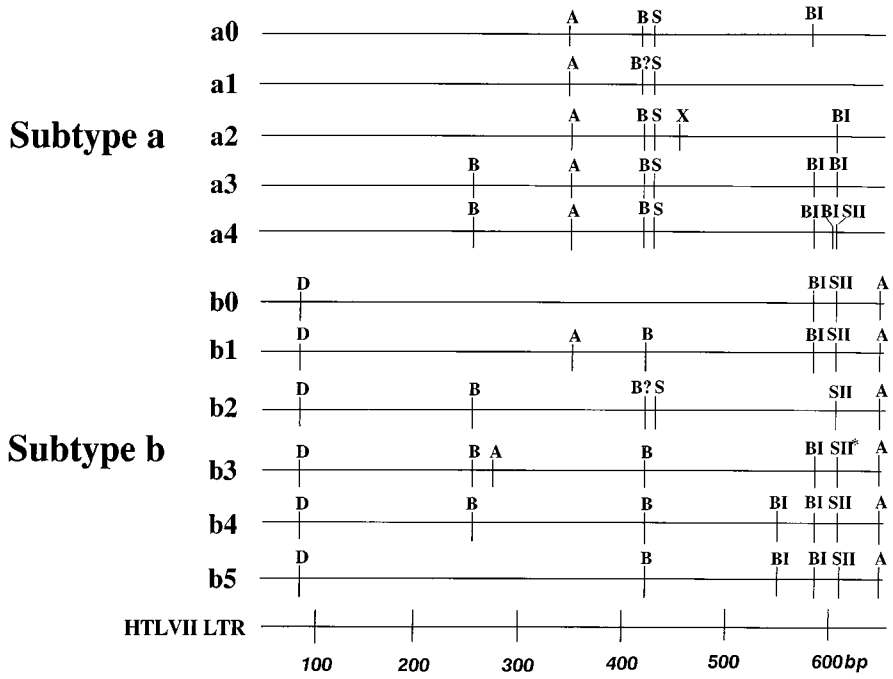

FIG. 1. Positions of selected restriction endonuclease sites of HTLV-II LTR sequences (positions 85 to 688, based on HTLV-II_{MoT}). Abbreviations: A, *Ava*II; B, *Ban*II; B?, potential *Ban*II site but sequence data unavailable; BI, *Bgl*I; D, *Dra*II; S, *Sau*I; SII, *Sac*II; X, *Xho*I. *, the restriction endonuclease site is absent in HTLV-II_{GU} but is present in HTLV-II_{VA}.

been demonstrated to distinguish between the IIa and IIb subgroups (20). A 100% correlation was observed when the results of the two enzyme digests were compared (Fig. 2). In view of the high correlation, the remaining 22 samples were subtyped as HTLV-IIa or HTLV-IIb, using only the *Dra*II enzyme. Of the 169 samples, 109 (64.5%) were HTLV-IIa and 60 (35.5%) were HTLV-IIb.

The LTR-based RFLP technique, using all seven restriction endonucleases, was then applied to 169 specimens to determine the relationship of a particular restriction type to a geographical location. Representative digests showing the expected RFLP of eight restriction types are shown in Fig. 3. Seven of the eleven possible restriction types were observed in the study population (Table 1). Of 109 HTLV-IIa specimens, IIa0 was identified in 87 samples (51.5%), IIa2 was identified in 7 samples (4.1%), IIa3 was identified in 13 samples (7.7%), and IIa4 was identified in 2 samples (1.2%). Of 60 HTLV-IIb specimens, IIb1 was identified in 12 samples (7.1%), IIb4 was identified in 23 samples (13.6%), and IIb5 was identified in 25 samples (14.8%). The HTLV-IIa1, HTLV-IIb0, HTLV-IIb2, and HTLV-IIb3 restriction types were not found in any specimens tested.

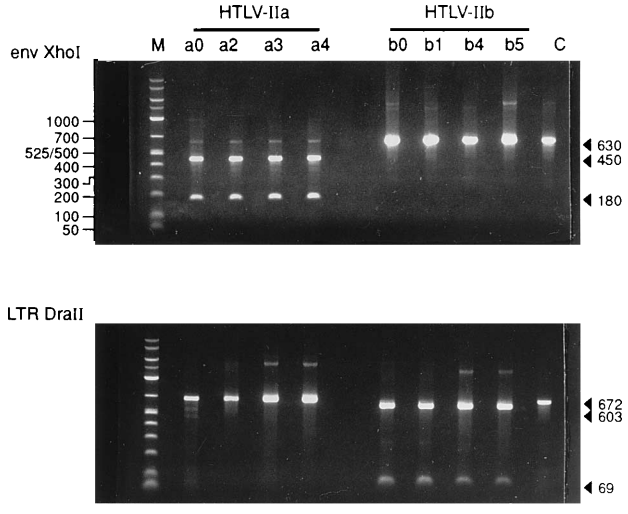

FIG. 2. Comparison between HTLV-IIa and HTLV-IIb subtyping by *Xho*I digestion of gp21 and *Dra*II digestion of the LTR. Included are representative samples from each restriction type. The expected fragment sizes (in base pairs) for each restriction digest are given at the right. Lane M, molecular weight marker (EXT+; AT Biochem, Malvern, Pa.); lane C, undigested DNA control.

