

# Genetically Related Human Immunodeficiency Virus Type 1 in Three Adults of a Family with No Identified Risk Factor for Intrafamilial Transmission

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**A small number of cases of human immunodeficiency virus (HIV) infection have been reported in individuals with no identified risk factors for transmission. We report on the seroconversion of the 61-year-old mother and the subsequent finding of HIV seropositivity in the 66-year-old father of a 31-year-old AIDS patient. Extensive investigation failed to identify any risk factor for intrafamilial transmission. We conducted a genetic analysis and determined the amino acid signature patterns of the V3, V4, and V5 hypervariable domains and flanking regions in the HIV-1 gp120 *env* gene of 26 clones derived from proviral DNA in peripheral blood mononuclear cells of the members of the family. *env* sequences of the viruses isolated from the patients were compared with sequences of HIV-1 subtype B viruses from Europe and local field isolates. Phylogenetic analysis revealed that the sequences of the viruses isolated from the patients were genetically related and formed an intrafamilial cluster of HIV-1 distinct from other subtype B viruses. Interindividual nucleotide variability in the C2-V3 and V4-C4-V5 domains ranged between 1.2 and 5.0% and between 2.2 and 7.5%, respectively, whereas divergence between HIV strains from the patients and control viral strains ranged from 6.6 to 29.3%. The amino acid signature patterns of viral clones from the three patients were closely related. In the C2-V3 region, two minor clones derived from the son's virus showed less nucleotide divergence (mean, 3.5 and 3.9%) than did the clones derived from the viruses of both parents or the seven other predominant clones derived from the virus from the son (mean, 5.4%). The top of the V3 loop of the last two clones and of all viral clones from the parents exhibited an unusual GPGG sequence. This is the first report of genotypic relatedness of HIV-1 in three adults of the same family in the absence of identified risk factor for transmission between the members of the family. Our findings suggest that atypical transmission of HIV may occur.**

A small number of cases of human immunodeficiency virus (HIV) infection have been reported in adults and children with no identified risk factors for transmission or an unproven mode of transmission despite thorough investigation (5, 6, 14, 34, 45). Two reports of such cases have included detailed genetic analyses of variable *env* sequences of the viral envelope in five patients of a dentist infected with HIV-1 (34) and in a child possibly contaminated through unrecognized exposure to blood by an HIV-1-infected child living in the same home (14). Although epidemiologic evidence argues against casual transmission of HIV (4, 12, 17, 19, 42), these few cases raise the possibility that atypical transmission by means other than those usually evidenced does occur.

We report on a cluster of HIV-1 infection in three adults of the same family whom we found to be infected with genetically related viruses and in whom no risk factor for intrafamilial viral transmission was identified.

## CASE REPORTS

Patient 1 was a 31-year-old man identified to be seropositive for HIV-1 in December 1994. The patient had used intravenous drugs between 1984 and 1989 and claimed to have been

free of drugs since then. The patient lived in his parents' home. He was admitted to our hospital on 4 December 1994 because of high fever, productive cough, hemoptysis, and respiratory distress. The chest X ray and thoracic computed tomogram showed extensive bilateral bullous lesions and a voluminous cavity in the apex of the right lung. *Pneumocystis carinii* pneumonia was diagnosed upon analysis of the bronchoalveolar lavage fluid. A diagnosis of tuberculosis was also suspected, although Ziehl's staining of bronchoalveolar lavage fluid gave negative results. The patient was treated simultaneously with high-dose trimethoprim-sulfamethoxazole and a quadruple combination of antituberculous drugs. Cultures and PCR for *Mycobacterium tuberculosis* remained negative. At the time of admission, the CD4 T-cell count was 10<sup>6</sup>/liter and the HIV RNA level in plasma was 209,700 equivalent (Eq) copies/ml as assessed retrospectively (Chiron, Emeryville, Calif.). Analysis of the pol 215 codon of proviral DNA from the patient's peripheral blood mononuclear cells (PBMC) by selective PCR (26) revealed a mixed sensitive and resistant genotype to zidovudine with a predominance of sensitive variants. In the next 16 months, the patient developed a disseminated form of Kaposi's sarcoma (KS). He died in April 1996.

Patient 2, the mother of patient 1, is a 61-year-old woman who had had no medical history until December 1994, except for mild hypertension and hypothyroidism. She was admitted to our hospital on 10 December 1994 with acute febrile meningitis. Cerebrospinal fluid analysis showed 30 lymphocytes/mm<sup>3</sup> and hyperalbuminorachia (1.34 g/liter). No bacteria, in-

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cluding *M. tuberculosis* and *Treponema pallidum*, and no viruses (herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, human herpesvirus 6) were found by PCR or isolated upon culture of cerebrospinal fluid and blood samples from this patient. However, because of her son's recent history, the patient was given antituberculous drugs. Antimycobacterial therapy was discontinued after 18 weeks because PCR for *M. tuberculosis* and cultures for mycobacteria remained negative. A first HIV screening serologic test performed on 12 December was negative. However, p24 antigenemia was found to be positive (64 pg/ml). On 19 December, Western blot analysis revealed an incomplete HIV-1 pattern, with the presence of bands at 24, 41, and 55 kDa. One week later, the Western blot showed a pattern typical of HIV-1 infection. Therefore, we interpreted the patient's meningitis as being associated with acute primary HIV infection. Analysis of the pol 215 codon in the patient's PBMC revealed a similar genotype for zidovudine resistance to that observed in her son. Patient 2 recovered from her initial symptoms within 2 weeks. She received a combination of zidovudine and zalcitabine for 11 months; the therapy was then switched to a combination of zidovudine and didanosine. In April 1995, the HIV RNA level in plasma was below the threshold of detection (<10,000 Eq copies/ml) and the CD4 T-cell count was  $600 \times 10^6$ /liter.

Patient 3, the father of patient 1 and the husband of patient 2, is a 66-year-old baker with a medical history of hypertension for the last 20 years. In April 1995, patient 3 volunteered for an HIV test, which was found to be positive for HIV-1. The CD4 T-cell count was  $220 \times 10^6$ /liter. The patient had no history suggestive of an acute retroviral syndrome. His plasma viral load at the time of diagnosis of the HIV infection was 683,700 Eq copies/ml. Analysis of the pol 215 codon revealed a similar genotype for zidovudine resistance to that observed in patients 1 and 2. Patient 3 received a combination of zidovudine and zalcitabine for 13 months. He remains clinically asymptomatic with a stable CD4 T-cell count of  $300 \times 10^6$ /liter.

