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Genetic Analysis of Human Immunodeficiency Virus Type 1 Envelope V3 Region Isolates from Mothers and Infants after Perinatal Transmission

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The human immunodeficiency virus type 1 (HIV-1) sequences from variable region 3 (V3) of the envelope gene were analyzed from seven infected mother-infant pairs following perinatal transmission. The V3 region sequences directly derived from the DNA of the uncultured peripheral blood mononuclear cells from infected mothers displayed a heterogeneous population. In contrast, the infants' sequences were less diverse than those of their mothers. In addition, the sequences from the younger infants' peripheral blood mononuclear cell DNA were more homogeneous than the older infants' sequences. All infants' sequences were different but displayed patterns similar to those seen in their mothers. In the mother-infant pair sequences analyzed, a minor genotype or subtype found in the mothers predominated in their infants. The conserved N-linked glycosylation site proximal to the first cysteine of the V3 loop was absent only in one infant's sequence set and in some variants of two other infants' sequences. Furthermore, the HIV-1 sequences of the epidemiologically linked mother-infant pairs were closer than the sequences of epidemiologically unlinked individuals, suggesting that the sequence comparison of mother-infant pairs done in order to identify genetic variants transmitted from mother to infant could be performed even in older infants. There was no evidence for transmission of a major genotype or multiple genotypes from mother to infant. In conclusion, a minor genotype of maternal virus is transmitted to the infants, and this finding could be useful in developing strategies to prevent maternal transmission of HIV-1 by means of perinatal interventions.

Infants born to mothers infected with human immunodeficiency virus type 1 (HIV-1) are at risk of acquiring HIV-1 infection and subsequently developing AIDS. Perinatally acquired infections account for the majority of all HIV-1 cases in children, with an estimated mother-to-infant transmission rate of more than 30% in the United States and worldwide (5, 10, 17, 26, 39, 42). While the actual mechanisms of perinatal transmission are unknown, the timing of HIV-1 transmission from mother to child can theoretically occur at three stages: prepartum (transplacental passage), intrapartum (exposure of infants skin and mucous membrane to maternal blood and vaginal secretions), and postpartum (breast milk). Postnatal transmission through breast milk feeding has been documented in several cases (22, 46, 51).

To date, there are no clearly defined factors, viral or host, associated with maternal transmission of HIV-1. Advanced clinical stage of the mother, low $CD4^+$ lymphocyte counts, maternal immune response to HIV-1 antigenemia, recent infection, high level of circulating HIV-1, and maternal disease progression have been implicated in an increased risk of mother-to-child transmission of HIV-1 (3, 4, 5, 10, 15, 38, 39). In addition, the possibility of viral factors affecting mother-infant transmission of HIV-1 cannot be ruled out, since more than half of the children born to HIV-1-infected mothers are uninfected. While some studies have demonstrated a direct association between the presence of maternal antibody against the variable region 3 (V3) domain of envelope and a lower rate of transmission of HIV-1 (7, 38), others have showed lack of correlation (14, 34). Mutations in the V3 region could potentially affect mother-infant transmission of HIV-1, since this region is an important determinant for cellular tropism and virus neutralization (16, 32, 43).

Genetic variability in HIV-1, especially in the V3 region of the envelope gene, has been observed within infected individuals (29). These variants arise during retroviral replication by error in reverse transcription (8, 35, 37). Several reasons for the existence of different genetic variants within an infected individual (immunologic pressure for change, alteration in cell tropism, replication efficiency, etc.) could be postulated (16, 23, 43, 44). Wolinsky et al. (48) compared the HIV-1 DNA sequences in the V3 and V4-V5 regions of envelope from mother and infant isolates and suggested that a minor subtype of maternal virus from the genetically heterogeneous virus population could be transmitted to the infant. In addition, there was a loss of the N-glycosylation site proximal to the first cysteine of the V3 loop in infants' sequence sets. Scarlatti et al. (41) showed, by comparing HIV-1 sequences derived from mother and infant isolates, that the transmitted virus represented either a minor or a major population present in mothers, with conserved N-glycosylation sites in infants' sequences. In a more recent study, transmission of multiple HIV-1 genotypes from mother to infant has also been reported (19).

To further elucidate the molecular mechanisms involved in mother-to-child transmission of HIV-1, we have undertaken a

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TABLE 1. Demographic, clinical, and laboratory parameters of HIV-1-infected mother-infant pairs

Patient	Age	Sex	Race	CD4 lymphocytes		Antiretroviral drug	Clinical evaluation[a]
				Per mm^3	%		
Mothers							
M1	31 yr		White	480		None	Asymptomatic
M2	23 yr		White	818		None	Asymptomatic
M3	23 yr		White	480		None	Asymptomatic
M4	26 yr		Black	395		ZDV[b]	Symptomatic AIDS
M5	36 yr		Black	706		None	Asymptomatic
M6	28 yr		Black	509		None	Asymptomatic
M7	23 yr		Black	692		None	Asymptomatic
Infants							
I1	28 mo	Male	White	46	8	ddC (zalcitibine)	Symptomatic AIDS, P-2A, B, F; failed to ZDV therapy
I2	14 mo	Female	White	772	26	ZDV	Symptomatic AIDS, P-2A, D1, 3, F
I3	24 mo	Female	White	4,379	34	ZDV	Asymptomatic, P-1B
I4	34 mo	Male	Black	588	34	ZDV	Symptomatic AIDS, P-2A, B
I5	6 wk	Female	Black	2,994	53		Asymptomatic, P1A
I6	4.75 mo	Male	Black	1,942	42		Asymptomatic, P1A
I7	1 wk	Male	Black	2,953	49	ZDV	Asymptomatic, P1A (on ZDV per ACTG 076 perinatal protocol)

[a] Evaluations for infants based on criteria in reference 5a.
[b] ZDV, Zidovudine.

systematic comparison of the sequences of the V3 region of the envelope gene of HIV-1 from seven infected mother-infant pairs following perinatal transmission in infants ranging in age from 1 week to 3 years. In this paper, we report a minor subtype of the maternal virus to be transmitted to the infants. Furthermore, we show that the virus populations of infants were more homogeneous than those of the corresponding mothers. In addition, the V3 region sequences were more homogeneous in younger infants' than in older ones, and the epidemiologically linked mother-infant viral sequences were more closely related than epidemiologically unlinked viral sequences. A comparative study that attempts to identify and characterize genetic variants involved in maternal transmission of HIV-1 should be performed immediately after birth because of the homogeneous nature of HIV-1 sequences present in newborn infants.

MATERIALS AND METHODS

Patient population and sample collection. Patients for this study were HIV-1-infected mother-infant pairs who were receiving medical care at the HIV Family Care Center at the Children's Hospital Medical Center, Cincinnati, Ohio. The study was approved by the Institutional Review Board of the Children's Hospital Medical Center, and written informed consent was obtained for participation in the study. We studied seven HIV-1-positive mothers and their infants. Blood samples were collected from mother-infant pairs, and the infants' ages at the time of specimen collection were 28 months (infant 1 [I1]), 14 months (I2), 24 months (I3), 34 months (I4), 6 weeks (I5), 4.75 months (I6), and 1 week (I7). The age range provided a wide spectrum of sequence heterogeneity as the virus evolved and also allowed us to find the age group of infants which would be appropriate for genotypic characterization. Demographic, clinical, and laboratory findings of HIV-1-infected mother-infant pairs are summarized in Table 1.

Isolation of DNA from PBMC. The peripheral blood mononuclear cells (PBMC) were isolated by a single-step Ficoll-Paque procedure (Pharmacia-LKB) from the whole blood of HIV-1-positive mothers and their infants. DNA was isolated by a procedure modified from that described by Oram et al. (31). Approximately 10^6 PBMC were centrifuged at 12,000 rpm for 2 min, and the cell pellet was resuspended in 0.5 ml of TNE buffer (0.5 M Tris-HCl [pH 7.5], 0.1 M NaCl, 1 mM EDTA). The suspension was treated with 0.50% sodium dodecyl sulfate and 10 μg of proteinase K (Boehringer) per ml at 60°C for 3 h and then subjected to several extractions with phenol and chloroform. The DNA was precipitated with ethanol, dissolved in 50 to 100 μl of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), and sheared by repeated pipetting.

