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Characterization of Human Immunodeficiency Virus Type 1 p17 Matrix Protein Motifs Associated with Mother-to-Child Transmission

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In order to determine if viral selection occurs during mother-to-child transmission of human immunodeficiency virus type 1 (HIV-1), we used a direct solid-phase sequencing method to sequence the p17 matrix protein-encoding regions of viral isolates from 12 HIV-1-infected mother-and-child pairs, 4 infected infants, 4 transmitting mothers, and 22 nontransmitting mothers and compared the sequences. The blood samples were collected during the delivery period for the mothers and during the first month of life for most of the children. The p17 nucleic sequences were distributed among several clades corresponding to the HIV-1 A, B, and G subtypes. At the amino acid level, no significant differences within the known p17 functional regions were observed among the subtypes. Statistical analyses could be performed with the B subtype. Within the major p17 antibody binding site, a constant KIEEEQN motif (amino acids 103 to 109) was found in all mother-and-child isolates from the B subtype. On the other hand, 9 of 17 nontransmitting mother isolates were variable in this 103 to 109 region. Thus, this motif was significantly associated with the transmitting status (chi square, $P = 0.0034$). A valine residue at position 104 was significantly associated with the nontransmitting phenotype (chi square, $P = 0.014$), suggesting that it has a protective role during vertical transmission. The C-terminal end of p17 was globally conserved among nontransmitting mother isolates (chi square, $P = 0.0037$). These results might improve the understanding of the pathogenesis of HIV-1 vertical transmission and might allow the screening of seropositive mothers by a rapid molecular or peptide test.

Mother-to-child transmission of human immunodeficiency virus (HIV) (3) is one of the major paths of AIDS epidemic spread. Since our first observation of vertical HIV type 1 (HIV-1) infection in 1986 (13), we have performed virological and clinical follow-ups on more than 180 infants born to HIV-infected mothers who attended three obstetrical and pediatric centers in the Paris suburban area (36, 40). The proportion of European and African women (50/50) is a specificity of this cohort, in which the transmission rate of 19.7% (41) remains close to the rate observed with other European cohorts. It is well known that the rate of HIV-1 vertical transmission goes from 14% in Europe and the United States to 39% in Africa (44). The proportion of HIV-1 transmission occurring in utero or during delivery is still a matter of discussion. Another unanswered question is whether vertical transmission occurs via cell-free virus or via infected cells from the mother or from the placental barrier. A third issue concerns the biological and molecular characteristics of transmitted viruses compared with those of nontransmitted viruses. Therefore, the pathogenesis of vertical transmission of HIV-1 is still poorly understood and seems likely to be multifactorial. It involves at least four main parameters: (i) the immune system of the mother, (ii) the placenta, (iii) the immune system of the fetus, and (iv) the virus. We will focus exclusively on the virus part. Scarlatti et al. (43) have shown that mothers with rapid/high viruses had a greater risk of transmission to their children than mothers with slow/low viruses and that in the former case, transmitted viruses were of either the rapid/high or slow/low type. Ometto et al. (34) have observed that viruses from transmitting mothers and infected children were better able than nontransmitted viruses to replicate on monocyte-derived macrophages. Most of the previous studies designed to elucidate the molecular characteristics of the transmitted and nontransmitted variants of HIV-1 have focused on the *env* gene and essentially on the V3 hypervariable loop (6, 30, 45, 51). An analysis of V3 nucleic and amino acid sequences and of other hypervariable regions showed that transmission could occur early or late during pregnancy (30) and that either a major or minor variant from the mother could be transmitted (1, 45, 51). The viral selection observed might reflect both selection during transmission and cell tropism selection in the child. Wolinsky et al. (51) have shown that transmitted variants found in three infants did not have an N glycosylation site that had previously been found in the isolate sequences from the mothers. The absence of this N glycosylation site in transmitted viruses was found only in the viral sequences of three of seven children by Ahmad et al. (1) and in one of three children by Mulder-Kampinga et al. (31). However, Scarlatti et al. (45) did not observe this molecular feature among 10 transmitted viruses. Lamers et al. (23) focused their work on the V1 and V2 regions and did not find any specificity related to vertical transmission in either. Therefore, in general, it seems that no specificity of the transmitted virus could be demonstrated by study of the *env* gene.

Other viral genes were demonstrated to play important roles in cellular tropism and viral phenotype. Among them, the p17 matrix protein gene was shown to interact with the *env* gene in defining the phenotype of the HIV-NDK virus from the D

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subtype (11). In addition, the p17-encoding region presents a low rate of variation in comparison with the *env* gene hypervariable regions and was suggested to be suited for epidemiological studies (2, 20, 22). Moreover, a high level of p17 antibodies is related to a better clinical state in both adults and children, suggesting a protective role for the immunological response against p17 (24, 49).

On the other hand, matrix protein p17 plays a major role during replication processes. (i) The myristoylated N terminus of p17 governs particle assembly at the plasma membrane (37, 47), and p17 interacts with transmembrane glycoprotein gp41 (25). (ii) p17 participates in the viral preintegration complex and in its active nuclear import (7, 21). This participation permits the virus to productively infect nondividing cells. Gallay et al. have demonstrated that the phosphorylation of a small amount of p17 would permit p17 to become a key regulator of the infection process of new cells (15, 16). In order to elucidate if viral selection occurs during the vertical transmission of HIV-1, we sequenced the p17 gene of viral isolates from HIV-1-infected transmitting mothers and their children and from nontransmitting mothers by a rapid direct solid-phase sequencing method.

MATERIALS AND METHODS

Patients. This study was conducted in accordance with French ethical guidelines. Mothers and infants were attending urban hospitals in Paris. Twelve HIV-1-infected mother-child pairs, 4 infants, 4 transmitting mothers, and 22 nontransmitting mothers were included. The coding for our samples involves three letters, and the codes for the transmitting mothers and their children are identical. M stands for mother, T stands for transmitting, NT stands for nontransmitting, and C stands for child. Seven mother isolates were kindly provided by G. Scarlatti (Laboratory of Immunobiology of HIV, Center St. Luigi, St. Raffaele Scientific Institute, Milan, Italy) and are coded with a number.