**Epidemiological investigation.** Extensive discussions conducted by three independent physicians, not involved in the patients' care, with both parents of patient 1 did not reveal any risk factor for HIV in patients 2 and 3, including sexual transmission, blood transfusion, usage of intravenous drugs, and identified contact with the blood of patient 1. Patient 1 lived at home, except for the periods when the disease required him to stay in hospital. While in hospital in the terminal phase of his illness, patient 1 received frequent visits from his parents. The patient died at hospital. The parents and their son had separate rooms and did not sleep together. They did not share toothbrushes or razors, but they did share eating utensils. The parents have not been involved at any time in the nursing care of their son. They claimed not to have been in contact with blood or other body fluids of their son, including stools, urine, vomitus, saliva, and nasal secretions. They did not use gloves at home. Patient 1 had no other skin lesions besides KS lesions. There was no bleeding associated with the KS lesions. There were no skin lesions on the hands of patients 2 and 3. Patient 1 had a poor dental condition and presented with oral lesions of KS. He did not present with nasal bleeding. He had had a severe cough and hemoptysis, while at home, prior to his first referral to hospital. Epidemiological investigation also did not evidence how transmission had occurred between the parents. Patient 2 works in her husband's bakery, which is located on the first floor of their lodging. Patients 2 and 3 claimed not to have had sexual intercourse for more than 5 years. Patient 3 had consulted one of us in previous years for sexual dysfunction associated with  $\alpha$ -methyl dopa therapy.

## MATERIALS AND METHODS

Blood samples were obtained on 2 January 1995 from patient 2, on 29 March 1995 from patient 1, and on 1 June 1995 from patient 3. Plasma and PBMC were frozen until use. We also analyzed PBMC obtained from all three patients 1 to 3 months after the initial sampling and plasma from two unrelated HIV-seropositive patients from the same ward, obtained at the same time as the 2 January 1995 sample from patient 2.

PBMC ( $2 \times 10^6$ ) were incubated overnight in lysis buffer containing proteinase K. DNA was extracted by the phenol-chloroform method. Amplification of proviral *env* DNA fragments in the hypervariable V3, V4, and V5 loops and their flanking interspersed constant (C) regions was performed by nested PCR with oligonucleotide primers derived from HIV-1 consensus *env* sequences, as described previously (10). The final amplified product of 667 bp was allowed to migrate on a low-melting-point agarose gel at 6.0%. Relevant bands were eluted, and DNA was extracted by the phenol-chloroform method. The amplified products were inserted into the pMOS blue T-vector plasmid (Amersham Life Science, Little Chalfont, United Kingdom). Clones carrying HIV-1 *env* sequences were identified by blue-white screening of recombinants and confirmed by direct-colony *env* PCR.

The nucleotide sequences were determined with the T7 sequencing kit from Amersham, using the dideoxynucleotide chain termination method. DNA sequences were aligned with CLUSTAL software (22) and corrected manually. Twenty-five *env* sequences of European isolates and 28 *env* sequences of local field isolates from Paris (8) were used as nucleotide and amino acid reference sequences for the HIV-1 subtype B C2-V3 region. The "local control" sequences that were used originated from patients in three hospitals in different areas of Paris from that where the family lives. The distance between those hospitals and the area where the family lives is less than 5 miles. We also used C2-V3 sequences from the previous studies of atypical HIV transmission reported by Ou et al. (GenBank accession numbers are as follows: M90847 for the dentist [US1.Dent], M90854 for patient A [US1.PtA], and M90964 [US1.LC9] and M90939 [US1.LC35] for two local controls) (34), and by Fitzgibbon et al. (GenBank accession numbers are as follows: L12752 for child 1 [US12.CHA], L19695 for child 2 [US12.CHB], and L12753 [US12.LC1] and L12754 [US12.LC2] for two local controls) (14). C2-V3 consensus sequences were generated from control European and Parisian HIV-1 sequences. Nine *env* sequences from Europe isolates were used as nucleotide and amino acid reference sequences for the V4-C4-V5 region. We also used V4-C4-V5 sequences from those previously published by Ou et al. (GenBank accession numbers are as follows: M91084 for the dentist [US1.Dent], M91090 for patient A [US1.PtA], and M91132 [US1.LC1] and M91133 [US1.LC2] for two local controls) (34). Sibling sequences (sets of viral sequences from the same patients) were not included in the reference sets. A V4-C4-V5 consensus sequence was generated from the control European HIV-1 sequences.

The genetic distances between the HIV-1 *env* sequences from the same patient and from those of one patient to those of another patient or set of reference sequences were defined as the average percentage of sequence divergence of all available pairs of C2-V3 and V4-C4-V5 nucleotide sequences. Only single-nucleotide differences were scored, and positions with gaps were excluded. The nonparametric U test of Mann-Whitney was used to evaluate the significance of the differences in the genetic variability between *env* sequences. The patients' sequences were also compared with all sequences accessible in the GenEMBL database by using the FASTA search tool (35).

Phylogeny construction and evaluation were performed with the Phylip software package (11), the neighbor-joining algorithm (36), the matrix distance Fitch and Margoliash method (13), and the Fitch and Wagner maximum parsimony method.

The hypervariable *env* amino acid sequences deduced from the patients' viruses were subjected to signature pattern analysis (14, 24, 34) with VESPA software (24). This method permits us to define particular sites in sequences at which residues are shared among certain groups of virus (24, 39). The amino acid C2-V3 or V4-C4-V5 sequences of patients' viruses were then scanned for amino acids that occurred at homologous positions in less than 50% of the reference set, defined by all the previously selected C2-V3 ( $n = 48$ ) and V4-C4-V5 ( $n = 9$ ) sequences of HIV-1 subtype B isolates from Europe and from Paris.

**Nucleotide sequence accession numbers.** The nucleotide sequences presented in this report have been deposited in the GenBank database under accession no. U87171 to U87221.

## RESULTS

Nucleotide *env* sequences were obtained from 26 clones of PCR products amplified from the PBMC of the patients. After truncating and gap-stripping, the sequences spanned 243 bp in the C2-V3 region, comprising the V3 loop flanked at the 5' and 3' ends by 87 and 51 bp, respectively; the sequences spanned 265 bp in the V4-C4-V5 region. All sequences belonged to the European/North American group M, subtype B.