PCR amplification. A two-step PCR amplification, first with outer primers and then with nested or inner primers, was performed to detect the presence of HIV-1 in infected patients' PBMC. The DNA oligonucleotide primers HIV-19 (5′-AATGTCAGCACAGTACAATGTACA, nucleotides [nt] 6935 to 6958, sense), HIV-20 (5′-CAGTAGAAAAAATTCCCCTCCACAATT, nt 7343 to 7368, antisense), HIV-21 (5′-CTGCTGTTAAATGGCAGTCTAGC, nt 6989 to 7011, sense), and HIV-22 (5′-TCTGGGTCCCCTCCTGAGGA, nt 7304 to 7323, antisense) were synthesized according to published HIV-1 sequences of pNL4-3 (29) by using a Pharmacia gene assembler. The PCRs were performed according to the procedure of Ahmad et al. (1) in a 50-μl reaction mixture containing 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 200 μM each dATP, dCTP, dGTP, and TTP, 0.2 to 1.0 μM each HIV-1 outer primer pair, and 2.5 U of *Taq* polymerase (Perkin-Elmer, Cetus Norwalk, Conn.). The reactions were carried out at 94°C for 1.5 min, 45°C for 2 min, and 72°C for 3 min for 35 cycles. The amplified DNA products were analyzed by electrophoresis on a 1.2% agarose gel. Negative controls consisting of DNA from PBMC of seronegative individuals were included in each set of reactions, which were negative in all assays. After the first round PCR, 1 μl of the product was amplified for 25 cycles with the corresponding inner primers at 94°C for 1.5 min, 50°C for 2 min, and 72°C for 3 min. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel. To avoid contamination, all samples, reagents, and first- and second-round PCR products were kept separately and dispensed in a laminar-flow hood in biosafety level 3 facility free from all laboratory-used DNAs.

Characterization of PCR products by Southern blot hybridization. To further characterize the PCR products, the DNAs were transferred to nitrocellulose filters and Southern blot hybridization was performed according to the procedure of Sambrook et al. (40) by using an oligonucleotide probe, HIV-33 (5′-CACAGACAATGCTAAAACCATAATAGTACAGC, nt 7045 to 7076, sense). The oligonucleotide probe does not overlap with any of the primers used in PCR amplification.

Cloning and DNA sequencing. The PCR products amplified by inner primer pair HIV-21/HIV-22 which were positive by Southern blot hybridizations were blunt ended by the large fragment DNA polymerase (Bethesda Research Laboratories, Gaithersburg, Md.), phosphorylated by T4 polynucleotide kinase (Bethesda Research Laboratories), and cloned into the *Sma*I site of pGem 3Zf (+) vector (Promega Corp., Madison, Wis.). Individual bacterial colonies were screened for the presence of recombinants by restriction enzyme analysis of plasmid DNA. The clones with the correct sizes of inserts were selected and propagated for single-stranded DNA, and nucleotide sequencing (10 to 30 clones for each patient) was performed according to the Sequenase protocol (U.S. Biochemical Corp., Cleveland, Ohio).

Computer alignment and analysis of HIV-1 sequences. The nucleotide sequences of the V3 region of the HIV-1 *env* gene from seven mother-infant pairs were translated to corresponding amino acids for 282 to 288 bases (94 to 96 amino acids); alignment of the sequences was performed easily by hand, as only two positions contained gaps. The nucleotide sequences were aligned in comparison with amino acid alignment. For each pair, the most frequent sequence within the mother's sequences was selected as the major sequence. Pairwise distances, defined as the percentage of mismatches between two aligned nucleotide sequences, were used to study the extent of genetic variation within a

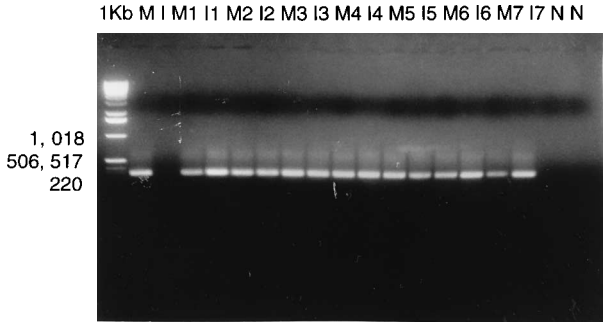

FIG. 1. PCR amplification of the V3 region of the envelope gene of HIV-1 from infected mother-infant pairs' PBMC DNA. The first-round PCR amplification was performed with primer pair HIV-19/HIV-20, and 1 μl of the PCR product was used for the second-round PCR by using primer pair HIV-21/HIV-22 to yield a 336-bp fragment analyzed on a 1.2% agarose gel electrophoresis. Lanes: 1Kb, DNA marker; M, mother sample; I, infant sample; M1 to M7 and I1 to I7 samples from M1 to M7 and I1 to I7; N, negative sample. Sizes are indicated in base pairs.

sequence set and between sets. For intrapatient variability (within mothers' and infants' sequence sets), pairwise distances were calculated between each pair of sequences within the set. For interpatient variability (between epidemiologically linked mother-infant sets and between epidemiologically unlinked individuals' sets), each sequence from one set was compared with each sequence of the other set. Within a sequence set, variant subtypes may be defined as subsets of sequences, each close to at least another sequence in a subset by less than a certain threshold, and all of them diverging from the sequences out of the subset by at least the same threshold. The value of the threshold depends on the variability of the region analyzed within the genome. For the V3 region, we have chosen a threshold of 4%, corresponding to the average pairwise distance between linked mothers and infants. To study the distribution of the variation within infants related to mothers, the sequences from both sets were compared with the major sequence, and distances were calculated as the percentage of mismatches relative to the major sequence. The selection pressure was calculated as the ratio of nonsynonymous to synonymous substitutions (30) by comparing the infants' sequences with the infants' major sequences. The phylogenetic analysis was performed by using the software PHYLIP, version 3.5 (11). The tree was built from a pyres distance matrix (function DNADIST) by using the neighbor-joining method (function NEIGHBOR). The robustness of the neighbor-joining tree was assessed by bootstrap resampling (50 data sets) of the multiple alignments (function SEGBOOT). The tree was rooted by using the reference HIV-1 sequence, MN (13).

Nucleotide sequence accession numbers. The sequences have been submitted to GenBank with accession numbers U16390 to U16652.

RESULTS

PCR amplification of the V3 region of the HIV-1 *env* gene from mother-infant PBMC DNAs. The PCR amplification of the V3 region was performed as a two-step procedure. The first round of amplification was performed by using primer pair HIV-19/HIV-20 followed by nested PCR amplification using primer pair HIV-21/HIV-22. These outer and inner primer pairs yielded 379- and 336-bp fragments, respectively. Figure 1 shows the result of the analysis of the second-round PCR products obtained by primer pair HIV-21/HIV-22 on a 1.2% agarose gel electrophoresis after staining with ethidium bromide. A 336-bp fragment was amplified in all infected mothers (mother 1 [M1] to M7) and linked infants (I1 to I7). HIV-1 was not detected in one infant who was negative for HIV-1 infection (lane I), whereas the corresponding mother was found to be positive (lane M). We used equal amounts of PBMC DNA from all patients, as visualized by agarose gel electrophoresis. We found the optimal annealing temperature for HIV-1 DNA and primer pair HIV-21/HIV-22 to be 50 to 55°C, and the maximum yield was obtained under the condition described above. We also confirmed the PCR result with another set of primers from the *gag* region (2), and the PCR results from the *gag* region correlated with those for the *env* region (data not shown). The PCR products were further characterized by Southern blot hybridization using an oligonucleotide primer (HIV-23) from the V3 region after transferring the DNAs on a nitrocellulose paper. The PCR products from all mothers (M1 to M7) and infants (I1 to I7) hybridized with the probe, whereas no signal was seen in the negative samples (data not shown). To determine the errors made by *Taq* polymerase, we included a known HIV-1 sequence (pNL4-3) for PCR amplification and sequencing.