Most of the mothers were asymptomatic and were classified CDC stage II (9). Four were CDC stage IV. Most of the European mothers were drug users or sexual partners of drug users. Heterosexual intercourse was the main route of transmission for African mothers (Table 1). Blood samples were collected at the delivery period for the mothers and within the first month of life for most of the

TABLE 1. Times of sampling and clinical and biological data for mother-child pairs studied

Status and patient code	Time of sampling[a]		CDC stage	CD4+ count (cells/mm^3)	Syncytium formation (HUT78)	Presence of p24	HIV-1 subtype	Source of HIV contamination[b]
	Mother	Infant						
Nontransmitting								
BOU	4		ND[c]	ND	ND	−	B	Hetero
HAR	−10		III	360	+	+	B	Hetero
HMI	4		II	323	−	−	B	Hetero
LOUB[d]	21		ND	ND	−	−	B	ND
RIV	1		ND	ND	−	ND	B	Hetero
VIL	−30		II	351	+	+	B	Hetero
CHET	−15		II	475	−	−	B	Hetero
GOB	9		ND	410	−	−	B	IVDU
BAR	7		IV	<200	+	+	B	Hetero
FAL	5		ND	ND	−	ND	B	ND
CEL	2		ND	ND	−	ND	B	ND
SIW[d]	4		ND	380	−	−	B	Hetero
MOE	3		ND	814	ND	−	B	ND
BAB[d]	7		II	360	−	−	A	Hetero
DOS[e]	1		II	315	−	−	A	Hetero
LAN[d]	6		ND	840	−	−	A	Hetero
FRA[d]	3		IV	166	−	−	A	Hetero
WAN[d]	18		ND	ND	−	ND	A	ND
Transmitting								
ABD	3	2	II	543	−	−	B	IVDU
ARI[f]	3	2	II	210	−	−	B	Hetero
BOIS	4	4	II	472	−	−	B	IVDU
DUM	30	32	I	873	+	−	B	
RYO	7	887	IV	<200	−	−	B	IVDU
PAL	4	5	II	323	−	−	B	IVDU
FLO	2	20	I	1,447	−	−	B	
DIA[d]	0	1	II	383	−	−	A	Hetero
MAM[d]	−60	4	I	461	−	−	A	Hetero
MPA[d]	3	3	II	216	−	−	G	Hetero
OSA[d]	10	10	II	220	−	−	G	Hetero
MOU	300	300	IV	94	ND	−	A	ND
LEN	−50	29	II	ND	−	ND	B	IVDU
LIN	ND	2	II	673	ND	ND	B	ND
AMO	ND	13	ND	ND	ND	ND	B	ND
DUB C	ND	7	ND	ND	ND	−	G	ND
IGN C[d]	ND	3,550	ND	220	ND	ND	G	ND

[a] The times of sampling are indicated in days preceding (−) or following delivery. The years of sampling for samples from which sequences were generated are as follows: 1990, DUB, DUM, MOU, and GOB; 1991, FLO, LEN, CHET, HMI, LOUB, VIL, and HAR; 1992, FRA, LAN, BAB, BOU, PAL, MAM, ARI, and BAR; 1993, ABD, OSA, MPA, DIA, BOIS, RYO, and CEL; and 1994, SIW, MOE, WAN, FAL, RIV, DOS, LIN, AMO, and IGN.

[b] Hetero, heterosexual activity; IVDU, intravenous drug user.

[c] ND, not determined.

[d] These patients are from sub-Saharan Africa. All other patients are from Europe and North Africa, except as noted.

[e] This patient is from Haiti.

[f] This patient is from the French West Indies.

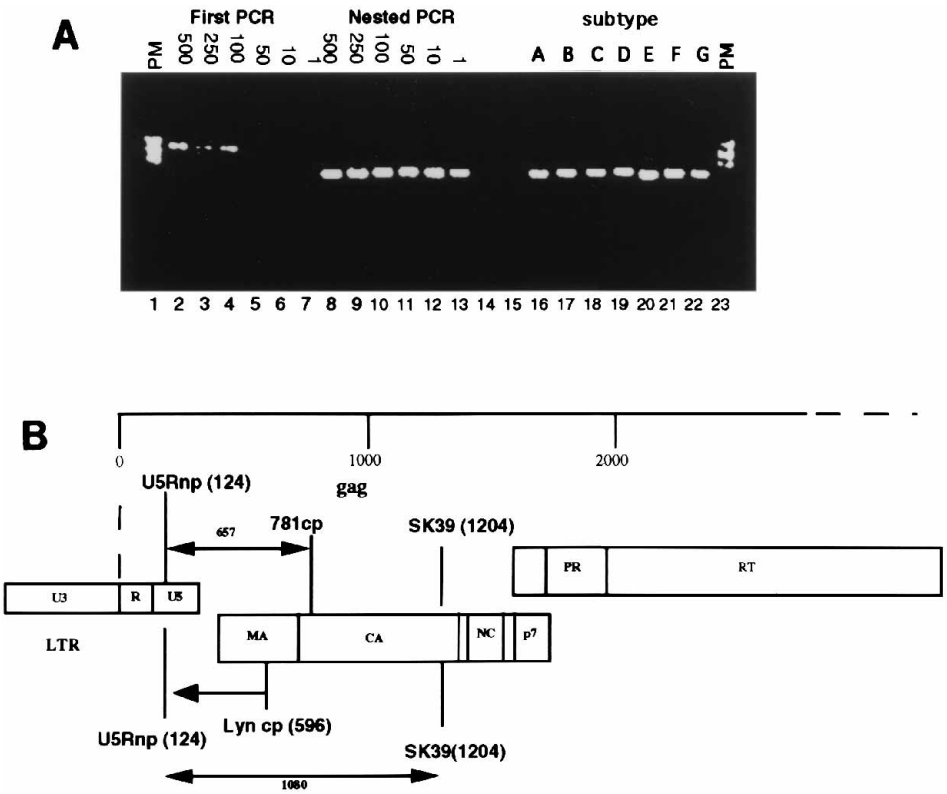

FIG. 1. (A) PCR products of the p17-encoding gene. Lanes PM, ϕX174 digested with *Hin*dIII; lanes 2 to 7, 500, 250, 100, 50, 10, and 1 copy, respectively, of integrated provirus DNA that was extracted from 85-14 F2 cells and amplified with the U5Rnp-SK39 primer set; lanes 8 to 13, nested PCR products of an outer PCR that were amplified with the BioU5Rnp-781cp primer set and that correspond to lanes 2 to 7, respectively; lanes 16 to 22, nested BioU5Rnp-781cp PCR products of viral DNA from, respectively, the HIV-1 A, B, C, D, E, F, and G subtypes. A, BAB MNT isolate; B, FAL MNT isolate; C, 15166 strain; D, NDK strain; E, Viet 26 strain; F, 1011 strain; G, MPA M isolate. (B) Locations of the primers used for nested PCR and sequencing processes within the p17-encoding gene of the HIV-1_{Lai} strain.

children. Child contamination was evidenced by viral isolation from peripheral blood mononuclear cell cultures and from peripheral blood mononuclear cell and healthy donor cord blood mononuclear cell cocultures and by PCR amplification of the *gag* and *pol* genes with specific primer sets as previously described (39).

DNA preparation. DNA for PCR amplification was prepared directly from patient peripheral blood mononuclear cells for two-thirds of the samples and from cells producing virus isolates after one passage on phytohemagglutinin-stimulated cord blood mononuclear cells from healthy donors and patient peripheral blood mononuclear cell cocultures for the rest of the samples. DNA was extracted by the phenol-chloroform method.

