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Similarity in *env* and *gag* Genes between Genomic RNAs of Human Immunodeficiency Virus Type 1 (HIV-1) from Mother and Infant Is Unrelated to Time of HIV-1 RNA Positivity in the Child

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Variation in the *env* (V3 region) and *gag* (p17 region) genes of genomic RNA of human immunodeficiency virus type 1 was studied in three mother-child pairs. One infant was human immunodeficiency virus type 1 RNA positive at birth (pair 114), one became positive 6 weeks after birth (pair 127), and one became positive 30 months after birth (pair 564). The first two children were born to seropositive mothers, and the last child was infected by breast-feeding following seroconversion of the mother after delivery. In both V3 and p17^{gag}, intrasample variability was much higher in the maternal samples, including the first seropositive sample of the seroconverted mother, than in the infants' samples. Variability was less in p17^{gag} than in V3, except in the postnatally infected child. In all three cases, infection of the child was established by variants representing a minority of the cell-free virus population in the maternal samples. For the two infants born to seropositive mothers, V3 sequences were more similar to the sequence populations of maternal samples collected during pregnancy than to those of samples collected at delivery or thereafter. However, in pair 114 a V3 variant identical to the child's virus was also detected in the sample collected at delivery. In contrast to the V3 region, p17^{gag} sequences of maternal samples of the first trimester of pregnancy and at delivery had comparable resemblance to the child's sequences in pair 114, while in pair 127, similarity to sequences of the sample collected at delivery was higher than that to sequences of the sample from early in pregnancy. In the last pair, V3 and p17^{gag} sequences from a maternal sample collected 18 months prior to the first RNA-positive sample of the child resembled the infant's sequences as much as the sample collected close to the presumed time of infection. Taken together, the evolutionary characteristics for genomic RNA *env* and *gag* genes did not point to a particular time of mother-to-child transmission.

Several studies have shown that following sexual, parenteral, or vertical transmission of human immunodeficiency virus type 1 (HIV-1), a highly or completely homogeneous *env* sequence population is observed in the recipient, contrasting with the heterogeneous population found in the donor (31, 34, 38, 49, 51, 53, 55, 56). Three models have been proposed to explain this feature: (i) the dilution model, in which the inoculum is small and the virus population observed in the recipient represents the progeny of only one infectious unit; (ii) selective penetration, in which certain variants may preferentially infect the cells lining the place of entry; and (iii) selective amplification, in which many viruses may enter the recipient but only a few viruses replicate efficiently in the new host (56). The observation of a more homogeneous sequence population in gp120 than in gp41, Nef, or p17 early in infection following sexual or parenteral transmission is in contrast to the heterogeneity that is commonly observed in chronically infected persons (55, 56). The gp120 of the envelope protein and in particular the V3 region is involved in tropism and replication efficiency of the virus (7, 8, 12, 18, 43). The apparently higher pressure to conserve sequences in gp120 early in infection indicates that selective mechanisms are involved in sexual and parenteral transmission, either at entry or during amplification.

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Evidence that selection may also play a role in mother-to-child transmission is provided by the observation that infection often appears to be established by a virus representing a minor variant in the maternal *env* sequence population (38, 49, 53). However, in previous studies no information about when the children became infected was available, and only one maternal sample was studied, collected at the time of delivery or some months thereafter. It has been demonstrated that the HIV-1 envelope shows genetic variation within a person over time (17, 50, 52). Therefore, the possibility that the virus population observed in the child represents a major variant in the mother at the actual time of transmission cannot be excluded.

Transmission from mother to child may occur in utero, intrapartum, or postnatally by breast-feeding (28, 40, 44, 46, 57). Although the absence of viral markers at birth, observed in 50 to 70% of the infected children (3, 5, 24), could also indicate transmission late in utero or may result from a very low level of virus replication before birth because of the presence of maternal antibodies, there is evidence that intrapartum transmission is more likely in these cases. First, the risk of infection for a firstborn twin is found to be twice as high as for a second-born twin (13, 14). The most plausible explanation for this observation is the fact that in general the first child is exposed for a longer time to contagious material in the birth canal than is the second-born child. Second, a recent analysis of the European Collaborative Study shows that the infection rate in children born by cesarean section was significantly lower than that in children born by vaginal delivery (44). Finally, trans-

mission from mother to child has been found to be associated with a lack of maternal neutralizing antibodies against autologous and the infant's isolates (22, 37). This suggests that the frequently observed absence of viral markers at the time of birth is unlikely to be caused by suppression of viral replication due to maternal antibodies.