Coding potential of the envelope open reading frame of mother-infant isolates. The multiple alignments of the amino acid sequences (amino acids 94 to 96) of V3 and the flanking region of HIV-1 *env* from PBMC DNA of seven mother-infant pairs are shown in Fig. 2. For pairs 3 to 6, the major sequence was identical to the consensus sequence that was deduced from the most frequent amino acid found at each position of the alignment of the sequences of the mother only. In case of multiple major sequences pairs 1, 2, and 7, the major sequence selected was the one minimizing the average distance with the other sequences of the mother. The coding potential of the envelope open reading frame was maintained in most of the sequences, with only four inactivating mutants (stop codons) in 92,253 bases sequenced. The two cysteine residues which sandwich the V3 loop at positions 296 and 330 (numbered according to sequence of the HXB2 clone [29]), involved in disulfide bridge formation (21), were mostly conserved with the exception of a substitution at position 296 to arginine for one sequence (m-7a [Fig. 2G]) and a substitution at position 330 to serine for two sequences (i-3c [Fig. 2C] and m-7k [Fig. 2G]) in a total of 324 different sequences comprising of 30,751 amino acids.

Comparison of the V3 region sequences of mother-infant isolates. The degree of variability of distances of the V3 region sequences of seven mother-infant sets is shown in Table 2. The amino acid sequence of the V3 region within each mother (M1 to M7) differed by 3.2, 5.3, 3.2, 7.4, 14.7, 3.2, and 8.5% (median values), ranging from 3.2 to 14.7%, with large variation reaching 21.1 and 20.2% between the two most divergent amino acid sequences in M5 and M7, respectively. By contrast, the variability within infant sets (I1 to I7) was 3.2, 5.3, 2.1, 6.3, 1.1, 2.1, and 1.1%, ranging from 1.1 to 6.3%, implying that the infants' sequences were more homogeneous than the mothers' sequences. The younger infants' sequences were even more homogeneous than the older infants' sequences (Table 2). Figure 3 also shows the distance distribution of V3 region sequences of the infants as they age. The heterogeneity within infant sets correlated with the progression of age with the exception of I3 at median values of 0.7 (pair 7), 0.7 (pair 5), 1.1 (pair 6), 2.5 (pair 2), 1 (pair 3), 1.8 (pair 1), and 3.1 (pair 4).

The epidemiologically linked mother-infant pairs (M1/I1 to M7/I7) differed in the V3 region amino acid sequences at median values of 5.3, 7.4, 4.2, 13.7, 15.8, 2.1, and 5.3%, respectively, with a range from 2.1 to 15.8%. Moreover, the median difference in nucleotide sequences (282 to 288 nt) of M1/I1 to M7/I7 were 2.5, 3.2, 1.8, 6.3, 7.4, 1.4, and 3.2%, respectively (range, 1.4 to 7.4%). Further analysis of pairwise sequences showed that the specific nucleotide sequences were highly conserved between each mother and her infant (M1/I1 to M7/I7), with minimum differences ranging from 0 to 3.5%. In addition to conservation of selected sequences between the mothers and their infants, there was a large variation in the sequences within these sets, with ranges of differences from 0 to 27.4% for protein sequences and 0 to 10.9% for nucleotides. Furthermore, Fig. 4 compares the distributions of V3 region amino acid sequence distances of the seven mother-infant pairs with

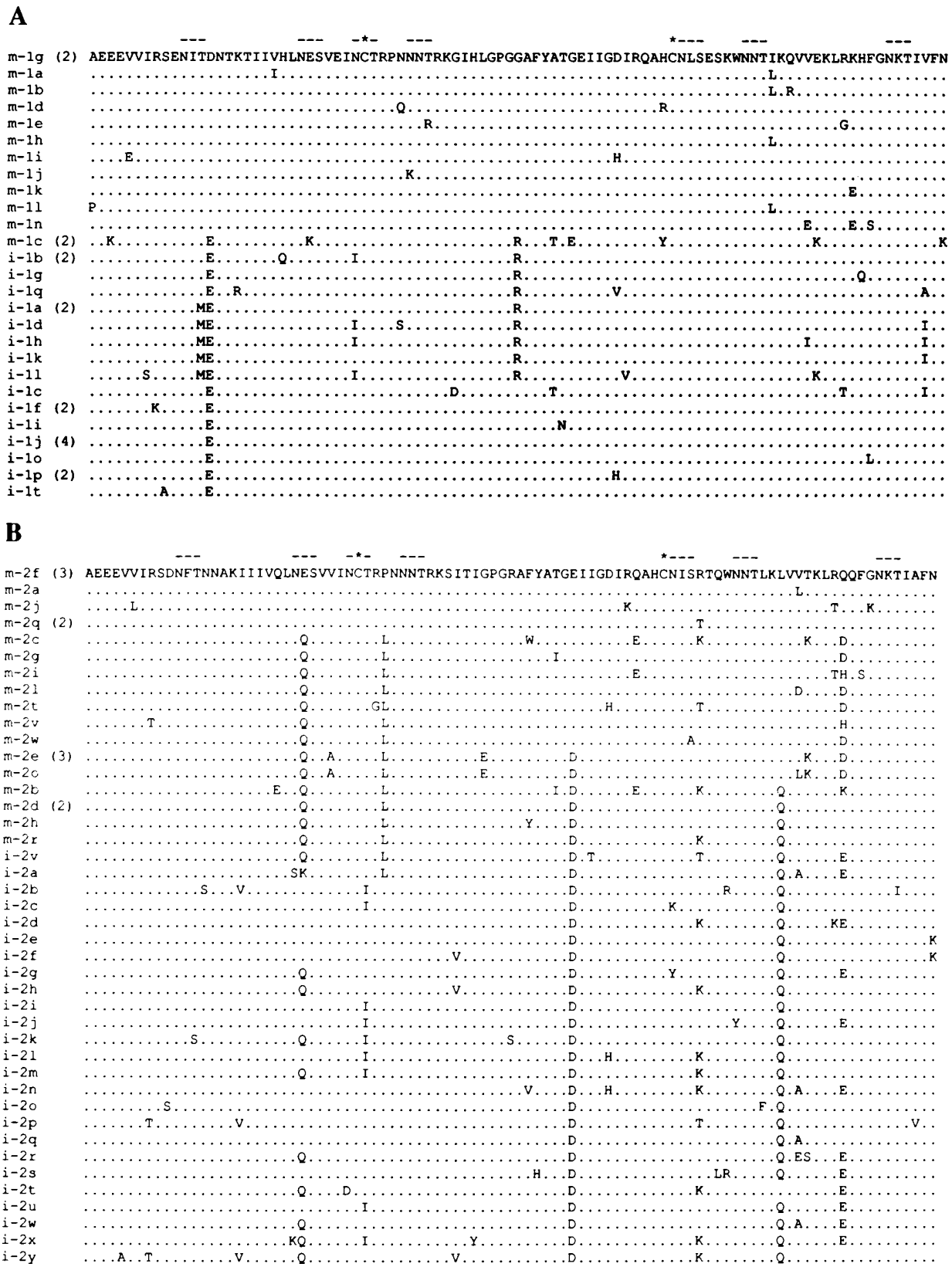

FIG. 2. Multiple alignments of deduced amino acid sequences of the V3 region of the envelope gene of HIV-1 from seven HIV-1-infected mother-infant pairs following perinatal transmission. (A) Mother-infant pair 1; (B) mother-infant pair 2; (C) mother-infant pair 3; (D) mother-infant pair 4; (E) mother-infant pair 5; (F) mother-infant pair 6; (G) mother-infant pair 7. In the alignment, the top sequence is the mothers' major sequence, dots replace amino acids identical to mothers' major sequence, dashes represent gaps, and asterisks represent stop codons. The number of identical clones is indicated in parentheses. Above the alignment, three continuous dashes indicate a putative glycosylation site and asterisks locate the two cysteine residues of the V3 loop.