PCR. DNA samples (1 μg) were amplified by nested PCR with primers specific for the p17 region of the *gag* gene. The outer primers were U5Rnp (5′-ACTCT GGTAGCTAGAGATCCCT-3′) and SK39 (5′-TTTGGTCCTTGTCTTAT GTCCAGAATGC-3′) (nucleotides 1 to 18 and 1204 to 1177 on the basis of the HIV-1_{Lai} strain genome, respectively). The inner primers were Bio U5Rnp and 781cp (5′-GGTGATATGGCCTGATGTACCAT-3′) (positions 781 to 759). Figure 1 shows the positions on the HIV-1_{Lai} strain genome of the primers used. The outer and inner primer sets were suitable for the amplification of all isolates from the HIV-1 A, B, C, D, E, F, and G subtypes (Fig. 1A). Both outer and inner HIV-1 *gag* PCRs were carried out in 100-μl reaction mixtures containing PCR buffer (Appligene) (1.5 mM $MgCl_2$, 0.2 mg of bovine serum albumin per ml, 0.1% Triton X-100, 10 mM Tris HCl [pH 9]), 0.25 μmol of each primer, 200 μmol of each deoxynucleoside triphosphate, and 0.625 U of *Taq* DNA polymerase (Appligene). For both PCR procedures, the amplification program consisted of one set of denaturation for 3 min at 94°C, 38 cycles of annealing at 57°C for 1 min, extension at 72°C for 1 min 10 s, denaturation at 94°C for 45 s, and a final set of annealing at 57°C for 2 min and extension for 10 min at 72°C. Two microliters of the first PCR mixture was used for the second PCR. The detection limits of the inner and outer PCRs were determined with an 85-14 F2 cell standard (41) and were, respectively, 1 and 10 copies. The *env* gene region encompassing the V3 hypervariable loop was amplified with the Bio H1-6541 (5′-GTCAGCACAGTACAATGTACACAT-3′) and H1-E4 7259 (5′-TTCTCC AATTGTCCCTCATAT-3′) primer set.

DNA sequencing. Single-stranded DNA was obtained by the immobilization of the nested biotinylated PCR products on streptavidin magnetic beads (Dynabeads M280-Streptavidin; Dynal AS) and then denaturation. Complete p17 sequences were achieved with the 781cp primer and the 5′ LYN p17 primer (5′-CTACTGTATTATATAATGAT-3′) (Fig. 1B), with the single-stranded Sequenase dye terminator kit (Applied Biosystems) being used. The V3 loop sequence from the *env* gene was achieved with H1-E3ac 6903 (5′-TTCTGGGTC CCCTCCTGAGGA-3′). The product was then loaded on a 6% polyacrylamide gel in an automated laser fluorescence apparatus (373A; Applied Biosystems). All isolates were sequenced in triplicate. Each sequence was considered valuable only if the three sequencings were strictly identical.

Sequence analysis and tree alignment. Multiple alignment was realized with the Seq-ed (Applied Biosystems) and CLUSTALW (48) programs. The degenerate alphabet DNA was introduced manually, and the alignment was adjusted by hand before phylogenetic analysis with version 3.56c of the Phylogeny Inference Package (PHYLIP) (14). Phylogenetic distances within the isolate p17 nucleic acid sequences of each mother-child pair and among all mother isolate sequences were calculated with the two-parameter Kimura algorithm (DNADIST from PHYLIP). Dendograms were created by the neighbor-joining, parsimony, and maximum-likelihood methods with the PHYLIP program. Tree diagrams were produced with PHYLIP's DRAWTREE program. Bootstrapping was performed with the SEQBOOT, DNADIST, and CONSENSE programs from the package. Differences in variations among groups were tested with nonparametric statistics (Mann-Whitney test).

RESULTS

Clinical statuses of patients. (i) Mothers. The mothers of our cohort can be divided into two groups: (i) mothers of African origin or who were partners of intravenous drug users and who have most likely been infected heterosexually and (ii) mothers who were formerly intravenous drug users. The clinical statuses of the mothers are presented in Table 1. The mothers from the Italian cohort were drug users (46).

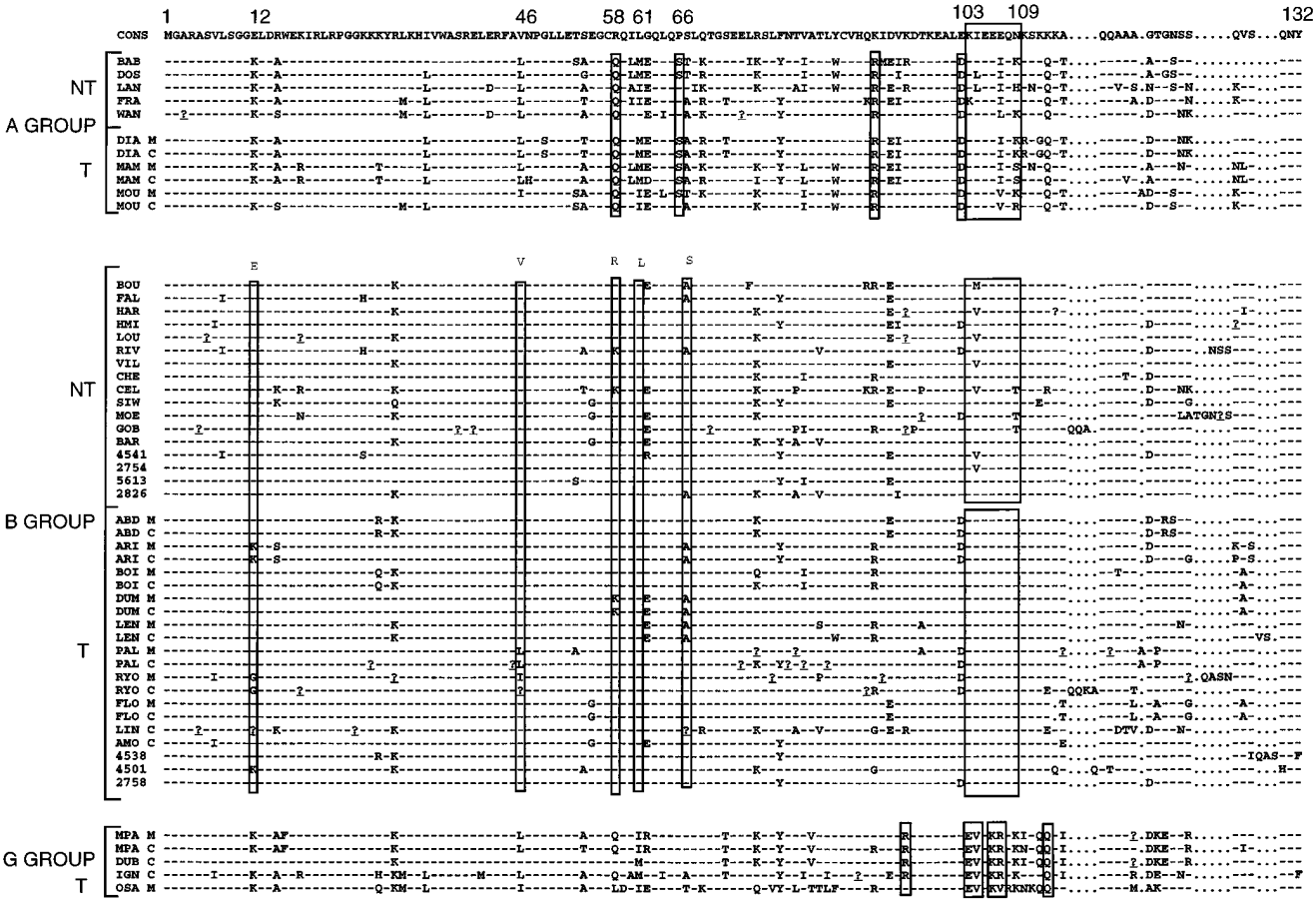

FIG. 2. Multiple alignment of the amino acid sequences of HIV-1 matrix protein p17 predicted for viral isolates associated with mother-to-child transmission. The p17 amino acid sequences were aligned by comparison with a consensus sequence. NT, nontransmitting; T, transmitting; M, mother; C, child; ?, no consensus. Dashes indicate identity with the consensus sequence; dots are introduced to maximize alignment. Sequences were classified among the HIV-1 A, B, and G groups. The narrow boxes correspond to amino acids with a subgroup signature (Fig. 3). The region from aa 103 to 109 (wide boxes) was highly variable among the groups. In the B group, a constant KIEEEQN motif was found in this region among all transmitted viruses but in only less than half of the nontransmitted viruses.