When the various restriction types within U.S. and non-U.S. specimens were compared, the predominant restriction type in the U.S. specimens was found to be IIa0 (MoT-like), at a prevalence of 61.7% (82 of 133 samples). Two samples were similar to restriction type HTLV-IIa2, 13 samples were similar to restriction type HTLV-IIa3, and 2 other samples were similar to restriction type HTLV-IIa4. Of the 36 non-U.S. specimens, only 10 were subtype HTLV-IIa. Five samples from South America were IIa0, and all five samples from Norway were IIa2. Of the 34 U.S. HTLV-IIb samples, 13 were like the Italian HTLV-IIb4 restriction type and 21 samples were identified as restriction type HTLV-IIb5. None of the U.S. samples were similar to the NRA isolate (restriction type IIb0) or to the HTLV-II J.G. sequence from New York (restriction type IIb2) or the Guaymi Indian restriction type IIb1. Among the non-U.S. specimens, seven Italian and four Spanish samples were all restriction type HTLV-IIb4 and three Mexican samples were typed as HTLV-IIb5.

A comparison of restriction types within Indian and non-Indian populations demonstrated that all Seminole and Guaymi Indian samples were HTLV-IIb, whereas the eight Navajo and Pueblo Indian samples were a mixture of five HTLV-IIa (63%) and three HTLV-IIb (37%) restriction types.

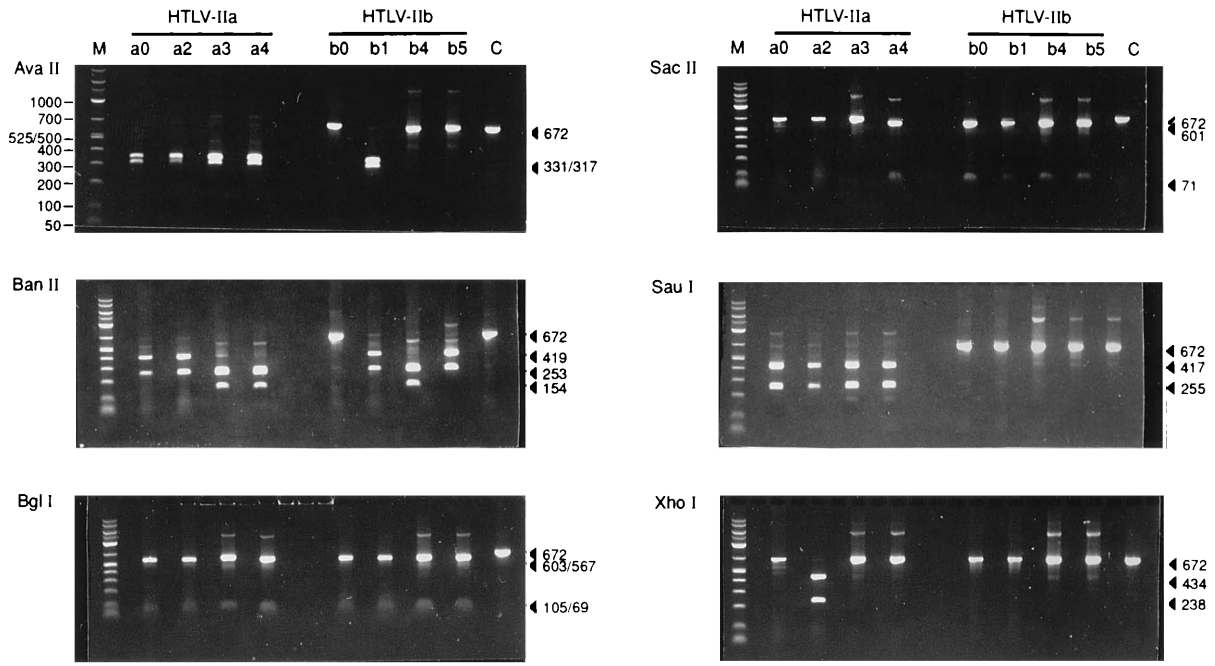

FIG. 3. RFLP patterns of representative samples from each restriction type, using the six endonucleases described in Materials and Methods. The expected fragment sizes (in base pairs) for each restriction digest are given at the right. Lane M, molecular weight marker; lane C, undigested DNA control.

While the majority of the Navajo and Pueblo Indian HTLV-IIa samples were restriction type IIa0, one sample from each group was restriction type IIa4. The non-Indian HTLV-IIa samples were a mixture of restriction types IIa0, IIa2, and IIa3. Within the HTLV-IIb group, the RFLP data show that HTLV-IIb4 and IIb5 are the only subtypes present in both the U.S. Indian and non-Indian populations. Interestingly, all of the North American Indian HTLV-IIb subtypes were HTLV-IIb5. All 12 samples from the Guaymi Indians from Panama were restriction type HTLV-IIb1.

Restriction type association with HTLV-II transmission. Four pairs of samples in the study population were epidemiologically linked: three from mother-child pairs in Louisiana and Mexico and one from sex partners in California. In two mother-child pairs, both the mother and child had restriction type HTLV-IIa0. The third mother-child pair had the HTLV-IIa3 restriction type. Similarly, both sex partners were type HTLV-IIa3. Samples from the mother-child pair from Mexico had been sequenced previously and shown to have a 100% identity in the U3 region of the LTR (31).