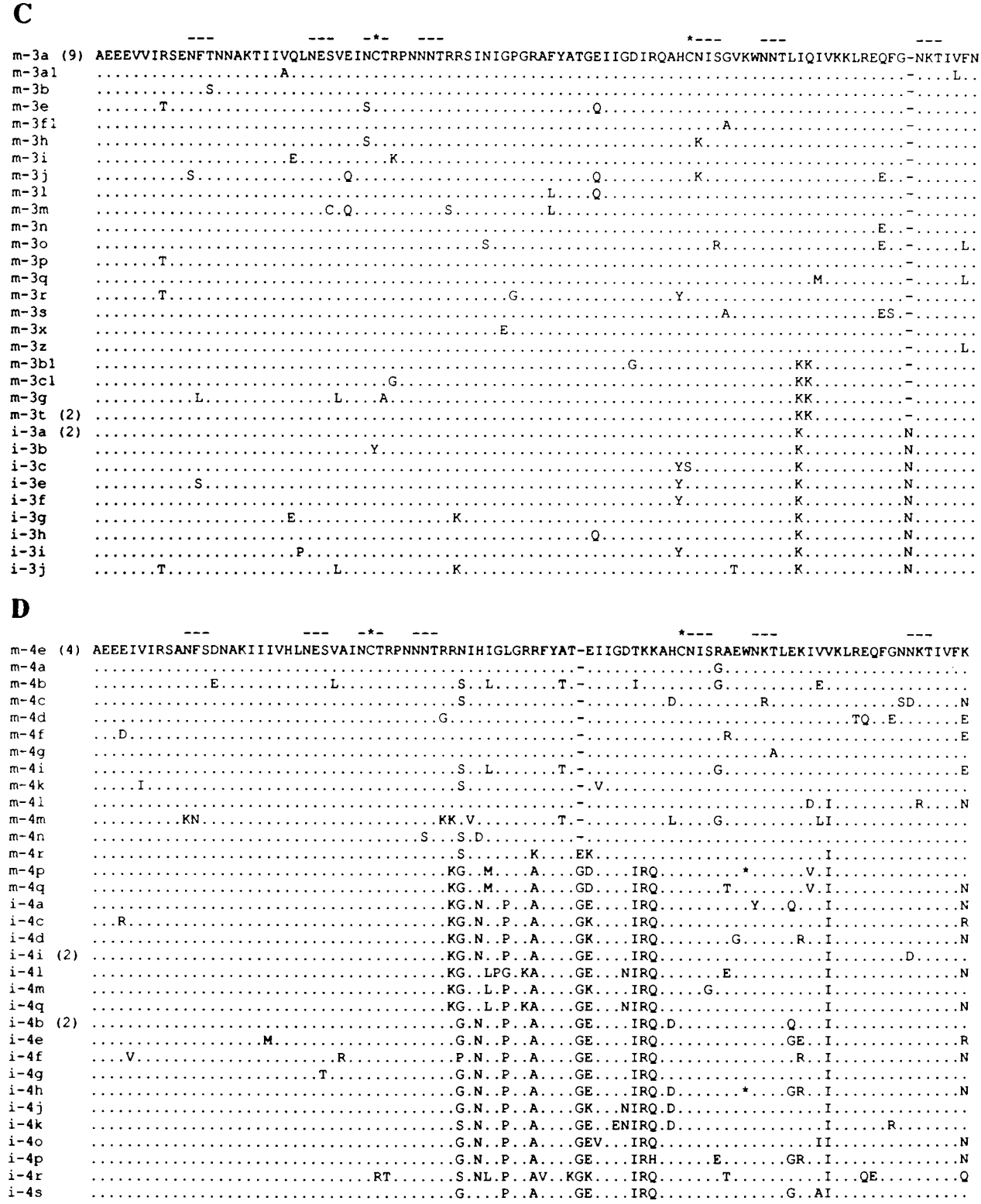

FIG. 2—*Continued.*

that of the mothers' major sequences. The data demonstrate that in most of the mother-infant pairs, the infants' sequences are far from the mothers' major sequences. However, despite the variability, the infants' sequences displayed amino acid patterns similar to those of their mothers. The maximum values of the ratio of nonsynonymous to synonymous substitution for the infants' sequences were 3.8 (I1), 3.8 (I2), 3.9 (I3), 3.0 (I4), 7.5 (I5), 1.3 (I6), and 7.8 (I7), suggesting that there was a

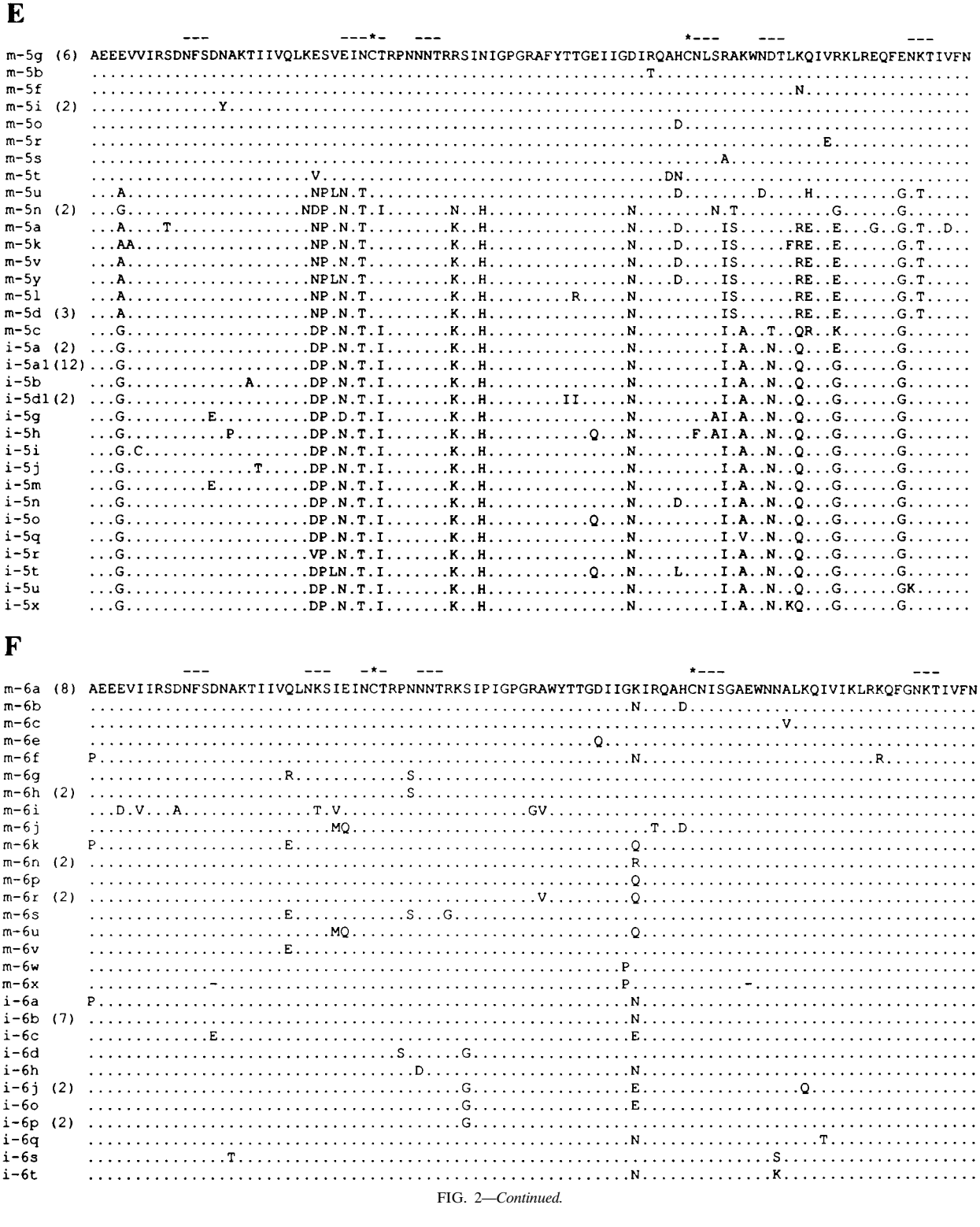

FIG. 2—*Continued.*

positive selection (>1) in the V3 region for amino acid changes in infants. The selection pressure was maximum in I7 and I5 and minimum in I6.