(ii) Newborns. Children were diagnosed as HIV positive (positive by PCR and viral isolation) during the first month of life. Infected children were classified P2A in terms of clinical status during the first year of life. Children were considered HIV negative if they were seronegative after 18 months of life.

Sequence diversity and phylogenetic analysis. Sequencing for the 54 isolates was completed. Figure 2 shows the multiple alignment of the deduced amino acid sequences. Since there were no cloning steps, each isolate sequence is the consensus for its major variant sequences. The predicted amino acid sequences of our isolates were, at first, classified among HIV-1 groups A, B, D, and G. Following Myers et al. and others (26, 32), we used specific amino acids as a signature for each subgroup (Fig. 2 and 3).

Genotype	E 12	V 46	Q 58	R 58	L 61	E 62	S 67	F 75	R 91	R 95	E 102	M 104	Q 113
A			■			■			■				
B	■	■		■	■		■						
G										■	■		■
D								■				■	

FIG. 3. Matrix protein signature of the sequences in the four HIV-1 genotypes. A black cell indicates that the amino acid is present in at least 75% of the isolates of a particular genotype. Residues are numbered according to the numbering shown in Fig. 2.

In Fig. 4, dendograms drawn by the neighbor-joining (Fig. 4A), parsimony (Fig. 4B), and maximum-likelihood (Fig. 4C) methods (14) show the distribution of the p17 consensus DNA sequences among several clades. In each dendogram, the first two clades corresponded exactly to the A and G groups of HIV-1 sequences, respectively. The third clade, on the basis of the three different methods used, included all isolates from the B subtype and the single isolate BOU-MNT, which was at first classified as a D subtype isolate on the basis of the amino acid pattern. A dendogram drawn with the BOU-MNT isolate sequence and reference sequences from the principal HIV-1 subtypes (32) demonstrated that BOU-MNT clustered with the B subtype (data not shown) and not the D subtype. We sequenced the V3 loop from the BOU-MNT isolate and confirmed the isolate as B subtype (data not shown). Therefore, Fig. 2 and 4 show the BOU-MNT isolate sequence falling within the group of sequences of isolates from the B subtype. This division in the subgroups allowed us to discard all sequence variations due to subtype.

(i) Variability among mother and child isolates. Mother isolate sequences were more heterogeneous than child se-

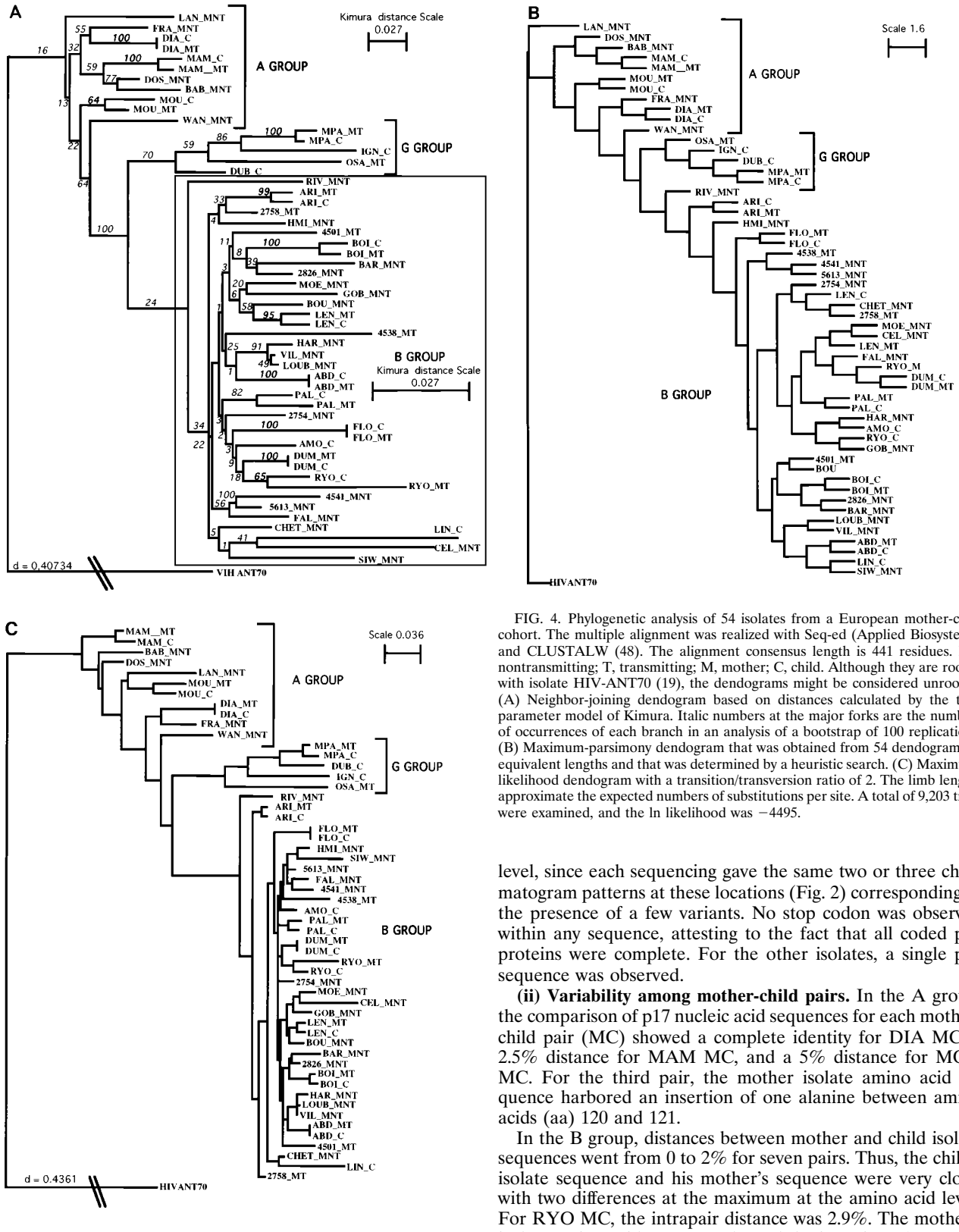

FIG. 4. Phylogenetic analysis of 54 isolates from a European mother-child cohort. The multiple alignment was realized with Seq-ed (Applied Biosystems) and CLUSTALW (48). The alignment consensus length is 441 residues. NT, nontransmitting; T, transmitting; M, mother; C, child. Although they are rooted with isolate HIV-ANT70 (19), the dendograms might be considered unrooted. (A) Neighbor-joining dendogram based on distances calculated by the two-parameter model of Kimura. Italic numbers at the major forks are the numbers of occurrences of each branch in an analysis of a bootstrap of 100 replications. (B) Maximum-parsimony dendogram that was obtained from 54 dendograms of equivalent lengths and that was determined by a heuristic search. (C) Maximum-likelihood dendogram with a transition/transversion ratio of 2. The limb lengths approximate the expected numbers of substitutions per site. A total of 9,203 trees were examined, and the ln likelihood was −4495.