Analysis of LTR sequences. Samples that had the newly defined HTLV-IIa2, HTLV-IIa3, HTLV-IIa4, HTLV-IIb4, and HTLV-IIb5 restriction types were analyzed further by DNA sequencing. The sequence data of these samples confirmed the presence of the restriction enzyme sites that were previously detected by the RFLP technique. For example, a C→T substitution at position 501 relative to MoT in the HTLV-II LTR sequences from Atlanta, Ga., and Norway resulted in the formation of a *Xho*I restriction site and the subsequent classification of these samples as HTLV-IIa2 (Fig. 3).

A comparison of the 7 previously published LTR sequences and the 15 LTR sequences from the current study revealed a range of nucleotide divergence of 3.4 to 6.4% between the HTLV-IIa and HTLV-IIb sequences. Within the HTLV-IIa sequences, the range of divergence was 0.5 to 1.6%, whereas that within the HTLV-IIb sequences was 0.2 to 3.0%. Analysis of the functionally important domains in the LTR region demonstrated that the transcriptional elements, the TATA box and the poly(A) signal, were highly conserved in all samples sequenced (Fig. 4). Similarly, all three 21-bp repeat elements within the U3 region of the LTR were conserved among the HTLV-IIa sequences and HTLV-IIb sequences, with the exception of a C→A substitution in the second direct repeat in all the HTLV-IIb samples that resulted in the formation of a *Dra*II restriction site. This *Dra*II restriction site was used in the RFLP method to distinguish between the HTLV-IIa and HTLV-IIb subtypes.

To examine in detail HTLV-IIa and HTLV-IIb genetic variations, an alignment of all new and previously published LTR sequences was generated. The length of the aligned sequences used for the phylogenetic analysis was 553 bp and comprised a total of 56 variable (or informative) sites. The *Dra*II, *Bgl*I, *Ava*II, *Sau*I, and *Sac*II restriction sites were located at 5 of these 56 characteristic informative sites. One of the *Ban*II sites is found in a variable region in this alignment, while the second potential *Ban*II restriction site was removed from the alignment during the editing of the sequences that was necessary for further phylogenetic analysis. At 15 of these variable sites, a characteristic HTLV-IIa or HTLV-IIb nucleotide pattern was observed (Fig. 4). Interestingly, the Navajo (NAV.DS) LTR sequence, which is restriction type IIa4, contained nucleotide substitutions at four other variable sites that were identical to those observed in the HTLV-IIb group (Fig. 4). To confirm the presence of these four nucleotides, four additional LTR clones from this sample were sequenced and were found to have the exact nucleotide substitutions (data not shown).

Phylogenetic analysis. The results of the phylogenetic analysis demonstrate that the HTLV-II sequences cluster into two separate groups, HTLV-IIa and HTLV-IIb (Fig. 5). Four separate phylogenetic clusters within the HTLV-IIb restriction types, termed B-I, B-II, B-III, and B-IV, were derived from the