Comparison of V3 region sequences of unlinked mother-infant isolates. Figure 5 shows the histograms of the distributions of nucleotide sequence distances within the same mother and same infant, between linked mother-infant pairs, and between two different mothers; the medians of distributions were 2.1, 1.4, 3.2, and 11.9%, respectively. The data suggest that the sequences from the epidemiologically linked mother-infant pairs were closer than those of the epidemiologically unlinked individuals. Interestingly, the infants' sequences were more

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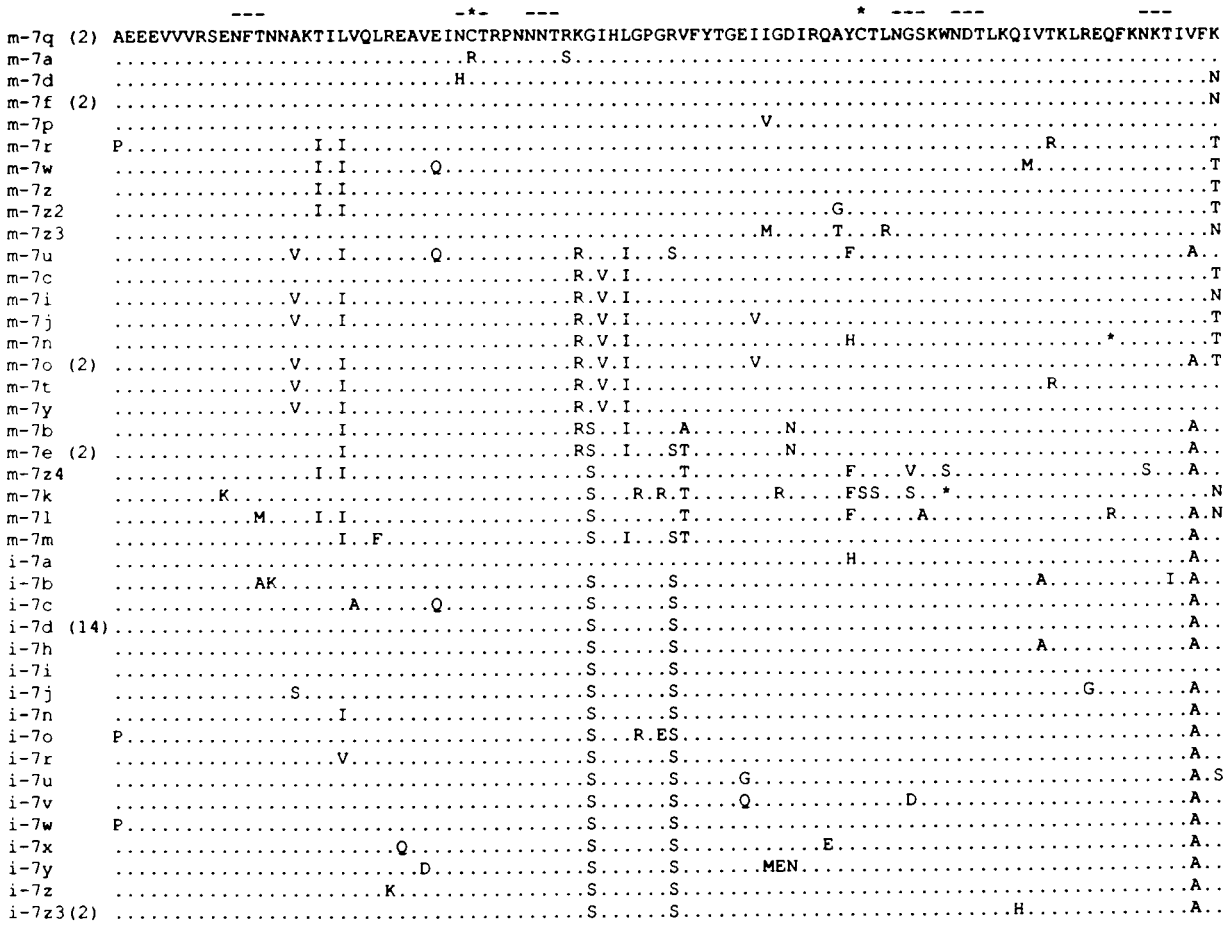

FIG. 2—*Continued.*

homogeneous than the related mothers' sequences, and the linked mother-infant sequences were closer even for those infants from whom the sample was taken as long as 34 months after birth.

Conservation and divergence of the central GPGR motifs of the V3 loop of mother-infant isolates. We observed both conservation and divergence in the GPGR motifs of the seven mother-infant pairs analyzed (Fig. 2). In mother-infant pairs 2 (Fig. 2B), 3 (Fig. 2C), 5 (Fig. 2E), and 6 (Fig. 2F), the GPGR motif was conserved in both mothers' and infants' sequences. In other mother-infant pairs, we found a mixed population of the central motif, with mostly GPGG in M1 and a mixed population of GPGR and GPGG in I1 (Fig. 2A), GLGR in M4 and GPGR in I4 (Fig. 2D), and GPGR in M7 and GPGS in I7 (Fig. 2G). This finding demonstrates that the GPGR motif is highly conserved in the infants' sequences with the exception of I7 compared with mothers' sequences.

Phylogenetic tree analysis of the V3 region sequences of mother-infant isolates. We traced the similarity relationships among the V3 region sequences of seven mother-infant pairs. Phylogenetic tree analysis performed as described in Materials and Methods revealed that the seven mother-infant pairs were well discriminated, divergent from each other, and confined within subtrees (Fig. 6). This finding was further emphasized by high bootstrap values obtained for seven mother-infant sequence sets. Bootstrap analysis performed by resampling the data sets 50 times formed the same clusters of the sequences all 50 times in all seven mother-infant pairs. The subtrees for pairs 2, 3, and 6 revealed a homogeneous virus population in which some mothers' and infants' sequences were intermingled. Moreover, the subtrees for pairs 1, 4, 5, and 7 showed distinct groups of tightly clustered mothers' sequences corresponding to different subtypes.

Selection of HIV-1 genotypes during mother-infant transmission. The mothers' virus populations of pairs 1, 4, 5, and 7 displayed two or more subtypes of variants or genotypes (Fig. 6) diverging at least by 4% and present with unequal frequencies. The infants' sequences of the linked pairs were closer to the mothers' minor subtypes (Fig. 2 and 6), suggesting the transmission of a specific variant appearing at a low frequency at the time of sample recovering. The minor genotypes of the mothers represented by m-1c (M1; Fig. 2A), m-4p, q (M4; Fig. 4D), m-5c (M5; Fig. 2E), and m-7e, m (M7; Fig. 2G) were found as the only subtype predominating in the linked infants. Mother-infant pairs 2, 3, and 6 showed a homogeneous sequence population. In addition, few sequences from the mothers' set shared amino acid similarities with the corresponding infants' sequences: m-2r (M2; Fig. 2B), m-3t, b1 (M3; Fig. 2C), and m6-b, f (M6; Fig. 2F). The bimodal distributions of distances between mother-infant sequences and the mothers' major sequence (Fig. 4) indicated that the infants' sequences were far from the mothers' major subtypes. Phylogenetic analysis

TABLE 2. Distances in the V3 region within the mother sets and infant sets and between mother-infant sets