Mean intrapatient diversity ranged between 1.1 and 2.6% in

TABLE 1. Nucleotide diversity in the C2-V3 and V4-C4-V5 regions of the gp120 *env* gene of clones derived from patients' proviruses

Patient	C2-V3 <sup>a</sup>						V4-C4-V5 <sup>a</sup>				
	No. of clones	Intraperson variation (%)	Interindividual variation (%)				No. of clones	Intraperson variation (%)	Interindividual variation (%)		
			To patient 3	To patient 2	To LC from Europe <sup>b</sup>	To LC from Paris <sup>c</sup>			To patient 3	To patient 2	To LC from Europe <sup>d</sup>
1	9	2.6 (0.4–6.0)	5.0 (3.8–9.2)	4.7 (2.3–9.7)	16.8 (13.1–28.4)	15.1 (12.1–21.3)	9	3.5 (0.0–7.3)	7.2 (5.7–9.6)	7.5 (6.8–10.2)	14.7 (7.9–21.1)
3	9	1.4 (0.0–6.0)		1.2 (0.0–7.8)	17.2 (13.2–29.3)	15.7 (11.1–22.2)	9	1.9 (0.7–4.1)		2.2 (1.1–4.5)	13.8 (6.6–21.8)
2	8	1.1 (0.0–3.8)			17.1 (10.0–26.9)	15.6 (8.9–21.3)	7	0.7 (0.0–1.9)			14.0 (7.2–21.0)

<sup>a</sup> Results are means and ranges of differences between sequences.

<sup>b</sup> Local controls (LC) from Europe: 25 C2-V3 sequences of unrelated subtype B strains from Europe (32), including 5 sequences from Paris (8).

<sup>c</sup> Local controls from Paris: 28 C2-V3 sequences of unrelated subtype B strains from Paris (8).

<sup>d</sup> Local controls from Europe: 9 V4-C3-V5 sequences of unrelated subtype B strains from Europe (32).

the C2-V3 region and between 0.7 and 3.5% in the V4-C4-V5 region (Table 1). In the C2-V3 region, inpatient diversity was higher in patient 1 than in patients 2 and 3 ( $P < 0.0003$ ). Genetic diversity in the V4-C4-V5 region was higher in patient 1 than in patients 2 and 3 ( $P < 0.0004$ ) and higher in patient 3 than in patient 2 ( $P < 0.0001$ ). The mean nucleotide distances in C2-V3 between clones PA7 and PA12, and all clones from viruses of patients 2 and 3 were 3.9 and 3.5%, respectively; the distances were significantly smaller than the mean divergence calculated between clones PA1, PA4, PA11, PA14, PA15, PA12, and all clones derived from viruses of patients 2 and 3 (5.4%;  $P < 0.0001$ ). Means of inter-patient diversity ranged

between 1.2% and 5.0% in the C2-V3 region, and between 2.2% and 7.5% in the V4-C4-V5 region (Table 1). In contrast, means of genetic diversity between the patients' cloned sequences and control sequences ranged between 16.8 and 17.2% for the C2-V3 regions and between 13.8 and 14.7% for the V4-C4-V5 regions. Means of genetic diversity in the C2-V3 region between patients' clones and local field isolates ranged between 15.1 and 15.7%. The results suggest that the viruses from patients 1 through 3 are genetically related. Moreover, the genetic diversity in C2-V3 was higher between patients' *env* sequences and European control sequences (mean, 17.0%; range, 10.0 to 29.3%) than between European sequences

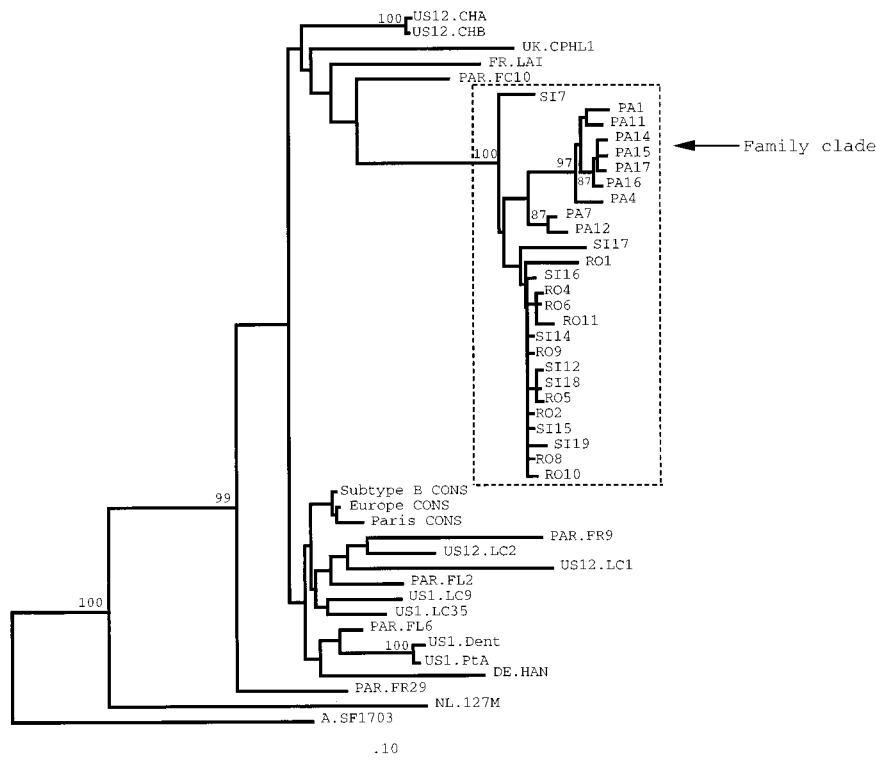


FIG. 1. Neighbor-joining phylogenetic tree of the coding sequences for the HIV-1 *env* C2-V3 region of 26 clones derived from proviruses of patients 1, 2, and 3 (isolates PA, SI, and RO, respectively), sequences of field isolates from Paris (PAR), published sequences from patients with atypical HIV transmission and from their local controls (US) (14, 34), and consensus sequences obtained from 25 unrelated controls from Europe (Europe CONS) (FR.LAI, IT.Sala1, IT.115, IT.193, UK.CPHL1, UK.CPHL2, UK.CPHL6, UK.CPHL7, UK.V77, UK.V87, CH.K11, CH.K16, DE.HAN, DE.31, BE.SIMI84, NL.H466, NL.B130, NL.127M, NL.114M, and NL.H36 [32], including 5 sequences from Paris [PAR.FC10, PAR.FL2, PAR.FL6, PAR.FR9, and PAR.FR29]), from 28 controls from Paris (Paris CONS), and the consensus sequence of HIV-1 subtype B (Subtype B CONS) (32). An HIV-1 subtype A consensus sequence (A.SF1703) was used as an outgroup. Vertical branches are for clarity only; the lengths of the horizontal branches are proportional to the single-base changes. Numbers at nodes represent the percentage of bootstrap samples for 100 replications, for which the corresponding cluster is depicted to the right. Only bootstrap values above 70% are indicated. Phylogenetic trees constructed by using the Fitch and Margoliash algorithm and by using the maximum-parsimony method resulted in similar branching patterns.