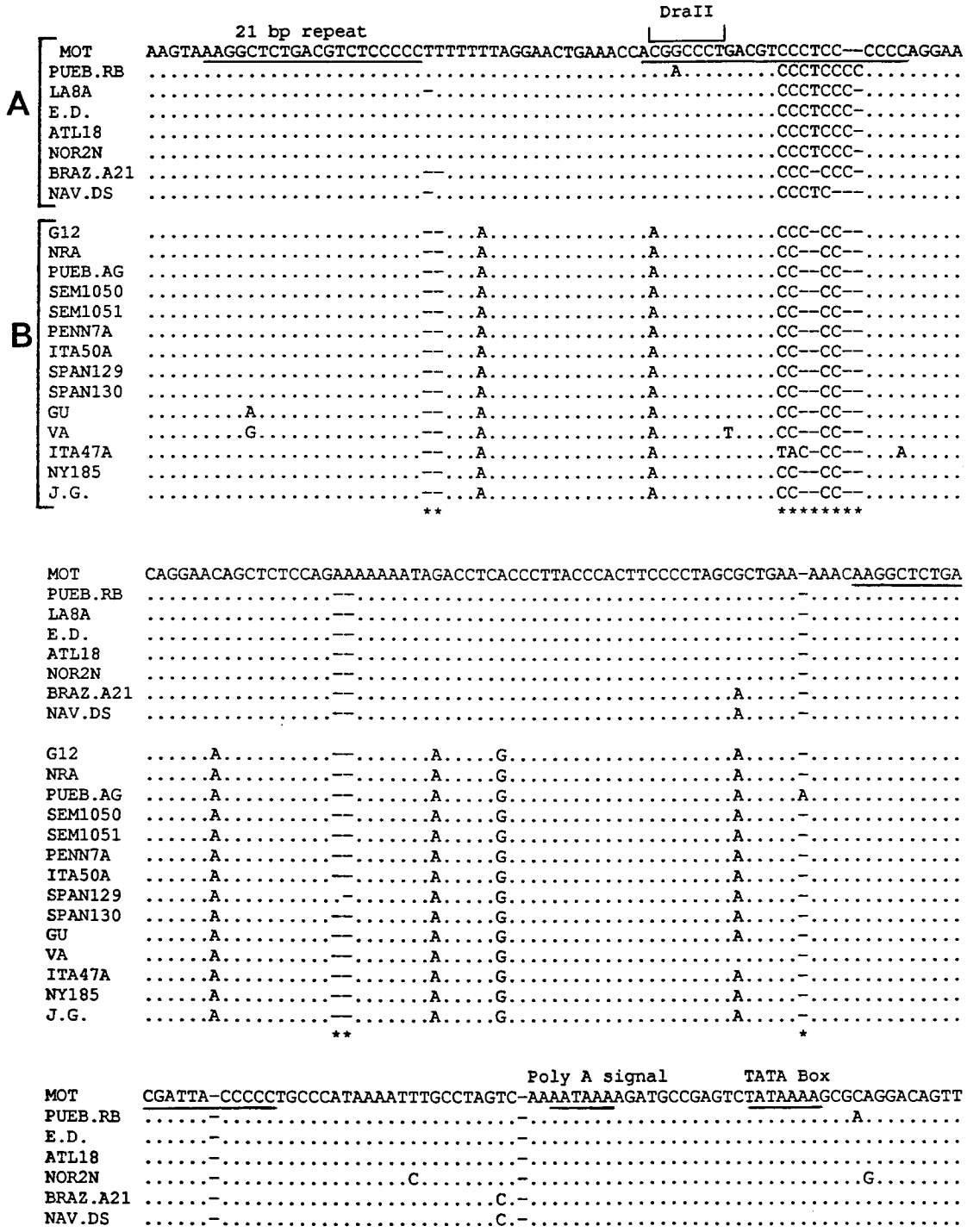

FIG. 4. Characteristic nucleotide patterns of aligned HTLV-II LTR sequences. The sequences are classified into the two major HTLV-IIa (A) and HTLV-IIb (B) subtypes. Dots represent nucleotides homologous to the MoT LTR sequence. Gaps introduced to maintain the alignment are indicated by dashes. Asterisks indicate the nucleotides that were removed from the sequences for the phylogenetic analysis. The location of the *Dra*II restriction site is indicated with brackets. The 21-bp direct repeats are underlined, and known regulatory motifs are underlined and labeled.

J.G. sequence from New York; the European sequences (ITA47A, ITA50A, SPAN129, SPAN130, GU, and VA) and one North American sequence (NY185); the NRA and North American Indian sequences and the sequence of one strain from Pennsylvania (PENN7A); and the Guaymi (G12) sequence, respectively. While the J.G. sequence from New York appears to be the ancestor of the other three phylogroups, another sequence from New York (NY185) clustered with the phylogroup created predominantly by European strains. Unlike the HTLV-IIb restriction type, the HTLV-IIa cluster showed less heterogeneity and formed three well-separated phylogenetic groups: A-I was the Navajo sequence, A-II was