It has been proposed to use viral diagnostic tests, like PCR and viral culture, to distinguish children infected in utero, defined as children with positive tests in the first 2 days of life, from those infected intrapartum, defined as children without detectable viral markers within the first week of life (4). Weiser et al. suggested that sequence analysis, too, could be helpful in timing of the moment of mother-to-child transmission (48). They studied the V3 region in a mother and her infected twins (born vaginally) by direct sequencing of proviral DNA. Results from sequence analysis were in agreement with the time of infection predicted on the basis of viral diagnostic tests. At birth, no proviral DNA was detectable in the firstborn child. The V3 sequence obtained from a sample collected at the age of 1 month resembled the predominant sequence in the maternal sample collected at the time of delivery. The secondborn child was positive for HIV-1 DNA at birth, and the viral sequence resembled the predominant sequence obtained from the mother in midpregnancy.

In this study we have examined three mother-child pairs. One child was positive for viral RNA at birth and therefore was infected in utero (31); one was negative for viral RNA and DNA by PCR and virus by culture at birth but HIV-1 RNA positive at the age of 6 weeks, suggesting intrapartum or late in utero transmission; and a third child was infected postnatally by breast-feeding following seroconversion of the mother after delivery (45). We tried to establish if selection is involved following different routes of mother-to-child transmission and if results of sequence analysis of cell-free virus in serum support the timing of transmission based on viral diagnostic tests. Genomic RNA-derived sequences of the V3 region of the *env* gene and the p17 encoding region of the *gag* gene were studied in sequentially collected maternal samples and the first RNA-positive samples of the children.

MATERIALS AND METHODS

Patients and sera. The samples examined in this study were collected from two mother-child pairs participating in a Dutch prospective study of HIV-infected mothers and their infants (pairs 114 and 127) and from one seroconverted mother-child pair (pair 564) from a Rwandese cohort of mothers and infants seronegative for HIV at delivery (45, 46).

The Dutch women were found to be HIV-1 seropositive during screening for HIV in the first trimester of pregnancy. They became infected heterosexually. The duration of infection in these mothers was unknown. Mother 114 was infected by a former partner from the Dutch Caribbean. She remained asymptomatic until the last follow-up at 38 months after delivery. The number of $CD4^+$ T-cells was between 0.5×10^9 and 1.0×10^9/liter during pregnancy and remained stable between 0.4×10^9 and 0.5×10^9/liter during follow-up. Mother 127 had a partner from Ghana for 4 years. Except for generalized lymphadenopathy (CDC-III) diagnosed at 11 months postpartum, she was asymptomatic until the last follow-up at 30 months after childbirth. The number of $CD4^+$ T-cells was between 0.2×10^9 and 0.3×10^9/liter during the study period. Both mothers were p24 antigen negative (Abbott Laboratories). The mothers have never been treated with zidovudine or other antiretroviral agents. Children 114 and 127 were born by vaginal delivery after 38 and 39 weeks of pregnancy, respectively, and were not breast-fed.

Serological data of mother-child pair 564 have been published previously (45). The mother had seroconverted between 9 and 12 months after delivery. She received a medical injection 9 months prior to seroconversion. However, it is not known if this was the source of infection or she had acquired the infection heterosexually. Except for generalized lymphadenopathy, she had no HIV-related symptoms until the last follow-up at 45 months after seroconversion. The number of $CD4^+$ cells and p24 antigen were not determined. The child was breast-fed since birth. Serum samples were collected every 3 months until the age of 18 months and thereafter with an interval of 6 months. The child remained seronegative until the age of 24 months. No proviral DNA was detectable by PCR at birth and at the ages of 3 and 18 months (45). However, HIV-1 antibodies as well as proviral DNA were detectable at the age of 30 months, 2 months after the mother had developed a severe breast abscess (45). Breast-feeding was stopped 1 week after the development of the breast abscess.

The serum samples used in this study were collected during the first, second, and third trimesters of pregnancy; at the time of delivery; and 10 months after childbirth from mothers 114 and 127. From their children cord blood samples as well as a venous sample collected from child 127 at the age of 6 weeks were examined. From mother 564, we studied the first seropositive sample and a sample collected 2 months after the development of the breast abscess, corresponding to 12 and 30 months after childbirth, respectively. For the child, the last seronegative sample and the first seropositive sample, collected at the ages of 24 and 30 months, respectively, were examined.