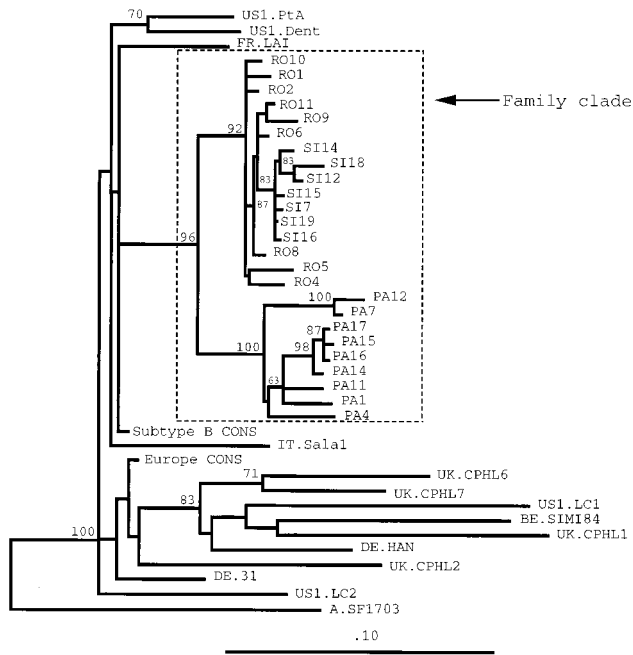


FIG. 2. Phylogenetic tree analysis (obtained by using the Fitch and Margoliash algorithm) comparing the coding sequences for the HIV-1 *env* V4-C4-V5 region of the 26 clones derived from proviruses of patients 1, 2, and 3 (isolates PA, SI, and RO, respectively), published sequences of viruses of patients with atypical HIV transmission and their local controls (US) (34), the consensus sequence obtained from nine unrelated controls from Europe (Europe CONS) (FR.LAI, IT.Sala1, UK.CPHL1, UK.CPHL2, UK.CPHL6, UK.CPHL7, DE.HAN, DE.31, and BE.SIMI84), and the consensus sequence of HIV-1 subtype B (Subtype B CONS). Phylogenetic trees constructed by using the neighbor-joining method and by using the maximum-parsimony method resulted in similar branching patterns.

(mean, 13.2%; range, 3.2 to 30.1%) ( $P < 0.0001$ ); similarly, genetic diversity in C2-V3 was higher between patients' *env* sequences and sequences from Parisian viruses than between the Parisian *env* sequences themselves ( $P < 0.0001$ ). Similar results were obtained upon analysis of the V4-C4-V5 region (data not shown).

Upon comparison of the patients' data with the GenEMBL database by using the FASTA search tool, the sequences of the patients' viruses did not appear related to previously published HIV-1 sequences; the less divergent sequences in C2-V3 (HIV-1 clone LC02; 10.6% divergence) as well as in V4-C4-V5 (HIV-1 clone RT1.21; 13.7% divergence) originated from North America.

The genetic relatedness of the patients' viruses was then confirmed by the analysis of phylogenetic trees. The sequences in C2-V3 (Fig. 1) and V4-C4-V5 (Fig. 2) of patients' viruses clustered with each other and diverged from control sequences of strains from Europe and Paris, as well as from published sequences of viruses from patients with atypical HIV transmission. The branch dividing the familial cluster from the other groups of sequences was resolved in 100% of bootstrap replicates in the C2-V3 domain and in 96% in the V4-C4-V5 domain, supporting the monophyletic grouping of the sequences of viruses from patients 1 through 3.

Phylogenetic analysis of the C2-V3 sequences showed two separate lineages (Fig. 1), one comprising all sequences of viruses from patient 1, including two predominant variants, among which sequences of clones PA7 and PA12 were minor variants, and the other comprising all sequences of viruses from patients 2 and 3. Although there was a distinctive branch

favoring a separate lineage for the C2-V3 sequences of the viruses from patient 1 within the family cluster, it was not strongly supported by the bootstrap value (which was only 56% in the neighbor-joining tree). Within the subcluster of variants of viruses from patients 2 and 3, the C2-V3 sequences did not discriminate between the patients.

Phylogenetic analysis of the V4-C4-V5 sequences clearly differentiated two lineages: the first subcluster comprised all sequences of viruses from patient 1, with 100% of bootstrap replicates; the second subcluster comprised all sequences of viruses from patients 2 and 3, with 92% of bootstrap replicates (Fig. 2). Within the subcluster of viruses from patient 1, there was a strong grouping of sequences of clones PA7 and PA12, with 100% of bootstrap replicates. In addition, all sequences of viruses from patient 2 formed a distinct subgroup in the subcluster of sequences from patients 2 and 3.

The phylogenies inferred by using the neighbor-joining, the Fitch and Margoliash, and the maximum parsimony methods were highly congruent. When hypervariable sites in the V4-C4-V5 domain were removed, the branching patterns were reproduced (data not shown). Taken together, the data demonstrate a strong genetic linkage among *env* sequences of viruses from patients 1 through 3 and an additional subgrouping of *env* sequences for viruses from patients 2 and 3.