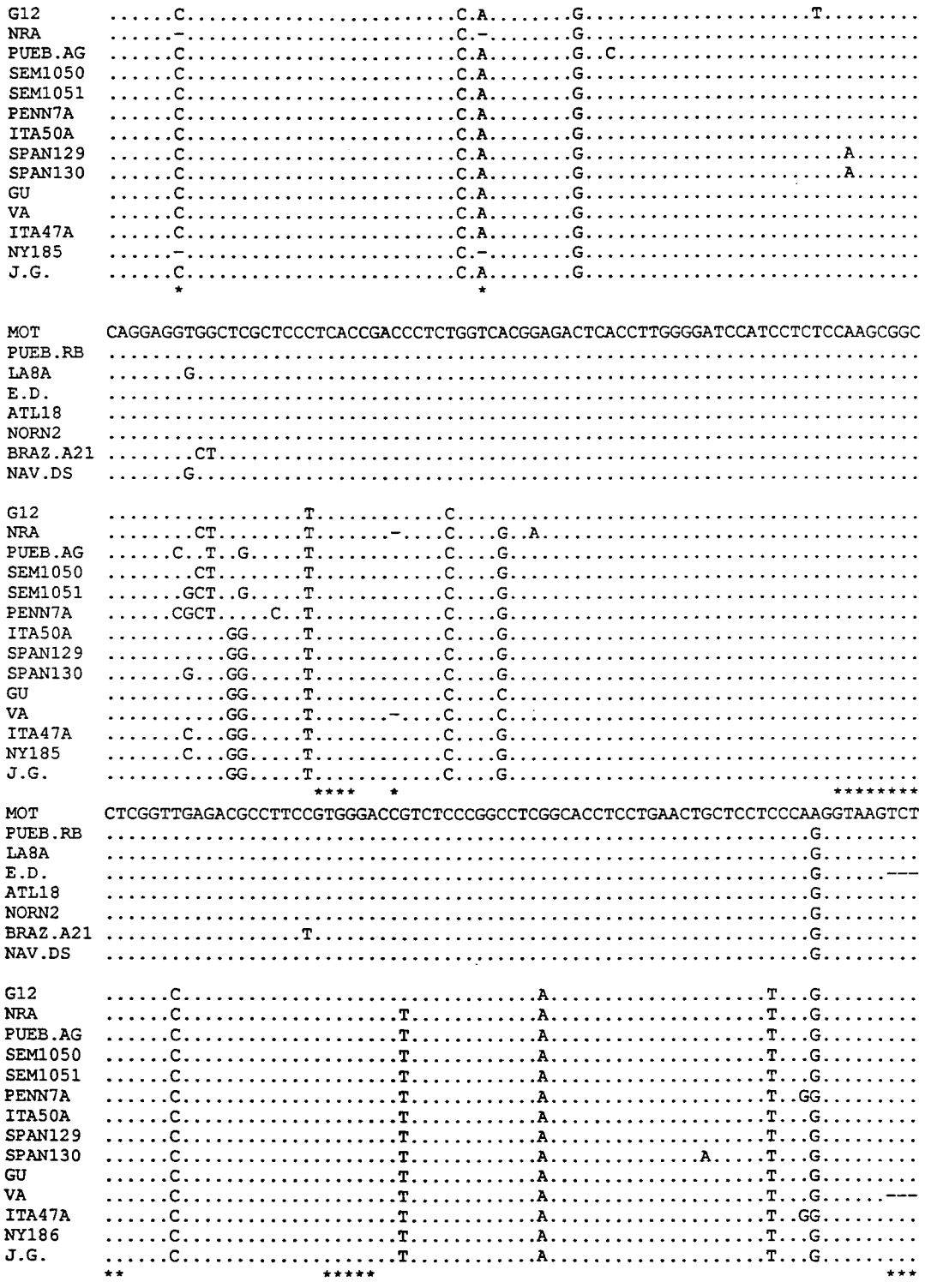

FIG. 4—*Continued.*

the Brazilian sequence, and A-III comprised MoT sequences and representative sequences from Los Angeles, Atlanta, and Norway. Statistical evaluation of the data provided by the maximum-likelihood analysis showed *P* values ranging from less than 0.05 to less than 0.01 for all final branching orders, indicating a high statistical significance for the final tree. The reliability of these phylogenetic results was validated by obtaining essentially identical topologies of the phylogenetic trees with both the maximum-likelihood and neighbor-joining methods. Furthermore, the topology of the final tree was found to be stable before and after bootstrapping with 100 rounds of replication as well as through pruning.

```
MOT       CCTCTCAGGTCGAGCTCGGCTGCCCCTTAGGTAGTCGCTCCCCGAGGGTCTTTAGAGACACCCGGGTTTCGCCTG
PUEB.RB   ....................................................................................
LA8A      ....................................................................................
E.D.      ----------------.....................................................T..............
ATL18     ........................................T...........................................
NORN2     ........................................T...........................................
BRAZ.A21  ....................................................................................
NAV.DS    ....................................................................................

G12       ........................T.................................................C.......
NRA       ........................T.................................................C.......
PUEB.AG   ........................T.................................................C.......
SEM1050   ........................T.................................................C.......
SEM1051   ........................T.................................................C.......
PENN7A    ........................T.................................................C.......
ITA50A    ........................T.................................................C.......
SPAN129   ........................T.................................................C.......
SPAN130   ........................T.................................................C.......
GU        ........................T.................................................C.......
VA        ------------------.......T.................................................C.....A
ITA47A    ........................T.................................................C.......
NY185     ........................T.................................................C.......
J.G.      ........................................................................C.......
          ****************  ****                          **

MOT       CGCTCGGCTAGACTCTGCCTT-AAACTTCACTTCGCGTTCTT-GTCTCGTTCTTTCCTCTTCGCCGTCACTGAAA
PUEB.RB   ......................-.....................-.............................
LA8A      ......................-.....................-.............................
E.D.      ......................-.....................-.............................
ATL18     ......................-.....................-.............................
NORN2     ......................-.....................-.............................
BRAZ.A21  ..................G...-.....................-..........A..................
NAV.DS    ..................G...-.....................-.............................

G12       ..................G...G....C...............G.....................T...........
NRA       ..................G...-....C...............-.....................T...........
PUEB.AG   ..................G...G.....................G.....................T...........
SEM1050   ..................G...G....C...............G.....................T...........
SEM1051   ..................G...G....C...............G.....................T...........
PENN7A    ..................G...G....CC..............G.....................T...........
ITA50A    ..................G...A....C...............G.....................T...........
SPAN129   ..................G...A....CC..............G.....................T...........
SPAN130   ..................G...A....CC............C.G.....................T...........
GU        ..................G...A....C...............G.....................T...........
VA        ..................G...A....C...............G.....................T...........
ITA47A    ..................G...G....C...............G.....................T...........
NY185     ..................G...A....C...............G.....................T...........
J.G.      ..................G...A....C...............G.....................T...........
                             *      ******          *

MOT       ACGAAACCTCAACGCCGCCC-TCCTGGCAGGC--GTCCCGGGGCCAACATAC-GCCGTGGA-GCGCAGCAAGGGC
PUEB.RB   ......................-TCC........TTGG.................T........GGC...........
LA8A      ......................-TCC........TTGG.................T........GGC...........
E.D.      ......................-TCC..A.....TTGG.................T........-GC...........
ATL18     ......................-TCC..A.....TTGG.A...............T........GGC...........
NORN2     ......................-TCC..A.....TTGG.................T........GGC...........
BRAZ.A21  ......................-TCC........TTGG.................T........GGC...........
NAV.DS    ......................-TCC........TTGG.................T....C...GGC...........

G12       .......T............TTC-........TTGG.................T....C...-GC....T.......
NRA       .......T............TTC-........--GG..........G.....-....C...-GC....T.......
PUEB.AG   .......T............CTC-........TTGG.................T....C...GGC....T.......
SEM1050   .......T............TTC-........TTGG.................T....C...GGC....T.......
SEM1051   .......T............TTC-........TTGG.................T....C...GGC....T.......
PENN7A    .......T............TTT-........TTGG.................T....C...GGC....T.......
ITA50A    .......T............TTT-........TTGG.................T....C...GGC....T.......
```