Molecular cloning and sequencing of the V3 and p17 region. Genomic RNA was isolated from 50 μl of sera for mother-child pair 114 and 100 μl of sera for mother-child pairs 127 and 564 according to the method of Boom et al. (2). Synthesis of cDNA and amplification by nested PCR of the V3 region were performed as previously described (31). For the p17 region, the conditions used were as described for the V3 region except for the use of specific Gag primers and optimization of the $MgCl_2$ and deoxynucleoside triphosphate (dNTP) concentrations in the PCR mixture. The first PCR was performed with the primers GAG-4 (antisense, 5′CATTCTGATAATGCTGAAAACATGGG; HXB2 positions 1296 to 1318 [31a]) and GAG-1 (sense, 5′CATGCGAGAGCGTCAG TATTAAGCGG; HXB2 positions 795 to 817), 2.0 mM $MgCl_2$, and 0.2 mM (each) dNTP. The second PCR was carried out with the primers GAG-2 (sense, 5′CATAAGCTTGGGAAAAAATTCGGTTAAGGCC; HXB2 positions 835 to 856) and GAG-3 (antisense, 5′CATGAATTCCTTCTACTACTTTTACCCA TGC; HXB2 positions 1248 to 1268), 1.5 mM $MgCl_2$, and 0.2 mM (each) dNTP or with the primers GAG-20 (sense, 5′CATAAGCTTCCCTTCAGACAGGAT-CAG; HXB2 positions 988 to 1005) and GAG-3, 2.0 mM $MgCl_2$, and 0.2 mM (each) dNTP. For direct sequencing, the second PCR was performed with primers extended at the 5′ end with an Sp6 site (sense primer) or T7 site (antisense primer).

The detection limit of the first PCR was between 10 and 100 molecules, using 1/10 (10 μl) of the PCR product for analysis on an ethidium bromide-stained agarose gel (31). Single molecules of DNA were detectable after nested PCR. Reconstruction experiments showed that the detection limit of the reverse transcriptase (RT) reaction followed by nested PCR was 10 to 50 molecules of RNA (5a). Since the amount of RNA used in the RT reaction corresponded to RNA isolated from 10 to 20 μl of serum, the detection limit of the RT-PCR was between 10^3 and 10^4 virions per ml.

For detection of proviral HIV-1 DNA, DNA was isolated from cryopreserved peripheral blood mononuclear cells according to the method of Boom et al. using diatoms (2). The concentration of DNA was determined by gel electrophoresis by comparison with standard amounts of human placental DNA. About 500 ng of DNA was used for amplification by PCR.

Negative controls consisted of a water sample (instead of serum or peripheral blood mononuclear cells), RT reaction mixtures without added avian myeloblastosis virus RT, and reagent controls run in parallel with the tested samples.

Cloning of the V3 fragments and plasmid isolation of mother-child pair 114 was previously described (31). Cloning of the p17 fragments and the V3 fragments of the other mother-child pairs was carried out with the T-A cloning kit (Invitrogen, San Diego, Calif.) according to the manufacturer's recommendations. Colonies were resuspended in 50 μl of brain heart infusion medium, and 2.5 μl of this suspension was amplified by PCR for 25 cycles in a reaction mixture containing 10 pmol of the primers Sp6 (5′GATTTAGGTGACACTATAG) and T7 (5′TAATACGACTCACTATAGGG), 2.7 mM of $MgCl_2$, 0.5 U of *Taq* (AmpliTaq; Roche Molecular Systems, Branchburg, N.J.), 50 mM of KCl, 0.2 mM (each) dNTP, and 0.1 mg of bovine serum albumin per ml (total volume of 25 μl). PCR products of the expected size were used for sequencing.

To calculate the rate of misincorporations introduced during PCR and cloning, 50 copies of a fragment of *env* of HXB2 (corresponding to positions 6953 to 7364, isolated from a molecular clone) or 50 copies of a molecular clone containing the *gag* gene of HXB2 (using the nested primers GAG-2 and GAG-3) were amplified and subsequently cloned in parallel with the patient samples in two separately performed experiments. Only point mutations were observed, corresponding to a misincorporation rate of 9 of 4,140 (0.22%) and 8 of 3,069 (0.26%) bp for the V3 region and 1 of 3,900 (0.03%) and 4 of 3,510 (0.11%) bp for the *gag* region.