Sequence	Pair	Infant age	Distance[a]								
			Within:						Between mother-infant sets		
			Mother set			Infant set					
			Minimum	Median	Maximum	Minimum	Median	Maximum	Minimum	Median	Maximum
Nucleotide	1	28 mo	0	1.8	5.3	0	1.8	4.2	0.4	2.5	5.6
	2	14 mo	0	2.5	5.6	0.35	2.5	4.6	1.1	3.2	6
	3	24 mo	0	1.4	3.9	0.35	1.0	3.1	0.3	1.8	4.9
	4	34 mo	0.4	2.1	8.8	0	3.1	7	3.5	6.3	10.9
	5	6 wk	0	6	9.1	0	0.7	3.2	1.8	7.4	10.5
	6	4.75 mo	0	1.4	5.6	0	1.1	2.1	0	1.4	4.6
	7	1 wk	0	3.6	9.2	0	0.7	3.6	0.7	3.2	8.2
Total[b]			0	2.1	9.2	0	1.4	7	0	3.2	10.9
Amino acid	1	28 mo	0	3.2	12.6	0	3.2	10.5	1.1	5.3	11.6
	2	14 mo	0	5.3	13.7	1.1	5.3	10.5	2.1	7.4	14.8
	3	24 mo	0	3.2	10.5	0	2.1	6.3	1.1	4.2	10.6
	4	34 mo	0	7.4	19	0	6.3	16.7	6.3	13.7	27.4
	5	6 wk	0	14.7	21.1	0	1.1	6.3	3.2	15.8	22.1
	6	4.75 mo	0	3.2	10.5	0	2.1	5.3	0	2.1	10.5
	7	1 wk	0	8.5	20.2	0	1.1	8.5	2.1	5.3	18.1
Total[b]			0	5.3	21.1	0	3.2	16.7	0	4.2	27.4

[a] Expressed as percent nucleotides (for nucleotide sequence) or percent amino acids (for amino acid sequence).
[b] Calculated from all the pairs taken together.

performed separately for each mother-infant pair also showed the mothers' minor genotypes to be closer to the infants' sequences (data not shown). There was no evidence of any major or multiple variants transmitted from mother to infant, as revealed by amino acid alignment and phylogenetic analysis (Fig. 2 and 6).

Role of N-linked glycosylation in mother-infant transmission. The N-linked glycosylation site (N-X-T or N-X-S) se-

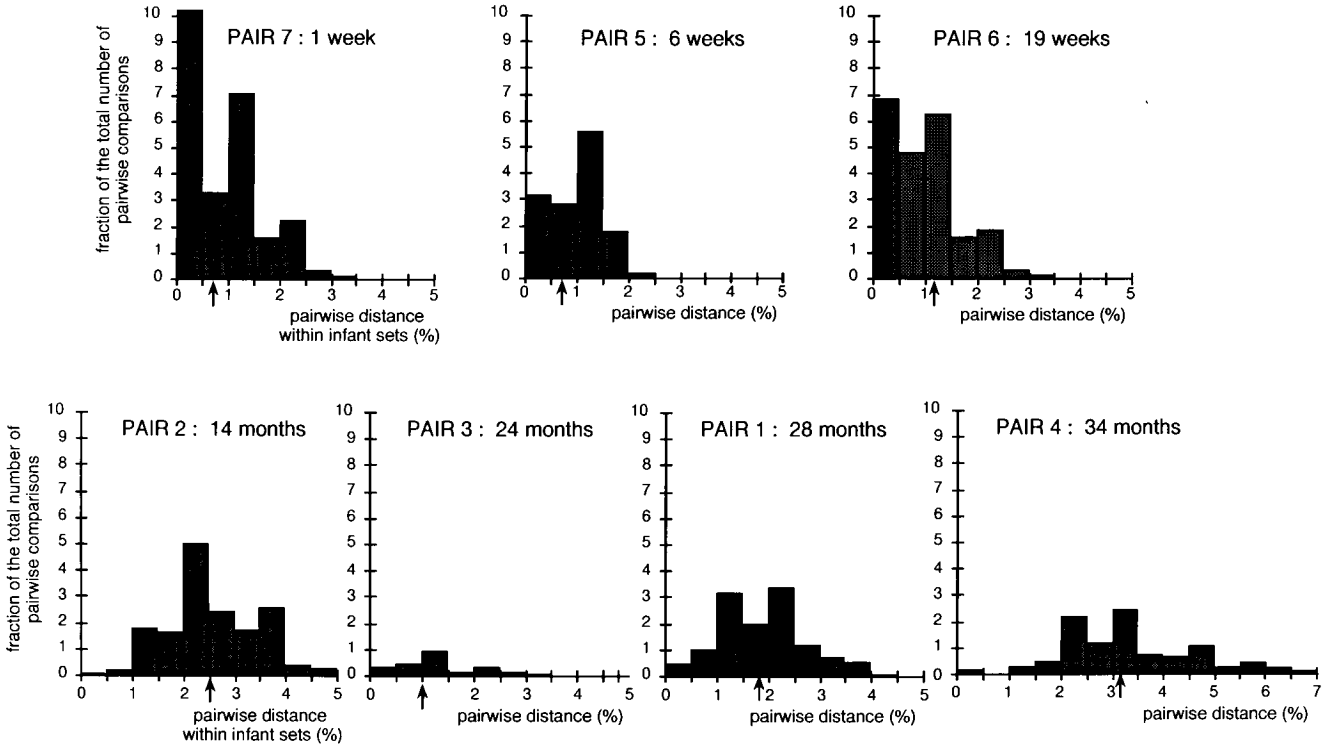

FIG. 3. Distributions of V3 region nucleotide sequence distances with varying infants' age. The number on each *y* axes represents the fraction of the total number of pairwise comparison that has given the corresponding percent nucleotide distance. The heterogeneity within infants' sets correlates with the progression of age. The medians of the distributions denoted by arrows on the x axes are 0.7 (pair 7), 0.7 (pair 5), 1.1 (pair 6), 2.5 (pair 2), 1 (pair 3), 1.8 (pair 1), and 3.1 (pair 4).