quences. For nine mothers (WAN, HAR, HMI, LOU, MOE, GOB, PAL M, MPA M, and RYO M) and five children (PAL C, RYO C, LIN C, IGN C, and DUB C), the isolate sequences were heterogeneous at one to six locations at the amino acid level, since each sequencing gave the same two or three chromatogram patterns at these locations (Fig. 2) corresponding to the presence of a few variants. No stop codon was observed within any sequence, attesting to the fact that all coded p17 proteins were complete. For the other isolates, a single p17 sequence was observed.

(ii) Variability among mother-child pairs. In the A group, the comparison of p17 nucleic acid sequences for each mother-child pair (MC) showed a complete identity for DIA MC, a 2.5% distance for MAM MC, and a 5% distance for MOU MC. For the third pair, the mother isolate amino acid sequence harbored an insertion of one alanine between amino acids (aa) 120 and 121.

In the B group, distances between mother and child isolate sequences went from 0 to 2% for seven pairs. Thus, the child's isolate sequence and his mother's sequence were very close, with two differences at the maximum at the amino acid level. For RYO MC, the intrapair distance was 2.9%. The mother's isolate amino acid sequence showed the insertion of a QASN tetrapeptide between aa 126 and 127, and the child's sequence showed a duplication of the QQKA tetrapeptide between aa 115 and 116. Figure 5 shows the nucleic distances among all MC pair isolate nucleic acid sequences from the B subtype. All

	ABD_M	ABD_C	ARI_M	ARI_C	BOI_M	BOI_C	DUM_M	DUM_C	LEN_M	LEN_C	PAL_M	PAL_C	RYO_M	RYO_C	FLO_M	FLO_C
ABD_M																
ABD_C	**0**															
ARI_M	0.047	0.047														
ARI_C	0.047	0.047	**0.01**													
BOI_M	0.058	0.058	0.058	0.058												
BOI_C	0.061	0.061	0.061	0.061	**0.013**											
DUM_M	0.05	0.05	0.044	0.044	0.05	0.052										
DUM_C	0.05	0.05	0.044	0.044	0.05	0.052	**0**									
LEN_M	0.052	0.052	0.047	0.047	0.05	0.052	0.034	0.034								
LEN_C	0.05	0.05	0.044	0.044	0.052	0.055	0.034	0.034	**0.015**							
PAL_M	0.05	0.05	0.05	0.05	0.059	0.061	0.042	0.042	0.045	0.048						
PAL_C	0.043	0.043	0.043	0.043	0.051	0.054	0.04	0.04	0.048	0.043	**0.024**					
RYO_M	0.061	0.061	0.061	0.061	0.061	0.058	0.031	0.031	0.058	0.057	0.043	0.048				
RYO_C	0.05	0.05	0.048	0.048	0.056	0.053	0.034	0.034	0.042	0.039	0.04	0.037	**0.029**			
FLO_M	0.058	0.058	0.061	0.056	0.056	0.058	0.047	0.047	0.061	0.061	0.051	0.054	0.062	0.053		
FLO_C	0.058	0.058	0.061	0.056	0.056	0.058	0.047	0.047	0.061	0.061	0.051	0.054	0.062	0.053	**0**	

FIG. 5. Kimura distances among the HIV-1 p17 DNA sequences of mother and infant isolates from the B subtype. The mean distance for mother-child pairs is 0.0114 ± 0.0111; the mean distance for mothers and nonrelated children is 0.0493 ± 0.0080 (Mann-Whitney, $P < 0.0001$). Bold-face values are the distances among the mother-child isolate sequences.

child isolate sequences were much closer to the sequences of their respective mothers than to any other sequence. The mean distance for mother-child pair isolate sequences was 0.0114 ± 0.0111 and was significantly different from the mean distance for mother and nonrelated child isolate sequences, which was 0.043 ± 0.008 (Mann-Whitney test, $P < 0.0001$).

In the G group, the distance between the child and mother isolate sequences for the only mother-child pair of this group was 2%.

(iii) Variability among mothers. In the A group, the distances among p17 isolate nucleic acid sequences went from 6 to 14% for the nontransmitting mother group. In the transmitting mother group, there were 10 to 13% distances, and among all mothers, the distances were 6 to 14%. In the B group, distances went from 3 to 11% for nontransmitting mother isolate sequences (Fig. 6). The transmitting mother isolate sequence distances went from 3 to 13%. Among all B group mother isolate sequences, there were 3 to 13% distances. Figure 6 shows that the mean distance for sequences for mothers with different transmitting statuses (0.056 ± 0.019) and the mean distance for sequences for mothers with the same status (0.050 ± 0.019 for nontransmitting and 0.058 ± 0.020 for transmitting) are almost exactly the same. In the G group, there was a 17% distance among the transmitting mother isolate sequences.

As was expected from the sequence distance comparison, the phylogenetic analysis showed that for all the mother-child pairs, the infant isolate sequence was closer to the mother isolate sequence than to any other sequence, attesting to the fact that the sequences are related for each MC pair (Fig. 4). The phylogenetic tree drawn on the basis of the mother-child pair isolate sequences (Fig. 7) shows that the MC pairs are distributed among their respective subtypes and that each child's isolate sequence is closely related to his or her mother's isolate sequence. Thus, the phylogenetic analysis demonstrates the epidemiological link between each child's isolate sequence and that of his or her mother.

The overall comparison of the p17 nucleic or amino acid sequences of our cohort did not evidence any specific grouping of transmitting mother isolates or of nontransmitting mother isolates for all HIV-1 subgroups (Fig. 4 and 7).

Comparison of p17 functional domains. During a comparison of the functional domains of all p17 amino acid sequences, no significant differences appeared in either the myristoylation site (aa 1 to 6 [MGARAS]) (18, 47) or the nuclear localization sites (K-18 to R-30) (7). However, a specific group sequence was observed at a putative site of p17 polymerization (residues 47 to 59) (10), and the phosphorylation site at aa 110 to 114 (8, 52) had the specific group sequences KSKQK, KSKKK, and ISQQK for the A, B, and G groups, respectively. The exception was the OSA M isolate from the G subtype, which had an NKQQK motif. The tyrosine phosphorylation site at Y-132 described by Gallay et al. (15) was conserved in all groups, except for the IGN C and 4538 isolates.