FIG. 4—*Continued.*

Comparison of RFLP and phylogenetic results. To evaluate the ability of the RFLP technique to predict the phylogenetic clustering of the HTLV-II samples, the results of the two methods were compared. Although only 7 of the 11 restriction types were found in the current study population, combinations of these 11 restriction types were dispersed into seven separate phylogroups (Fig. 5). Phylogroups B-I through B-IV contained strains subtyped as HTLV-IIb, while phylogroups

```
SPAN129  .......T............TTC-........TTGG.................T...AC...GGC....T......
SPAN130  .......T............TTC-........TT-C....T...........T....C...GG-....T......
GU       .......T............TTC-........TTGG.................T........GG-....T......
VA       .......T.......G....TTT-........TTGG.................T....C...GGC....T......
ITA47A   .......T............TTC-........TTGG.................T....C...GGC....T......
NY185    .......T............TTC-........TTGG.................T....C...GGC....T......
J.G.     .......T............TTCT........TTGG.............G.T....C...-GC....T......
                             ****        ****                 *         ***

MOT      TAGGGCTTCCTGAACCTCTCCGGGAGAGGTC
PUEB.RB  ..............................
LA8A     ..............................
E.D.     ..............................
ATL18    ..............................
NORN2    ..............................
BRAZ.A21 ..............................
NAV.DS   ..............................

G12      ..............................
NRA      ..............................
PUEB.AG  ..............................
SEM1050  ..............................
SEM1051  ..............................
PENN7A   ..............................
ITA50A   ..............................
SPAN129  ..............................
GU       ..............................
VA       ..............................
ITA47A   ..............................
NY185    ..............................
J.G.     ..............................
```

FIG. 4—*Continued.*

A-I through A-III contained strains subtyped as HTLV-IIa. The HTLV-IIb2 restriction type clustered in the B-I phylogroup, HTLVIIb-3 and HTLV-IIb4 restriction types clustered in the B-II phylogroup, the HTLV-IIb0 and HTLV-IIb5 restriction types clustered in the B-III phylogroup, and the Guaymi HTLV-IIb1 restriction type formed the B-IV phylogroup. The HTLV-IIa4 restriction types clustered into the A-I phylogroup, the Brazilian HTLV-IIa0 restriction types clustered into the A-II phylogroup, and the remaining HTLV-IIa0 restriction types as well as restriction types IIa1 through IIa3 formed the A-III phylogroup. These results suggest that RFLP can be used to reliably predict the phylogroup of an HTLV-II-infected sample. However, it may be necessary to confirm by DNA sequencing any unexpected or new RFLP patterns obtained with certain samples.

DISCUSSION

Phylogenetic analysis of the LTR region provides a powerful tool with which to examine the evolutionary relationships between HTLV-IIa and HTLV-IIb. Several observations can be derived from this analysis. First, the LTR-based classification of the samples as HTLV-IIa and HTLV-IIb fully correlated with the results obtained for the gp21 region. Second, the high sequence divergence in the HTLV-IIb group led to the recognition of four phylogroups that were predominantly resolved on the basis of geography and/or ethnicity and corresponded predominantly to sequences from a New York IDU (B-I), European IDU (B-II), North Amerindians (B-III), and a Guaymi Indian (B-IV). The ethnic variation among the American Indians may explain the different HTLV-II forms originally present in these Indian populations before or during their migration from Asia to North America. The Seminole, Pueblo, and Guaymi are believed to be descended from the Paleo-Indian (ancestral Amerind) migration, while the Navajo are thought to be descended from the more recent Nadene migration from Asia (54). Since the Guaymi have been living in relative isolation, it is possible that the B-IV phylogroup is a true representative of the HTLV-II form endemic in this group. However, the Navajo have extensively mixed genetically and culturally with the neighboring Pueblo (54). Therefore, it is hard to determine whether the B-III phylogroup truly represents the HTLV-II form present in the Pueblo. Third, all HTLV-IIa sequences grouped into a distinct cluster. Despite the low sequence divergence in this cluster, three phylogroups were identified and corresponded to the sequences from a Navajo (A-I), Brazil (A-II), and MoT (A-III). Fourth, a current phylogenetic tree suggests that the HTLV-IIa phylogroup has evolved from the HTLV-IIb phylogroup.

This observation contrasts with the previous findings which indicated that the HTLV-IIa and HTLV-IIb lineages originated from a single ancestor (8). The use in the present study of the maximum-likelihood method of phylogenetic inference may explain the differences seen between the phylogram derived by our analysis and that obtained by Dube et al., who used the parsimony method (PAUP) (8). To verify this point, we have analyzed our LTR sequences by using the DNA parsimony method in the Phylip 3.5c package. This method yielded a tree that is similar to that of Dube et al. (8) (data not shown). Therefore, these data clearly indicate that the use of different phylogenetic methods accounts for the differences seen in the respective trees. However, the topology of trees obtained by using the parsimony method may be influenced by this method's sensitivity to homoplastic sites and the so-called long-branch attraction (41, 51). Because of these inherent limitations in the parsimony method, we adopted the maximum-likelihood method for our phylogenetic analysis. The high statistical significance ($P < 0.01$) of the branching pattern between the HTLV-IIa and HTLV-IIb phylogroups in our tree and the stability of the tree, which was confirmed by pruning, support the reliability of our analysis.