Cross-contamination between patients' samples upon amplification was ruled out by the following lines of evidence. First, samples from patients 1, 2, and 3 were not amplified, cloned, and sequenced in the same week. Second, we have used a second blood sample obtained from each of the patients at 1 to 3 months after the initial sample, cloned the PCR product from the *env* gene, and sequenced three clones from the samples from each patient in a different laboratory from that in which the original sequencing work was performed. Minor changes from the initial sequences were observed (data not shown). The newly obtained sequences in C2-V3 and V4-C4-V5 fitted within the phylogenetic subgroup corresponding to the family cluster (Fig. 3). The only difference in C2-V3 observed with the second samples from the patients, compared with the initial samples (Fig. 1), was that one clone from patient 2 belonged to the PA subcluster (clones obtained from patient 1) and vice versa (Fig. 3A). This difference, however, was not significant. Thus, the clones from the second samples behaved similarly to the clones from the initial samples upon phylogenetic analysis. The results of the phylogenetic analysis of the second samples in V4-C4-V5 (Fig. 3B) were similar to those of the analysis of the initial samples (Fig. 2), with distinct subgrouping according to the patients. The fact that similar features have been found in the initial samples and the second samples, which have been independently analyzed, rules out the possibility that our results correspond to cross-contamination between samples. Third, we further sequenced the amplification product of viruses from PBMC of two unrelated HIV-1-seropositive patients (patients 272 and 374) obtained at the same time as the first sample from patient 2. Unexpectedly, HIV-1 from patient 374 was found to be of subtype A. C2-V3 as well as V4-C4-V5 sequences from the viruses derived from patients 272 and 374 were clearly distinct from the family cluster (Fig. 3).

The relatedness of patients' viruses was further assessed by analyzing the deduced amino acid sequences of the V3 and V4 hypervariable regions of gp120. The deduced amino acid sequences obtained from patients 1 through 3 were aligned (Fig. 4 and 5). The top of the V3 loop harbored the GPGR motif in five of nine clones derived from patient 1. GPGR is the most common motif in HIV-1 subtype B sequences (32). The other clones harbored the GPGG and GPDR motifs. Both clones

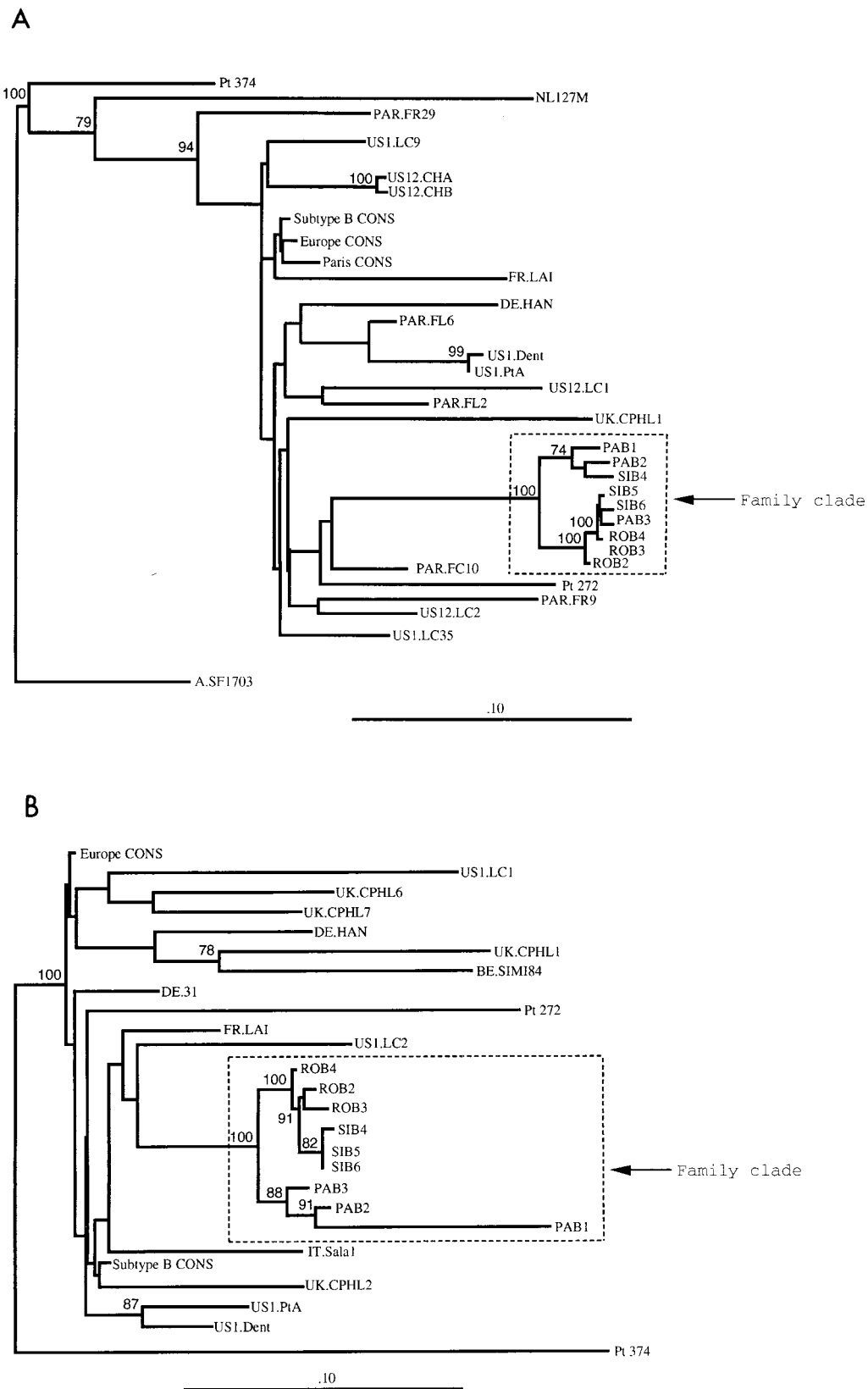


FIG. 3. Phylogenetic tree analyses comparing the coding sequences for the HIV-1 *env* C2-V3 region as obtained by the neighbor-joining method (A) and the *env* V4-C4-V5 region as obtained by the Fitch-Margolias method (B) for the 9 clones derived from proviruses of patients 1 (clones PAB1, PAB2, and PAB3), 3 (clones ROB2, ROB3, and ROB4), and 2 (clones SIB4, SIB5, and SIB6) obtained at a second blood sampling; published sequences of viruses from patients with atypical HIV transmission and their local controls (US) (14, 29); the consensus C2-V3 sequence from 28 controls from Paris (Paris CONS); the consensus C2-V3 and V4-C4-V5 sequences obtained from unrelated controls from Europe (Europe CONS); and the consensus C2-V3 and V4-C4-V5 sequences of HIV-1 subtype B (Subtype B CONS) (32), as previously defined in the legends to Fig. 1 and 2, respectively. An HIV-1 subtype A consensus sequence (A.SF1703), and the V4-C4-V5 sequence of HIV-1 of patient 374 were used as outgroups for the phylogenetic analysis in C2-V3 and V4-C4-V5, respectively.