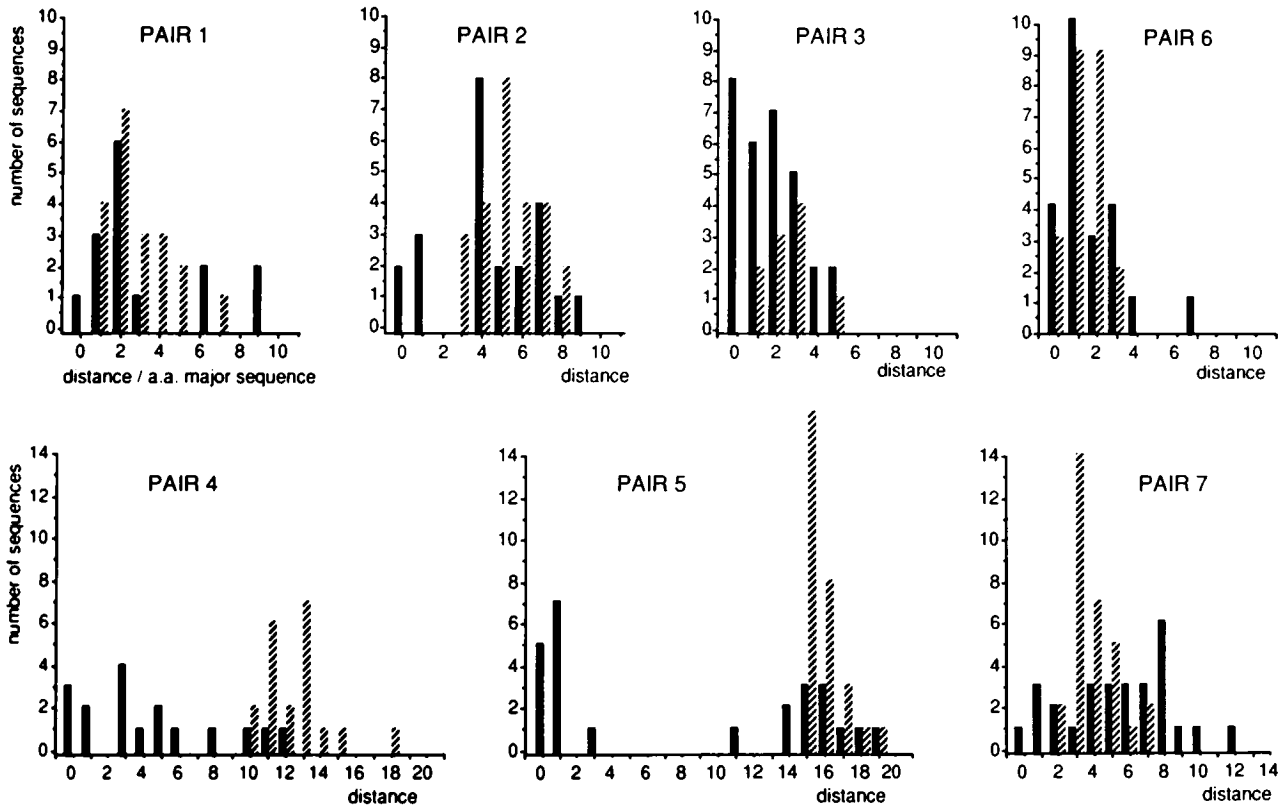

FIG. 4. Distributions of V3 region amino acid sequence distances between seven mother-infant pairs' sequences and the mothers' major sequence. The distributions of the mothers' and infants' distances from the major sequences are represented by dark bars and dashed bars, respectively. The distances are calculated as the number of amino acid mismatches between the major sequence and each other sequence of mother-infant sets. The number on each *y* axis represents the number of sequences that show the corresponding distance.

quons (36) proximal to the first cysteine of the V3 loop was completely absent only in one infant's sequence set (I5; Fig. 2E), absent in 5 of 22 sequences (I1; Fig. 2A), and absent in 9 of 25 sequences (I2; Fig. 2B). In I3, I4, I6, and I7, the glycosylation site was conserved. It was present in all mothers' sequences except 3 of 31 sequences in M3 (Fig. 2C), 2 of 26 sequences in M5 (Fig. 2E), and 1 of 28 sequences in M7 (Fig. 2G). Interestingly, the unglycosylated m-5c minor variant of the mother (M5; Fig. 2E) predominated in her infant, but this pattern of transmission was not seen in all mother-infant pairs. In other mother-infant pairs, the conserved N-linked glycosylation site in the V3 loop was present in most of the mothers' and infants' sequences. The possibility that some of the unglycosylated variants seen in I1 and I2 at the time of sampling could have been the major variants in the infants after transmission or at a younger age cannot be ruled out. The other N-linked glycosylation sites were fairly conserved within a given patient sample. We observed a variation in both number and position between patient samples (Fig. 2).

DISCUSSION

We have performed a systematic comparison of the V3 region sequences of the envelope gene from seven HIV-1-infected mother-infant pairs, with infants varying in age from 1 week to 3 years at the time of sampling following perinatal transmission. The V3 sequences directly derived from the DNA of uncultured PBMC from mothers showed a significant degree of heterogeneity compared with their corresponding infants, who displayed a homogeneous virus population, confirming the earlier published reports (41, 48). The younger infants' sequences were more homogeneous than the older infants' sequences (Fig. 3), which suggests that HIV-1 becomes more diverse as the infants grow older (28). Our results also show that the V3 region sequences of the individual infants were different but displayed patterns similar to those seen in their mothers.

Our findings indicate the selection of minor genotypes or subtypes from the heterogeneous virus population of mothers which are transmitted to their infants and are in agreement with the results described elsewhere (41, 48). Wolinsky et al. (48) reported that a proviral form infrequently found in a mother predominated in her infant and that the conserved N-glycosylation site within the V3 region was absent in all infant sequences, which was also the characteristic of the transmitted virus. Our results comparing the V3 sequences of seven mother-infant pairs with those of three younger infants (1 week, 6 weeks, and 4.75 months) and four older infants (14, 24, 28, and 34 months) at the time of sampling are in general agreement with the results of Wolinsky et al. (48). We observed that the minor genotypes found in the mothers at the time of sampling predominated in their infants. This particular feature was more obvious in mother-infant pairs 1, 4, 5, and 7 than mother-infant pairs 2, 3, and 6 (Fig. 2 and 6). Nevertheless, the infants' sequences were still closer to their mothers' sequences than to unlinked sequences (Fig. 2 and 5). Phylogenetic tree analysis performed together for seven mother-infant pairs (Fig. 6) and separately for each mother-infant pair (not shown) revealed the presence of multiple subtypes or geno-

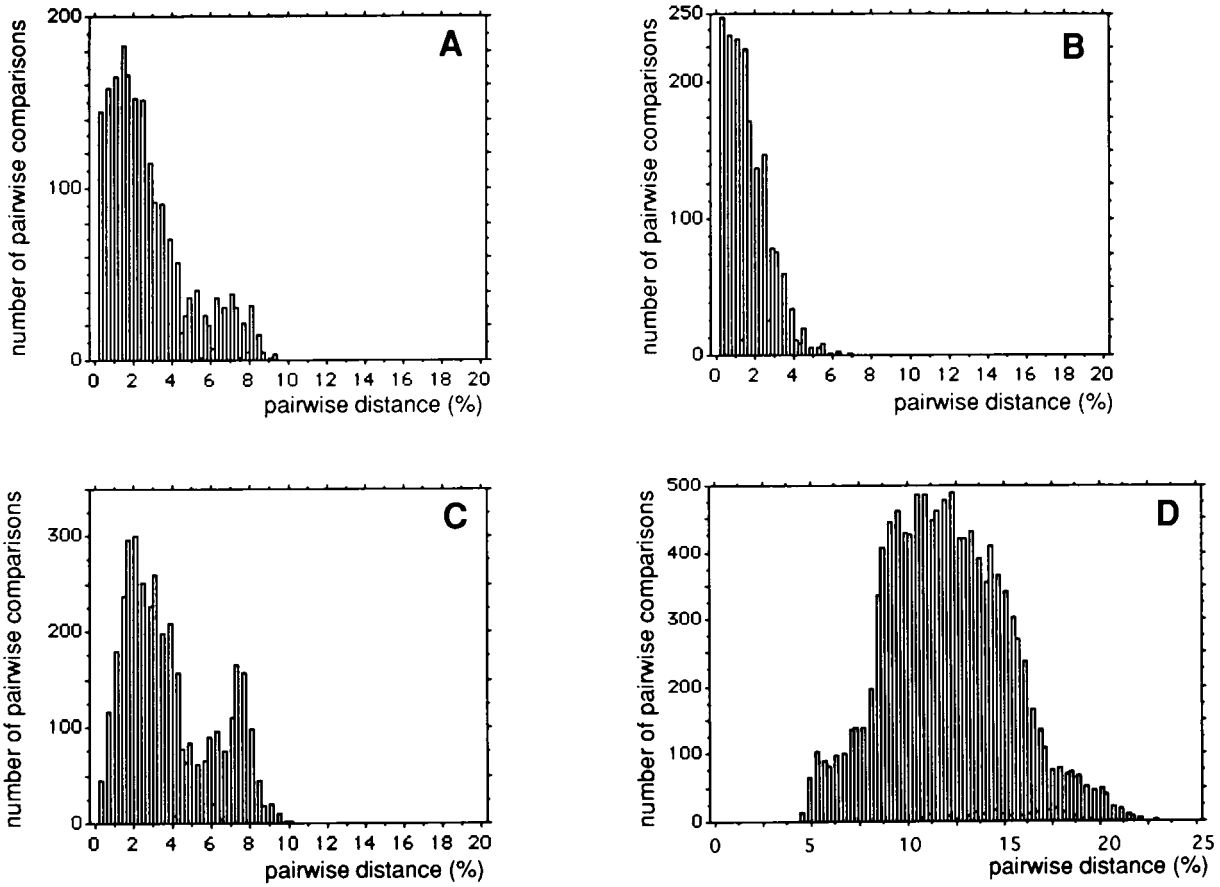

FIG. 5. Distributions of V3 region nucleotide sequence distances from mother-infant pairs. The percentages of mismatches were calculated between nucleotide sequences within the same mother's set (A), within the same infant's set (B), between epidemiologically linked mother-infant sets (C), and between unlinked mothers' sets (D). The distance percentages were rounded off to the nearest decimal. The number on each *y* axis represents the total number of pairwise comparisons that had given the corresponding percent distance range. The medians of distributions are 2.1% for 1,753 comparisons (A), 1.4% for 1,890 comparisons (B), 3.2% for 3,333 comparisons (C), and 11.9% for 10,830 comparisons (D).