The ability of the present RFLP system to subtype the HTLV-II specimens into seven restriction types demonstrated the discriminatory capability of this method. The successful use of *Dra*II to differentiate the HTLV-IIa and HTLV-IIb subgroups is advantageous because it obviates the need for simultaneous envelope-based subtyping. The phylogenetic analysis revealed the evolutionary relationship of the various restriction types and placed them among seven phylogroups. Therefore, the RFLP method can now be used to accurately predict predefined phylogroups. Of all of the phylogroups, only A-II was not represented by a distinct restriction type and is currently typed by RFLP as IIa0. This HTLV-II sequence from Brazil was not available when the present RFLP method was initially developed. However, there are several restriction enzymes, such as *Eco*47III and *Bbv*II, that distinguish the Brazilian sequence from IIa0. Therefore, it is possible that a fraction of the IIa0 specimens in our study population may actually belong to the A-II phylogroup.

The predominant subtype in the study population was HTLV-IIa, with a prevalence of 64.5%. This result may be due

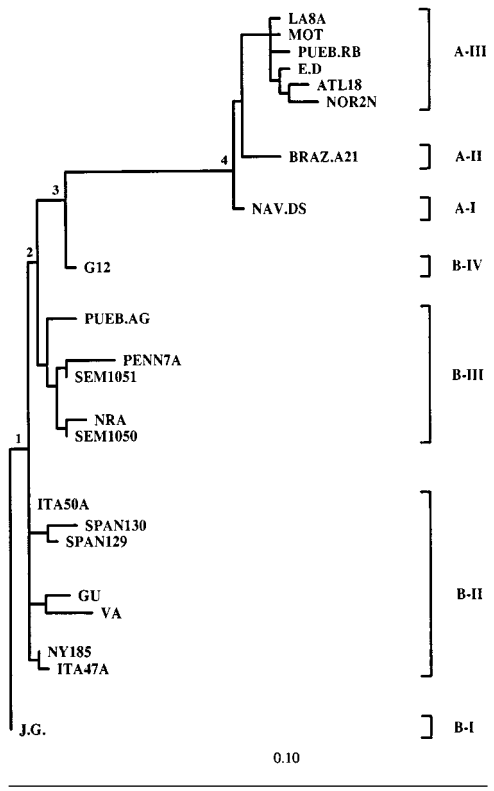

FIG. 5. Phylogenetic tree of HTLV-II strains. The tree was constructed by using the maximum-likelihood method after alignment of 556 bp of 7 previously published and 15 new HTLV-II LTR sequences (for details, see Materials and Methods). The scale bar shows the ratio of nucleotide substitution for a given horizontal branch length. Vertical distances are for clarity only. All major branches of the tree (numbered 1 to 4) had *P* values of less than 0.01. All other branch points had *P* values of less than 0.05 to less than 0.01. The outgroup sequence HTLV-I_{ATK} LTR (not shown on the phylogram for simplicity) branches from the line going from strain J.G., which is an ancestor to all other HTLV-II strains. Proposed phylogenetic groups within the major HTLV-IIa and HTLV-IIb subtypes are shown. J.G., E.D., and NY185 are from New York, N.Y.; ITA47A, ITA50A, GU, and VA are from Italy; SPAN129 and SPAN130 are from Spain; SEM1050 and SEM1051 are from Seminoles; NRA is HTLV-II_{NRA}; PENN7A is from Pennsylvania; PUEB.AG and PUEB.RB are from Pueblo Indians; G12 is HTLV-II_{G12} from a Guaymi Indian in Panama; NAV.DS is from a Navajo Indian; BRAZ.A21 is from Brazil; NOR2N is from Norway; ATL18 is from Atlanta, Ga.; MOT is HTLV-II_{MoT}; and LA8A is from Los Angeles, Calif.

to the presence of a large number of U.S. samples (n = 133), of which 74.5% were HTLV-IIa. Pardi et al. have also found a high prevalence (81.2%) of HTLV-IIa in 22 U.S. IDU samples (42). HTLV-IIa may represent the predominant group of HTLV-II which has initially spread among the non-Indian U.S. population. Alternatively, HTLV-IIa may be transmitted more efficiently than HTLV-IIb. It is conceivable that differences in the sequences between the two HTLV-II groups play a biological role affecting their transmission. The LTR sequence data demonstrated a pattern of at least 15 different nucleotides that was specific for each HTLV-II group. For instance, we identified a nucleotide substitution in the second 21-bp repeat of the U3 region which could potentially affect the function of this motif in transactivating viral expression, thereby altering the viral load and subsequently affecting the transmission of the virus.