V3 loop

	272		1	35	353						
Subtype B CONS	EEEVVIRSEN	FTDNAKTIIV	QLNESVEIN	C TRPNNNTRKS	IHIGPGR-APY	TTGELIGDIR QAHC	NLSRAK	WNNTLKQIVI	K	V3 loop charge	
Europe CONS	.....?	.....	.....	.....	.....	.....	.....	.....	.....		
Paris CONS	.....*	.....*	.....*	.....*	.....*	.....*	.....*	.....*	.....*		
PA1	.K...VK.A.	.....V.....	.....N..V..	.....G.....	.PM...-TZ.	A..A...-	.....	.I.AS.	..D...H.A.	N	+4
PA4	.K...V.A.	.....V.....	.....N..V..	.....G.....	.PM...-TW.	A..A...-	.....	.I.GS.	..E...H.A.	N	+6
PA7	.....V.A.	.....V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...-	.....	.I.GS.	..E...H.I.	N	+6
PA11	.K...VT.A.	.....V.....	.....N..V..	.....G.....	.PM...-TW.	A..A...-	.....	.I.AS.	..E...H.A.	N	+6
PA12	.....A.	.....V.....	.....N..V..	.....G.....	.PM...GGR.L.	A..A...-	.....	.I.GS.	..E...H.T.	N	+6
PA14	.K...V.A.	.....V.....	.....N..AV..	.....G.....	.PM...-TW.	A..A...-	.....	.I.AS.	..E...H.I.	N	+6
PA15	.K...V.A.	.....V.....	.....R..N..V..	.....G.....	.PM...-TW.	A..A...-	.....	.I.AS.	..E...H.I.	N	+6
PA16	.K...V.A.	.....V.....	.....N..V..	.....G.....	.PM...-TW.	A..A...-	.....	.I.AS.	..E...H.I.	N	+6
PA17	.K...V.A.	.....V.....	.....N..V..	.....G.....	.PM...D.-TW.	A..A...-	.....	.I.AS.	..E...H.I.	N	+6
RO1	.....A.	.....N..V.....	.....N..V..	.....G.....	VPM...G-RL.	A..A...F.T	.....	.I.GT.	LEZNF.THSY	.	+6
RO2	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
RO4	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
RO5	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
RO6	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.WN.	MEZNF.THSY	.	+6
RO8	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.WN.	MEZNF.THSY	.	+6
RO9	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
RO10	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	R.....	.I.GT.	..E...H.A.	.	+6
RO11	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...L.G-RL.	A..A...N.	.....	.I.WN.	MEZNF.THSY	.	+6
SI7	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
SI12	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
SI14	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
SI15	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
SI16	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...RH.A.	.	+6
SI17	.....K.A.	.....N..V.....	.....N..V..	.....G.....	.PM...D-RL.	A..A...N.	.....	.I..T.	Z.K...H.A.	.	+5
SI18	.....A.	.....N..V.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
SI19	.....A.	.....MN...D.....	.....N..V..	.....G.....	.PM...G-RL.	A..A...N.	.....	.I.GT.	..E...H.A.	.	+6
				Mac CONS	C TRPNNNTRKS	IHIGPGR-APY	<sup>£</sup> TTGELIGDIR QAHC				
				T-cell CONS	C TRPNNQTRRS	IHIGPGR-APY	<sup>£</sup> TRQIGDIR QAHC				

FIG. 4. Multiple alignments of the inferred amino acid sequence of the HIV-1 *env* C2-V3 region coding sequences of clones from patients 1, 2, and 3 (isolates PA, SI, and RO, respectively). The consensus C2-V3 amino acid sequence of European and North American HIV-1 subtype B (Subtype B CONS), that from 25 unrelated controls from Europe (Europe CONS), and that from 28 local controls from Paris (Paris CONS) are shown at the top. The macrophage (Mac CONS) and T-cell (T-cell CONS) consensus sequences of the V3 loop defined by Chesebro et al. (9) are shown at the bottom. Positions 11, 24, 25, and 32, in the consensus T-cell CONS defined as amino acid positions that may encode basic residues in T-cell-tropic isolates, are indicated in bold and underlined (15, 31). Positions 21 and 25, which has been identified as important in macrophage tropism by Westervelt et al. (44), are indicated by the symbol £. The V3 loop is boxed; its top is in bold. The V3 loop charge of each sequence is indicated on the right. Numbers within the box refer to amino acid position in the consensus sequence of the V3 loop of HIV-1 subtype B *env* gp120 (32), counting from the cysteine at the amino terminus. Amino acids are numbered according to their position in the HIV-1 LAI sequence (32). Dots indicate sequence homology, and dashes indicate gaps introduced for optimal alignment. Stop codons in the sequence are indicated by Z. Asterisks designate the noncontiguous amino acids of the signature pattern in the C2-V3 region for patient 1 (Table 2). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

with the GPGG motif belonged to the phylogenetic subcluster of clones PA7 and PA12 (Fig. 1). The GPGG motif also represented the amino acid sequence of the top of the V3 loop in most clones derived from the viruses from patients 2 and 3. As previously reported for the V4 region of HIV-1 (32, 40), multiple insertions of 3 nucleotides were observed in the V4 loop, thus maintaining the reading frame downstream. Two V4 motifs, YSNGTW and KGSNYTGVND, that are not present in HIV-1 subtype B, were frequently observed, indicating a high degree of relatedness among the V4 loop sequences of the viruses from these three patients. Of 13 residues that constituted the signature pattern of patient 1, 11 were found with high frequencies (0.96 to 1.0) in clones derived from patients 2 and 3 (Table 2). Only one amino acid residue in clones from patients 2 and 3 was present with a 100% frequency in the 13 amino acids of the reference set of unrelated isolates of HIV-1 subtype B at the homologous positions of the signature pattern of patient 1, supporting the conclusion that the viruses of patients 1 through 3 were placed together within the cluster. Only 3 of 10 positions of the common signature pattern of the three patients corresponded to residues in the same position in the signature pattern reported by Ou et al. (34), and only 2 positions corresponded to residues in the signature pattern reported by Fitzgibbon et al. (14). The amino acid patterns in the V4-C4-V5 region were similar for the three patients (Table 2).