types of HIV-1 in mothers. The minor subtypes were closer to the infants' major sequences despite variability in the ages of the infants. In addition, the distributions of distances between mother-infant pair sequences and mothers' major sequences (Fig. 4) show that the infants' sequences are far from the mothers' major sequence subtypes. Our results are also consistent with the findings of selective transmission of HIV-1 found in transmitter-recipient partners involving sexual transmission, including a homogeneous sequence population present in the recipients (6, 24, 33, 47, 49, 50). Moreover, in this study, we failed to find a mother-infant pair in which a major (41) or multiple (19) variant of the maternal virus was transmitted to the infant. This difference could be attributed to the time of sampling, clinical stage, or geographic origin. However, in the present study, the possibility that the minor variant of HIV-1 found in maternal blood at the time of sampling was the major variant at the time or in the source of transmission cannot be discounted. Therefore, it will be important in the future to perform comparative sequence analysis on the source of transmission such as vaginal secretions or placenta.

The complete loss of the conserved N-glycosylation site proximal to the first cysteine to the V3 loop was observed only in one infant's sequence set (I5), and a partial loss was found in two other infants' sequences (I1 and I2). This site is highly conserved among all HIV-1 isolates except two (ELI and Z321) in the Human Retroviruses and AIDS Database (29). Interestingly, the minor subtype in M5 (m-5c) which predominated in her infant (I5; Fig. 2E) was also unglycosylated at the first cysteine of V3 loop, which is clearly in agreement with the results of Wolinsky et al. (48). In contrast, we failed to observe similar features of transmission in mother-infant pairs 1, 2, 3, 4, 6, and 7, in which infants I1 to I4 were older and I6 and I7 were younger (4.75 months and 1 week). The sequences for I3, I4, I6, and I7 had the conserved N-glycosylation site proximal to the first cysteine of the V3 loop intact as described elsewhere (28, 41). In mother-infant pairs 1 and 2, in which we find some infants' sequences missing the glycosylation site, it is possible that all of the sequences were unglycosylated at the time of birth or at a younger age. The same pattern of transmission as seen in mother-infant pair 5 cannot be ruled out for mother-infant pairs 1 and 2. This suggests that both glycosylation and unglycosylation sites proximal to the first cysteine of the V3 loop may play an important role in selective transmission of HIV-1 variants from mother to infant. Moreover, this may be associated with the clinical stages of the mother, virus transmission at different stages of pregnancy, immunological pressure for change, alteration in cell tropism, and/or replication efficiency. The other glycosylation sites were fairly conserved but varied in position and number. The N-linked glycosylation sites could be important in the formation of conformational or reduction of linear epitopes (18), which can

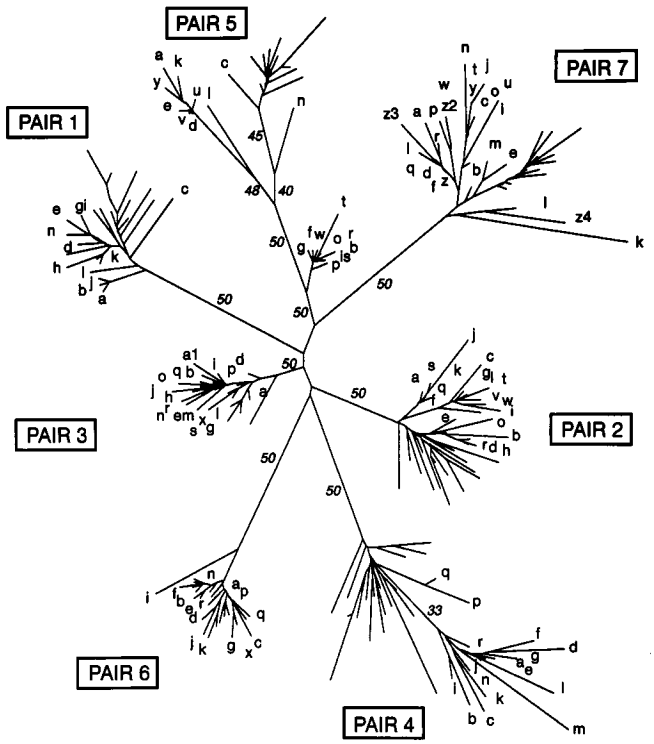

FIG. 6. Phylogenetic tree of the V3 region sequences from seven mother-infant pairs. The distances were calculated between the nucleotide sequences from the seven mother-infant pairs. Each leaf of the tree represents one V3 region sequence. The mothers' sequences in each pair are labeled with the letters of the corresponding clones (a to z; Fig. 2), whereas the infants' sequences are unlabeled. The branches' lengths were calculated from the nucleotide sequences (DNADIST, PHYLIP version 3.5). The value shown on each branch represents the occurrence number of branches over 50 bootstrap resamplings of the data sets. The tree reveals that the seven mother-infant pairs are well discriminated and that the sequences of each pair are confined within a subtree. The branching of subtrees of pairs 2, 3, and 6 are bush-like, and some of the mother-infant sequences are intermingled. The subtrees of pairs 1, 4, 5, and 7 show distinct subtypes among the mother sequences. For example, in pair 5 the minor subtype of M5 (sequence c) is closer to the infant's sequences (unlabeled leaves).

also influence cell tropism and host range in several viral systems (36).

The coding potential of the envelope gene was maintained in almost all of the sequences (92,253 bases) except four sequences containing stop codons. The other important features of the V3 loop, including two cysteines at the proximal and distal ends of the V3 loop and the central tetrapeptide GPGR motif, were also fairly conserved in the majority of the sequences (20, 25, 29). As far as the GPGR motif is concerned, the infants' sequences were more conserved than the mothers' sequences. Sequence diversity within the V3 region including the GPGR motif has been observed more in African isolates than in North American isolates (31). The alteration in the GPGR motif, mainly to GPGG, GLGR, GPGS as seen in some of our mother-infant sequences, was also observed by others (48). It is not clear whether the central motif GPGR could affect the selection or transmission of HIV-1 variants from mother to infant. Our data show a positive selection pressure for change in the infants' V3 sequences. The difference in selection pressure could be attributed to the presence of maternal antibodies in infants (38) because the samples were collected from the infants at 1 week to 34 months after birth.

The actual mechanism of HIV-1 transmission from mother to child is not known. Several studies indicate that mother-to-child transmission occurs at or close to delivery (9, 12). By comparing HIV-1 sequences, a recent study indicates that transmission can take place during the first and second trimesters of pregnancy (28). The data presented here and elsewhere (48) suggest that a minor genotype from the mother is selected and transmitted to the infant, predominating initially as a homogeneous population and then becoming diverse as the infant grows (28). This is the first step in understanding the molecular mechanism underlying mother-infant transmission of HIV-1. It will now be easier to develop strategies to inhibit the replication of minor variants in mothers or a homogeneous population in infants. In addition to identification of specific genotypes in mothers that are transmitted to infants, research should also focus on the replication efficiency and cell tropism of the variants. HIV-1 infection has been documented in placental tissue (27, 45). Since placental tissue is rich in monocytes and macrophages, macrophagotropic viral isolates are likely involved in mother-to-child transmission of HIV-1 through placental passage. The minor genotypes, which probably escape a critical immune response, may be less virulent, highly cytopathic, lymphotropic, or macrophagotropic. These unanswered but important questions should be addressed in our attempt to understand the molecular mechanisms of mother-to-infant transmission of HIV-1.

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