The N-terminal half of p17 was globally conserved in each subtype. On the other hand, several variability domains were observed in the C-terminal portion of p17. These variability domains and several variable amino acids for isolates from the B subtype, which was the largest of our cohort, were compared. These comparisons of the nontransmitting and transmitting groups were made by the chi-square test. Table 2 shows the corresponding results.

Two amino acids and two domains seemed to be sufficient by themselves to distinguish the nontransmitting group from the transmitting group. (i) The single glutamic acid residue at position 93 was significantly associated with transmitting status (chi square, $P = 0.0338$). (ii) The first statistically significant domain was the last C-terminal 6-mer, QVSQNY. Indeed, in the B subtype, it was significantly associated with the nontransmitting group (chi square, $P = 0.0037$) (Fig. 2). Figure 8B shows the distribution of this C-terminal 6-mer within the nontransmitting and transmitting groups. (iii) In the region (positions 103 to 109) of the major antibody site [(KE)ALD KIEE(EQ)] for the B subtype described by Boucher et al. (5), the KIEEEQN motif was always found among the 10 transmitted viruses from children. Moreover, this motif was constant among the 11 transmitting isolates from the mothers. On the other hand, among the 17 nontransmitting mother viruses,

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1.BOU_NT	0																													
2.FAL_NT	0.0363	0																												
3.HAR_NT	0.0363	0.0419	0																											
4.HMI_NT	0.0471	0.0419	0.0419	0																										
5.LOUB_NT	0.0311	0.0366	0.0077	0.042	0																									
6.RIV_NT	0.0607	0.0470	0.0611	0.0584	0.0613	0																								
7.VIL_NT	0.0310	0.0365	0.0102	0.0419	0.0026	0.0611	0																							
8.CHET_NT	0.0414	0.0471	0.0389	0.0418	0.0417	0.0498	0.0389	0																						
9.CEL_NT	0.0779	0.0956	0.0728	0.0896	0.0728	0.1044	0.0726	0.0807	0																					
10.SIW_NT	0.0658	0.066	0.0499	0.0525	0.0528	0.0802	0.0499	0.0524	0.0956	0																				
11.MOE_NT	0.0366	0.0422	0.0367	0.0502	0.0288	0.0765	0.0261	0.0418	0.0672	0.0529	0																			
12.GOB_NT	0.0397	0.0565	0.0508	0.0593	0.0455	0.065	0.0453	0.0423	0.0784	0.0818	0.0371	0																		
13.BAR_NT	0.0551	0.0608	0.0553	0.0579	0.0501	0.0749	0.0499	0.0658	0.0956	0.0662	0.042	0.0619	0																	
14.4541_NT	0.0524	0.0362	0.0391	0.0418	0.0419	0.0635	0.0391	0.0417	0.0954	0.0634	0.0584	0.0676	0.0716	0																
15.2754_NT	0.0389	0.0391	0.0337	0.0418	0.0285	0.058	0.0284	0.0387	0.0837	0.0661	0.0392	0.0478	0.0524	0.0444	0															
16.5613_NT	0.0336	0.0284	0.0337	0.0285	0.0285	0.0582	0.0284	0.0283	0.0865	0.0551	0.0393	0.0425	0.0524	0.0284	0.031	0														
17.2826_NT	0.0335	0.039	0.0337	0.0445	0.0285	0.0471	0.0284	0.044	0.0865	0.0632	0.0338	0.0451	0.0363	0.0551	0.0362	0.0363	0													
18.ABD_T	0.0442	0.0498	0.0337	0.039	0.0311	0.0553	0.0284	0.0442	0.0837	0.0578	0.0365	0.0535	0.0634	0.0551	0.0414	0.0416	0.0416	0												
19.ARI_T	0.0390	0.0445	0.0500	0.0392	0.0501	0.0499	0.0500	0.0390	0.0957	0.0659	0.0528	0.0481	0.0689	0.0553	0.0471	0.0417	0.0469	0.0471	0											
20.BOI_T	0.0498	0.0608	0.0471	0.0636	0.0445	0.0691	0.0417	0.0441	0.0952	0.0718	0.0500	0.0534	0.0524	0.0661	0.0468	0.0470	0.0362	0.0578	0.058	0										
21.DUM_T	0.0311	0.0313	0.0420	0.0476	0.0367	0.0501	0.0368	0.0417	0.0719	0.0720	0.0288	0.0451	0.0501	0.0529	0.0338	0.0339	0.0337	0.0500	0.0446	0.0499	0									
22.LEN_T	0.0283	0.0444	0.0444	0.0554	0.0392	0.058	0.0391	0.0388	0.0726	0.0688	0.0313	0.0397	0.0551	0.0607	0.0415	0.0417	0.0335	0.0524	0.0470	0.0496	0.0337	0								
23.PAL_T	0.0476	0.0480	0.0478	0.0478	0.0425	0.0644	0.0424	0.0475	0.0989	0.0727	0.0424	0.0481	0.0643	0.0589	0.0395	0.0369	0.0422	0.0503	0.0504	0.0586	0.0422	0.0449	0							
24.RYO_T	0.0723	0.0672	0.0670	0.0698	0.0616	0.0839	0.0614	0.0720	0.1142	0.0951	0.0587	0.0824	0.0838	0.0839	0.0584	0.0641	0.0666	0.0777	0.0780	0.0779	0.0479	0.0751	0.0592	0						
25.FLO_T	0.0554	0.0558	0.0555	0.0476	0.0503	0.0671	0.0500	0.0446	0.0987	0.0640	0.0533	0.0652	0.0668	0.0639	0.0447	0.0474	0.0555	0.0582	0.0614	0.0556	0.0422	0.0611	0.0508	0.0788	0					
26.LIN_C	0.0896	0.1017	0.0785	0.0899	0.0816	0.1052	0.0814	0.0671	0.1025	0.0873	0.0906	0.1029	0.0927	0.0958	0.0928	0.0869	0.0755	0.0869	0.0932	0.0813	0.1016	0.0842	0.1054	0.1206	0.0991	0				
27.AMO_C	0.0362	0.0363	0.0417	0.0364	0.0364	0.0608	0.0364	0.0414	0.0894	0.0632	0.0312	0.0451	0.0442	0.0469	0.0335	0.0283	0.0388	0.0496	0.0442	0.0549	0.0311	0.0389	0.0422	0.0611	0.0474	0.0954	0			
28.4538_T	0.0608	0.0610	0.0555	0.0610	0.0502	0.0864	0.0474	0.0660	0.1104	0.0888	0.0613	0.0705	0.0745	0.0664	0.0580	0.0471	0.0580	0.0636	0.0691	0.0745	0.0612	0.0635	0.0673	0.0599	0.0751	0.1227	0.0551	0		
29.4501_T	0.0417	0.0528	0.0419	0.0528	0.0366	0.0609	0.0365	0.0416	0.0832	0.0892	0.0420	0.0530	0.0608	0.0636	0.0443	0.0445	0.0390	0.0445	0.0446	0.0444	0.0470	0.0444	0.0528	0.0753	0.0585	0.0754	0.0470	0.0637	0	
30.2758_T	0.0442	0.0338	0.0391	0.0284	0.0392	0.0445	0.0391	0.0231	0.0838	0.0469	0.0311	0.0452	0.0577	0.0390	0.0362	0.0257	0.0415	0.0362	0.0257	0.0577	0.0338	0.0416	0.0394	0.0610	0.0501	0.0813	0.0335	0.0579	0.0444	0

FIG. 6. Kimura distances for all isolate p17 sequences of the B group. The mean distance for transmitted isolate sequences and nontransmitted isolate sequences is 0.056 ± 0.019, and the mean distances for isolate sequences with the same transmission statuses (i.e., nontransmitted and transmitted isolates) are 0.050 ± 0.019 and 0.058 ± 0.020, respectively.