DISCUSSION

We report on a family in which the parents of an HIV-positive adult man became HIV positive with a virus that was phylogenetically linked to their son's virus. We identified no risk factor for intrafamilial viral transmission and no unproven mode of transmission despite conducting a thorough investigation. Our attention was initially drawn by the unexpected seroconversion of the mother of a 31-year-old HIV-infected drug addict and the subsequent finding of HIV seropositivity in his father. We initiated a molecular analysis of cell-associated viruses of the three family members. A similar genotypic pattern of the pol 215 codon was observed, suggesting some similarity between the viral populations of the three patients. We then documented the genotypic relatedness between the viruses of the patients by three separate approaches, including assessment of genetic distance, phylogenetic analysis, and analysis of amino acid signature patterns within the hypervariable regions of the gp120 *env* gene. Analysis of the nucleotide sequences in the hypervariable *env* regions of viruses of the three family members showed an interpatient diversity of 1.2 to 5.0% and 2.2 to 7.5% in the C2-V3 and V4-C4-V5 regions, respectively. Interpatient diversities below 5.0% in C2-V3 region (3, 7, 14, 29, 30, 34, 46) and below 8.5% in the V4-C4-V5 region (3, 34) have been reported previously in the setting of epidemiologically linked sexual and parenteral HIV transmission.

### V4 loop

Subtype B CONS	386													444		
	FFY	CNTTQLF	NSTW??????	????N?T?N	?TITLPC	RIK	QIINMWQEVG	KAMYAPPI								
PA1	...	...	YSNGTW	-KGS.Y.EV.	G.....	K..	.....	..AP.-								
PA4	...	...	YSNGTW	-KGS.Y.GV.	D..S...	K..	.....	..L-								
PA7	...	D..K..	YNNGTW	-KGS.Y.GV.	D.....	K..	.....	.....								
PA11	...	...	YSNGTW	-KGS.Y.GV.	D...P..	K..	.....	.....								
PA12	...	D..K..	YNNGTW	-KGS.Y.GV.	D.....	K..	.....	..N								
PA14	...	...	YSNGTW	-KGS.H.GV.	D.....	K..	.....	.....								
PA15	...	...	YSNGTW	-KGS.Y.GV.	D.....	K..	.....	.....								
PA16	...	...	YSNGTW	-KGS.Y.GV.	D.....	K..	.....	.....								
PA17	...	...	YSNGTW	-KGS.Y.GV.	D.....	K..	.....	.....								
RO1	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	.....								
RO2	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	.....								
RO4	...	...	YSNGTW	VKGS.YAGV.	D.....	K..	.....	..H...								
RO5	...	...	YSNGTW													
RO6	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	.....								
RO8	...	...	YSNG													
RO9	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	..I...S.								
RO10	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	..R.....								
RO11	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	.....								
SI7	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	.....								
SI12	...	...	YSNGTZ	E.GS.Y.GV.	D.....	K..	.....	.....								
SI14	...	...	YSNGTW													
SI15	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	.....								
SI16	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	.....								
SI18	...	...	YSNSTZ	E.GS.Y.GV.	D.....R	K..	.....	..L.....								
SI19	...	...	YSNGTW	VKGS.Y.GV.	D.....	K..	.....	.....								

FIG. 5. Multiple alignments of the inferred amino acid sequence of the HIV-1 *env* V4 loop coding sequences and its flanking regions of clones from patients 1, 2, and 3 (isolates PA, SI, and RO, respectively). The consensus V4-C3 amino acid sequence of European and North American HIV-1 subtype B (Subtype B CONS) is shown at the top. The V4 loop is boxed. Amino acids are numbered according to their position in the HIV-1 LAI sequence (32). The question marks indicate hypervariable sites at indicated positions. Asterisks designate the noncontiguous amino acids of the signature pattern in the V4-C4 region for patient 1 (Table 2). The amino acid region located after the frame shift in clones RO5, RO8, and SI14 was kept blank. Other symbols are defined in the legend to Fig. 4.

Interpatient diversity in the V4-C4-V5 domain was in a similar range to that estimated in the analysis of an epidemiologically linked cluster of infection reported by Ou et al. (34). In contrast, nucleotide sequences yielded more than 13.5% divergence between the C2-V3 and V4-C4-V5 sequences studied and sequences of unrelated local field isolates. Similar degrees of genetic divergence have been reported in nonepidemiologi-

cally linked HIV infections (2, 3, 14, 18, 34, 46, 47). These findings strongly suggested that the viruses from the three members of the family originated from a common source of infection. The genetic relatedness of the viruses was further confirmed by the finding that all sequences clustered tightly within the same monophyletic group; the sequences diverged significantly from selected reference sequences, as well as from sequences

TABLE 2. Amino acid signature patterns of viruses of patients 1 through 3 with reference to unrelated HIV-1 subtype B local controls

Signature pattern and virus	Amino acid frequency or reference amino acid <sup>a</sup>																
	C2-V3												V4-C4-V5				
	A	V	N	V	G	P	M	A	A	S	H	I	N	Y	W	V	K
Patient 1 signature pattern <sup>b</sup>	A	V	N	V	G	P	M	A	A	S	H	I	N	Y	W	V	K
Patient 3 viruses	1.0	0.96	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	0.56	0.0	0.0	1.0	1.0	1.0	1.0
Patient 2 viruses	1.0	0.96	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	1.0	0.0	0.0	1.0	0.62	1.0	1.0
Unrelated local field isolates <sup>c</sup>	0.13	0.02	0.04	0.22	0.04	0.07	0.07	0.44	0.05	0.05	0.00	0.08	0.0	0.00	0.13	0.00	0.07
Unrelated local field isolates	E	T	E	E	N	H	I	T	E	A	Q	K	K	—	—	—	R
Patient 1 viruses	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	—	—	—	0.0
Patient 3 viruses	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	—	—	—	0.0
Patient 2 viruses	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	—	—	—	0.0
Common signature patterns of patients 1-3	A	—	N	V	G	P	M	A	A	—	H	I	—	Y	W	V	K
Unrelated local field isolates	0.13	—	0.04	0.22	0.04	0.07	0.07	0.44	0.05	—	0.00	0.08	—	0.0	0.0	0.0	0.0
Reference 34 <sup>d</sup>	A	—	A	—	—	—	—	A	—	—	—	—	—	M	W	E	R
Reference 14 <sup>e</sup>	E	—	—	—	W	—	—	—	—	—	—	—	—	NA	NA	NA	NA

<sup>a</sup> Numbers depict the frequency at which the same amino acid is found at a given position within the signature pattern. —, absence of amino acid signature at that position. NA, not available. Top panel: frequencies of the amino acids defining the signature pattern of patient 1 in viruses of patients 2 and 3, as well as in the reference set of unrelated local field isolates of HIV-1 subtype B; middle panel: frequencies of the amino acids of the reference set of unrelated local field isolates of HIV-1 subtype B at the homologous positions of the signature pattern of patient 1, in viruses of patients 2 and 3; bottom panel: frequencies of the amino acids of the reference set of unrelated local field isolates of HIV-1 subtype B at positions of the common amino acid signature pattern of viruses of patients 1 to 3, and signature patterns previously reported by Ou et al. (34) and Fitzgibbon et al. (14).