The recognition of the HTLV-IIb phylogenetic group (B-III) that is specific to the North American Indians and is represented by restriction type HTLV-IIb5 allowed us to investigate the hypothesis that the North American Indians are the source of the HTLV-II in the non-Indian U.S. population (23). This theory is supported by the fact that unlike other Indians, such as the Guaymi, these groups have not been living in isolation and have had significant interactions, including blood donation and needle sharing, with non-Indian U.S. populations (23). Our data show that 42.8% of the HTLV-IIb non-Indian U.S. samples were HTLV-IIb5 while 57.2% were HTLV-IIb4. These results suggest that the HTLV-IIb5 infections in the non-Indian U.S. samples may have originated from the endemically infected North American Indians. The absence of Guaymi-like HTLV-II in the U.S. non-Indian samples was not surprising given the lack of interaction between the geographically isolated Guaymi Indians in Panama and the U.S. population (30). Although our data on the prevalence of the HTLV-IIb4 (B-II phylogroup) among the Spanish and the Italian IDU may suggest a European origin for the B-II phylogroup, the small number of European HTLV-II samples tested and the association of intravenous drug use with these infections make it difficult to determine with certainty the direction of spread of HTLV-IIb4 between Europe and the United States. In view of the close phylogenetic relationship of the B-II phylogroup to the Amerindian phylogroups (B-III and B-IV), it is tempting to speculate that the origin of the B-II phylogroup may be Amerindian. Nevertheless, sequences similar to those in the B-II phylogroup have yet to be identified among Amerindians to support such a speculation.

The origin of HTLV-IIa in the non-Indian population may be harder to resolve than that of HTLV-IIb because an endemic HTLV-IIa population has yet to be identified. The understanding of the evolution of HTLV-IIa should take into consideration the possible worldwide presence of this subtype, which was seen in IDU from Norway in this study and previously identified in a prostitute from Ghana (24). Whether HTLV-IIa originated from the HTLV-IIa-infected Navajo or Pueblo is still uncertain for the following reasons: (i) HTLV-IIa has been observed in only a fraction of the HTLV-II-infected Navajo and Pueblo (23, 42) and (ii) two HTLV-IIa restriction types were seen in the HTLV-II-infected Navajo and Pueblo. The first was similar to the predominant HTLV-IIa0 restriction type (MoT-like), and the second was the HTLV-IIa4 restriction type, which was seen in only one Navajo and one Pueblo. Taken together, our findings do not necessarily support the spread of these HTLV-IIa subtypes from the Navajo or Pueblo to the non-Indian U.S. population. On the contrary, the presence of HTLV-IIa0 among the Navajo and Pueblo can be interpreted as evidence for HTLV-IIa infections originating from the non-Indian U.S. populations. However, this question may be resolved with more certainty by the restriction typing of additional American Indian and non-Amerindian samples of known risk factor status.

Unlike HTLV-I, which is thought to have originated from Africa or Asia in the Old World (16, 44, 55), the origin of HTLV-II is still not clear. The endemicity of HTLV-II among the Amerindians, the recent isolation of a simian T-lymphotropic virus type II (STLV-II) from Central American spider monkeys (4), and the lack of evidence of STLV-II infection in Old World monkeys (46) suggested a New World origin for HTLV-II (10). However, the reports of HTLV-II infection in Gabon (17) and in isolated Pygmies in Africa (19) do not favor this hypothesis. As sequence information on the newly described STLV-II (4) or on HTLV-II from indigenous populations in different geographic areas such as Africa or Asia becomes available, definite conclusions about the origin of HTLV-II may be reached.

It is important to note that our RFLP-based results depend largely on the choice of the restriction endonuclease. Despite

the fact that a total of 169 samples were examined in this study and seven restriction endonucleases were used to differentiate among the restriction types, we were unable to find any of the IIa1, IIb0, IIb2, or IIb3 restriction types in this study population, although they were representative of previously published LTR sequences. These results may truly reflect the prevalence of these restriction types in our study population. Alternatively, these data may be due to the geographic sequestration of these types or to the choice of highly strain specific restriction endonucleases used to define these particular types. However, as additional HTLV-II LTRs (i.e., from Africa and/or Asia) are sequenced in the future, the current RFLP system will have to be modified to accommodate distinct subtypes. Quasispecies development in an individual may complicate the RFLP results, especially if sequence changes occur at the restriction sites used in the present RFLP technique. However, in the RFLP analysis of all 169 samples in the present study, no unexpected restriction patterns or incomplete digestion of PCR-amplified LTR products were seen to indicate the presence of quasispecies that differ at the restriction endonucleases used for their differentiation. In addition, RFLP analysis of 10 clones from two samples (NY185 and NAV.DS) showed identical results for all clones from each sample (data not shown). Also, analysis of LTR sequence heterogeneity of the two to three clones available on the samples sequenced in this study revealed a range from zero to three base substitutions for the 629-bp fragment (giving a divergence range of 0.0 to 0.5%) at positions that did not affect the RFLP testing. Taken together, these data suggest that quasispecies development within HTLV-II-infected individuals is very limited and is unlikely to alter the RFLP testing results.

Because the LTR-based RFLP method is simple, rapid, and discriminatory, it will be useful in molecular epidemiologic investigations and provide a viable alternative to the more laborious nucleotide sequencing technique, especially if a large number of samples are examined. Also, our data on four pairs of samples (three mother-child and one sex partners) revealed concordant restriction typing results to provide evidence for the vertical and horizontal transmission of HTLV-II in these individuals. This finding further illustrates the usefulness of the LTR-based RFLP method in the study of the transmission of HTLV-II.

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