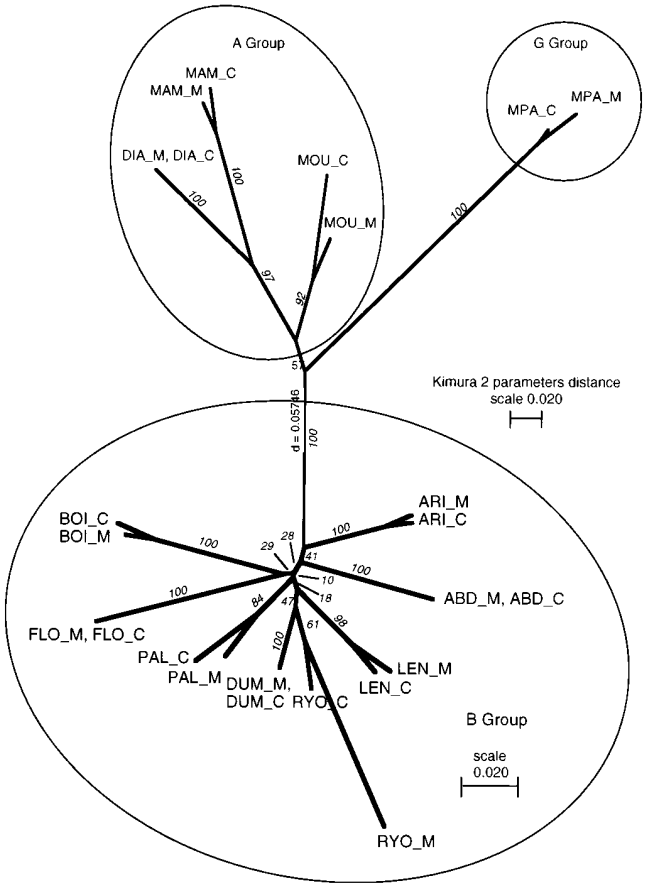

FIG. 7. Phylogenetic tree of the p17 DNA sequences of isolates from mother-child pairs. The neighbor-joining tree is based on the distances calculated with the two-parameter model of Kimura. The multiple alignment was realized with CLUSTALW (48). The alignment consensus length is 426 residues. The scale of distances is different for the B subgroup. Italic numbers at the major forks are the numbers of occurrences of each branch in an analysis of a bootstrap of 500 replications.

only 8 presented this KIEEEQN motif. Five nontransmitting mother isolate sequences had a KVEEEQN motif, two had a KIEEEQT motif, one had a KMEEEQN motif, and one had a KVEEEQT motif (Fig. 2). On the basis of the presence of the KIEEEQN motif, there was a significant difference between the transmitting mother isolate group and the nontransmitting mother isolate group (chi-square test, $P = 0.0034$) (Fig. 8A). (iv) On the other hand, the single valine residue at position 104 was associated with the nontransmitting phenotype (chi square, $P = 0.0014$). It is noteworthy that this region (positions

TABLE 2. Statistical analysis of HIV-1 B subtype p17 variable amino acids and motifs

Amino acid or motif	No. in the 11 transmitted isolates	No. in the 17 nontransmitted isolates	Chi-square value	Chi-square value corrected for continuity
Arginine-30	6	8	0.6957	1
Arginine-75	7	8	0.395	0.637
Glutamic acid-93	9	7	0.0338	0.083
Glutamic acid-102	6	14	0.1117	0.245
KIEEEQN	11	8	0.0034	0.012
Valine-104	0	7	0.014	0.044
Glycine-120	8	11	0.657	0.976
QVSQNY	5	16	0.0037	0.014

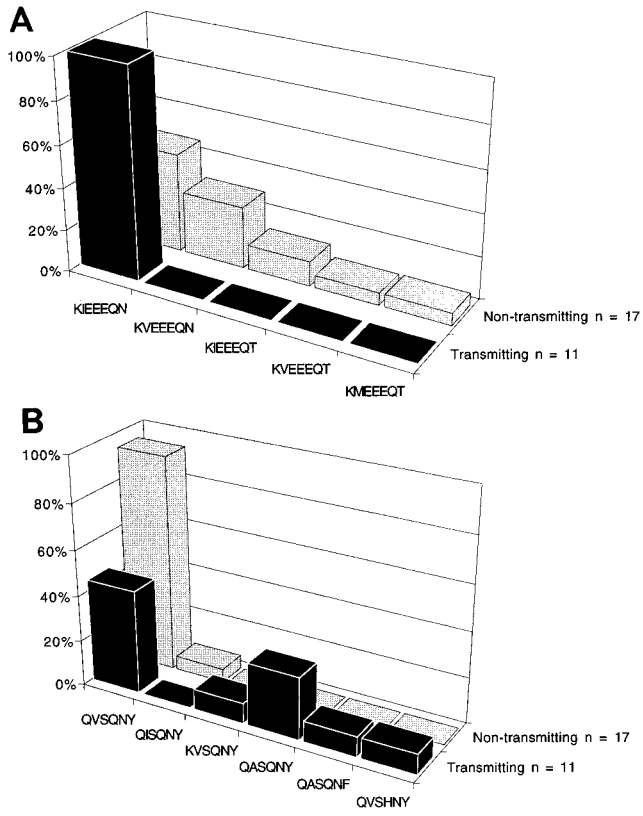

FIG. 8. (A) Relationship between the amino acid sequence (positions 103 to 109) of the p17 matrix protein from HIV-1 and the transmission statuses of the mothers from the B subgroup. The percentage of each motif at positions 103 to 109 is indicated for nontransmitting and transmitting mothers. A constant KIEE EQN motif was found in viral isolates from transmitting mothers (chi-square test, $P = 0.0034$). The valine at position 104 is associated with a nontransmitting status (Mann-Whitney test, $P = 0.048$). (B) Relationship between the amino acid C-terminal 6-mer sequences of HIV-1 p17 and the transmission statuses of the mothers from the B subgroup. The QVSQNY motif was associated with the nontransmitting status (chi-square test, $P = 0.0037$).

103 to 109) was quite different among the A, B, and G subtypes.

DISCUSSION

The mechanism of mother-to-child transmission of HIV-1 is complex and is presumably multifactorial. One of the crucial questions concerning HIV-1 pathogenesis is whether there is a viral selection during the vertical transmission of HIV-1. Analysis of *env* gene variability and prospective epidemiological studies of a mother-child cohort throughout pregnancy and at delivery and postpartum have failed to evidence an association between any particular molecular feature of the virus and vertical transmission.

p17, the HIV-1 matrix protein, is essential for the HIV-1 viral cycle, in particular for cell tropism and the viral phenotype. We used direct sequencing of the p17 genes from a mother-child cohort in order to detect viral selection during the vertical transmission of HIV-1.