<sup>b</sup> The signature pattern corresponds to amino acids which differ by more than 50% of those present in the same positions in the reference set of C2-V3 and V4-C4 sequences of local HIV-1 subtype B isolates from Europe or Paris.

<sup>c</sup> Forty-eight distinct sequences in the C2-V3 domain and nine sequences in the V4-C4-V5 domain of field isolates from Europe and Paris.

<sup>d</sup> Amino acid signature pattern of the viruses of the dentist reported by Ou et al. (34).

<sup>e</sup> Common amino acid signature pattern of the viruses of the children reported by Fitzgibbon et al. (14).

from patients with atypical HIV transmission (14, 34). In addition, phylogenetic analysis demonstrated that within the familial HIV cluster, variants of the virus from patient 1 belong to a subcluster that is phylogenetically distinct from a subcluster comprising the variants of viruses from patients 2 and 3. In the subcluster of variants from patient 1, the C2-V3 and V4-C4-V5 sequences of two minor clones, PA7 and PA12, were strongly subgrouped. Obviously, one cannot totally exclude that the fact that PA7 and PA12 appear as minor strains is not due to a sampling bias. In the C2-V3 region, these clones showed less nucleotide divergence than did the clones derived from the viruses from both parents and the other predominant clones of the virus from the son. Analysis of the amino acid sequences of the latter two clones further revealed the presence of an unusual GPGG motif at the top of the V3 loop, reported to occur with a frequency of only 2.1% among the published HIV-1 sequences of North American and European isolates (32), that was shared with viruses from patients 2 and 3. Furthermore, the majority of the viruses from patients 1 to 3 showed several similar amino acid changes in the C2-V3 region compared with the HIV-1 B consensus sequence. Signature patterns were almost identical for the clones from the three patients but distinct from reference sequences, supporting the notion that the viruses from these patients harbored very similar quasispecies (20, 32), with marked similarities in highly functionally relevant domains of the *env* gp120 gene including the V3 loop (15, 27, 41). Indeed, the viruses from all three patients had the same predicted macrophage-tropic, non-syncytium-inducing (NSI) phenotype; i.e., all studied V3 sequences showed a serine at position 11 and an alanine at position 25, resulting in global electrostatic neutrality at these positions, compatible with an NSI phenotype (15) and highly characteristic of macrophage-tropic viruses (9, 44). Each of the patients' variants exhibited more homology to the consensus V3 sequence of macrophage-tropic variants than to the consensus V3 sequence of T-cell-line-tropic variants (9) (Fig. 4). Only one of the variants encoded a basic amino acid at one of the four positions in the V3 loop that are associated with T-cell tropism (31).

The mode of cross-infection of HIV among members of the family remains unknown. The clinical features of HIV disease and the genetic analysis of viruses suggest a chronology of infection where patient 1 was infected before patient 3, who, in turn, was infected before patient 2. Sequences obtained from patient 2 demonstrated a limited nucleotide and amino acid genetic diversity, as previously documented in primary infections (1, 25, 32, 48, 49). The relatively high homogeneity of the viral populations in patient 3 is compatible with a recent infection (46). In contrast, the C2-V3 and C4-V4-V5 domain nucleotide sequences of the variants from patient 1, who had most probably been infected while using intravenous drugs, i.e., at least 5 years before sampling, were significantly more heterogeneous than those derived from patients 2 and 3. Analysis of the intraindividual variations in the V4-C4-V5 domain confirmed the higher divergence of viral strains from patient 1 than of those from patients 2 and 3 and further revealed that strains from patient 3 were significantly more divergent than those from patient 2. The results are consistent with patient 1 having been infected for a longer time than patients 2 and 3 and with patient 3 having been infected before patient 2. In addition, patient 3 already presented with a high HIV load in plasma and low CD4 cell counts 4 months after patient 2 had seroconverted. Since the majority of the variants from patients 2 and 3 were genetically related among themselves and were related to minor variants from patient 1, one may speculate that patient 1 first contaminated patient 3 and later contami-

nated patient 2; in the latter case, the same minor variants from patient 1 would have represented the common source of infection of patients 2 and 3, and therefore should have been selected twice during intrafamilial transmission. There is evidence that minor strains are commonly transmitted from HIV-positive donors to the recipients (49, 50). Alternatively, patient 1 may have infected patient 3, who, in turn, contaminated patient 2; in this case, minor variants of the virus from patient 1 should have been initially selected during transmission to patient 3; major variants of the virus from patient 3 would then have been the source of infection in patient 2.

Despite extensive epidemiological investigation, no risk factor for cross-infection was identified among family members. The risk of transmission of HIV in the household in the absence of sexual or percutaneous exposure has been extensively investigated (4, 12, 17, 19, 28, 33, 37, 38, 42, 43). It is known that viral transmission has not occurred after tens of thousands of days of sharing eating utensils, towels, combs, toilets, bathtubs, and beds and of hugging and kissing between family members (16). A meta-analysis of several studies in the United States and Europe has revealed no case of infection in the follow-up of more than 1,700 person-years after household contact with HIV-infected people (42). However, a small number of cases of infection has been reported in which no risk factor could be identified despite extensive investigation (5, 6, 14, 34, 45). The contact of cutaneous or mucous membranes with infected blood or body fluids has been considered to be the explanation for most such cases of HIV transmission (38, 43). The estimated risk of transmission after mucous and cutaneous exposure to HIV-infected blood in prospective studies of health care workers is below 0.1% (21, 23). Transmission of the virus from the son to one of his parents through casual contact as the parents were caring for their son is highly unlikely to have occurred on two independent occasions. These findings raise the issue of whether the virus exhibited distinct phenotypic properties or the recipients exhibited specific genetic susceptibility to infection. Taken together, our observations suggest that atypical transmission of HIV may occur.

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