Multiple alignment of the 54 deduced amino acid sequences enabled us to distribute 53 of them among the HIV-1 A, B, and G subtypes, according to the classification of Myers et al. (32). Therefore, in most cases, direct sequencing of the p17 gene seems to be a convenient way to classify HIV-1 viral isolates into their respective HIV-1 subtypes. Our observation is in agreement with the V3 European subtype distribution (32): the B subtype is the most frequent in Caucasian patients, and the A and G subtypes are less frequent among Caucasians and are mainly found in African patients.

The phylogenetic analysis showed that the studied sequences are distributed among several clades which correspond to their respective HIV-1 subtypes. Moreover, for all the dendogram-building methods used (neighbor joining, parsimony, and maximum likelihood), all of the mother-child isolates were closely linked. The phylogenetic tree drawn with all mother-child isolates demonstrated that each child's isolate sequence was closely related to the mother's sequence. The overall comparison of the direct sequences for our mother-child cohort did not evidence any particular feature for either the transmitting or the nontransmitting group.

The distance analysis showed that for all the MC pairs, the child's isolate sequence was very close to the mother's sequence, suggesting that the transmitted virus was not a minor variant. This result is in agreement with the observations that infant viral subtypes are always found previously in the mother (30, 51). Moreover, as for the V3 loop, at which low intrapatient variation was observed for children during the first month of life (30), p17 did not seem to vary during this period of time, during which no clinical or biological evolution was detected (data not shown). Therefore, the direct sequencing of p17 appears to be a good tool for epidemiological studies as it has been assessed by other authors (20, 22), in particular for the study of mother-to-child transmission.

Detailed analysis of the functional domains of p17 was more informative. No p17 sequence was truncated or substantially deleted. The p17 matrix protein domains which are functionally important were conserved in all isolate sequences. The myristoylation site (aa 1 to 6 [MGARAS]) required for membrane targeting and viral assembly (18, 47) was conserved. The nuclear localization sites (K-18 to R-30) (7) were also conserved. Therefore, the p17 genes of transmitted viruses do not seem to lack any major functional domain. Moreover, the phosphorylation site (Y-132) described by Gallay et al. (15) as being an essential domain was present in almost all sequences, except two.

Analysis of the C-terminal half of p17 amino acid sequences was of major interest. A statistical comparison of all the variable motifs from the nontransmitting and transmitting groups from the majority B subtype showed that two amino acids and two regions were likely to distinguish the two groups.

The C-terminal part of the p17 sequence was rather conserved in the nontransmitting group and was more variable in the transmitting group. In particular, the C-terminal 6-mer QVSQNY was conserved among 16 of 17 nontransmitting mother isolates. However, the relative variability of the previous motif among the transmitting group makes this motif a poor candidate for the discovery of a selective molecular pattern for p17 that may be associated with mother-to-child transmission.

On the other hand, within the p17 major antibody binding site (5) for the B subgroup, the KIEEEQN motif (aa 103 to 109) was conserved in all mother-child isolates but in only 8 of 17 nontransmitting mother isolates. The two transmitting groups were significantly different for the presence of this motif (chi square, $P = 0.0034$). Moreover, six transmitted viruses from the B group that were sequenced elsewhere (42a and data not shown) have the same KIEEEQN p17 motif. Therefore, the KIEEEQN motif appears to be necessary but insufficient for vertical transmission in the B group. This hypothesis is in accordance with a previous result published by Mulder-Kampinga et al. (31), who found the same KIEEEQN

motif in all the sequenced clones from one child's isolate and from his mother's isolate. However, this domain was highly divergent among all HIV-1 subgroups of our cohort (Fig. 2). Hence, a larger cohort is needed to determine whether there is a similar correlation with transmission in the A and G groups.

This correlation of the presence of KIEEEQN motif with vertical transmission may be explained in three ways.

(i) Nontransmitting mothers may have seric p17 antibodies that could prevent vertical transmission. Naylor et al. (33, 42) have found that a 30-aa synthetic peptide from p17, named HGP-30, induced high titers of neutralizing antibodies in rabbits. Boucher et al. (5) identified the sequence (KE)ALDK IEE(EQ) as the HIV-1 p17 major antibody binding site. Moreover, a decline in HIV-1 p17 antibodies has been shown to be related to disease progression in both children and adults, suggesting that p17 antibodies may be protective (24, 49). This property might also be valuable for vertical transmission. The results of numerous studies aimed at identifying a correlation between the absence of vertical transmission of HIV-1 and the presence of antibodies directed against the gp120 Env glycoprotein in maternal serum (12, 17, 35, 38, 46) are controversial. Therefore, this type of correlation may exist with antibodies specific to epitopes less variable than those of Env proteins, such as the p17 matrix protein. The search for such protective anti-p17 antibodies is now in progress in our laboratory.

(ii) The heptameric peptide, which is exposed in an alpha helix (27, 28), might be involved in the tropism phenotype. Indeed, a construction of HIV-1 from which the IEEEQN motif has been deleted is impaired for viral entry into Sup-T1 cells (53, 54), and a mutation close to this region was suspected to cooperate with other viral genes to confer the syncytium inductor phenotype (11). The in utero route of HIV-1 vertical transmission might then involve the infection of nondividing cells of the placental barrier by viruses presenting the KIEE EQN motif. Efficient infection may then depend on other viral factors and/or host factors, possibly intracellular factors or cytokines, for example. Placenta cell infection models are being investigated to test this hypothesis.

(iii) The difference between transmitted and nontransmitted viruses sharing the KIEEEQN motif might be due to differences in viral loads or in the stage of disease in the mothers. Indeed, it has been demonstrated that a high risk of mother-to-child transmission is correlated with a high viral burden in the mother (39, 50) or with an advanced stage of HIV infection (4).

The presence of valine at position 104 in the isolates from the B subtype was correlated with the nontransmitting status. This residue may prevent vertical transmission of HIV-1 by at least three potential mechanisms. (i) The apparent protection might reflect a specific maternal antibody response directed against isolates presenting a valine at position 104 of the p17 amino acid sequence, just inside the p17 major antibody site, as discussed above. Moreover, this potential protective role of p17 antibodies is more plausible if, as we have shown in our cohort, variations in the p17 major antibody binding site for the B subtype appear only in nontransmitting mother isolates and not in transmitting mother isolates. (ii) The valine residue at position 104 might confer particular tropism properties to the virus. (iii) The presence of this valine instead of an isoleucine may impair the dimerization kinetics of p17 protein that were demonstrated by Morikawa et al. (29) to be essential for particle assembly and viral production. Nevertheless, the p17 region at positions 103 to 109 for isolates from the B subtype seems to be involved in mother-to-child transmission of HIV-1. If the relationship between this domain and vertical transmission is confirmed, the screening of seropositive mothers by a rapid test using molecular probes or peptide-specific antibodies may be valuable in assessing the likelihood of vertical transmission.

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