Sequencing was performed with dye-labelled Sp6 and T7 primers (*Taq* dye primer cycle sequencing kit; Applied Biosystems). The products were analyzed on an automatic sequencer (Applied Biosystems).

Virus culture. Virus culture of cryopreserved peripheral blood mononuclear cells was done by biological cloning as described previously (39). Briefly, 10,000 to 40,000 cells were cocultivated with 3-day phytohemagglutinin-stimulated donor cells in 96-well plates for 4 weeks. Totals of 1.4×10^6 and 2.4×10^6 cord blood cells of child 127 were cocultivated in two separately performed experiments. Supernatant was tested weekly by an in-house p24 antigen capture enzyme-linked immunosorbent assay.

Analyses. For the V3 region, a 276- to 282-bp fragment (HXB2 positions 7031 to 7312), including the encoding region of the V3 loop, was analyzed. The fragment of the p17 region analyzed consisted of 242 or 390 bp (respectively, HXB2 positions 1006 to 1247 and 858 to 1247). A consensus sequence was

TABLE 1. Characteristics of serum samples studied

Pair	Sample[a]	Code	Moment of infection of the child	Mean intrasample variation [% (range)] in[b]:	
				V3 region of *env*	p17 region of *gag*
114	Mother				
	−6 mo	M−6	Intrauterine	2.4 (0–4.7)	0.7 (0–2.1)
	−4 mo	M−4		2.7 (0–5.4)	ND
	−2 mo	M−2		3.9 (0–7.6)	ND
	At delivery	M0		4.2 (0–8.3)	0.9 (0–2.5)
	10 mo	M10		3.6 (0–6.6)	ND
	Child, cord blood			0.7 (0–2.5)	0.4 (0–0.8)
127	Mother				
	−7 mo	M−7	Probably at delivery (no viral DNA or RNA	3.3 (0–5.7)	0.6 (0–1.3)
	−4 mo	M−4	or virus detected in cord blood)	3.3 (0.4–5.4)	ND
	−2 mo	M−2		4.1 (0.7–7.0)	ND
	At delivery	M0		1.8 (0–4.0)	0.7 (0–1.3)
	10 mo	M10		4.7 (0.4–8.1)	ND
	Child, 1.5 mo			0.9 (0.4–2.1)[c]	0.4 (0–1.0)
564	Mother				
	12 mo[d]	M12	Postnatally, probably 28 mo after birth	2.8 (0.4–4.7)	1.0 (0–2.3)
	30 mo	M30		3.3 (0.7–5.4)	1.4 (0–2.6)
	Child, 30 mo[d]			0.0	0.3 (0–0.8)

[a] The time of collection of the samples is expressed with regard to the time of birth of the child.
[b] Calculated for nucleotide sequences (mean Hamming distance). For the V3 region a 276- to 282-bp fragment was analyzed, and for the p17 encoding region a 242-bp (mother-child pair 114) or 390-bp (mother-child pairs 127 and 564) fragment was analyzed. ND, not determined.
[c] From this sample, sequences were obtained from two separately performed RNA extractions, PCR amplifications, and cloning experiments. Shown is the mean of the variation observed within the two tested aliquots (0.7% [range, 0 to 1.8%] and 1.1% [range, 0.4 to 2.1%]).
[d] First seropositive sample collected within 3 months (M12) or 6 months (child) after the last seronegative sample.

derived by assigning to each position the nucleotide or deduced amino acid most frequently found (at least in 50%) in the individual clones. Consensus sequences were based on 10 to 22 clones.

All calculations were carried out with nucleotide sequences. Pairwise comparisons were performed to establish nucleotide distances (Hamming distances [16]) between sequences. Positions where an alignment gap had to be introduced in one of the sequences were excluded from calculations (pairwise gap deletion). Intrasample sequence variation is expressed as the mean nucleotide distance of all pairwise comparisons between sequences obtained from a sample.

Phylogenetic analysis was done with the neighbor-joining method (36) in the PHYLIP package (11), using the two-parameter estimation method described by Kimura (21) for calculation of distances. The bootstrap resampling (100 replications) method implemented in the program MEGA (25) was employed to place approximate confidence limits on individual branches.

Calculation of the proportion of synonymous (K_s) and nonsynonymous (K_a) substitutions was performed according to the method described by Nei and Gojobori (33) implemented in the program MEGA. Every sequence within a sample was compared to every other, and K_s and K_a were averaged to calculate the K_s/K_a ratio.

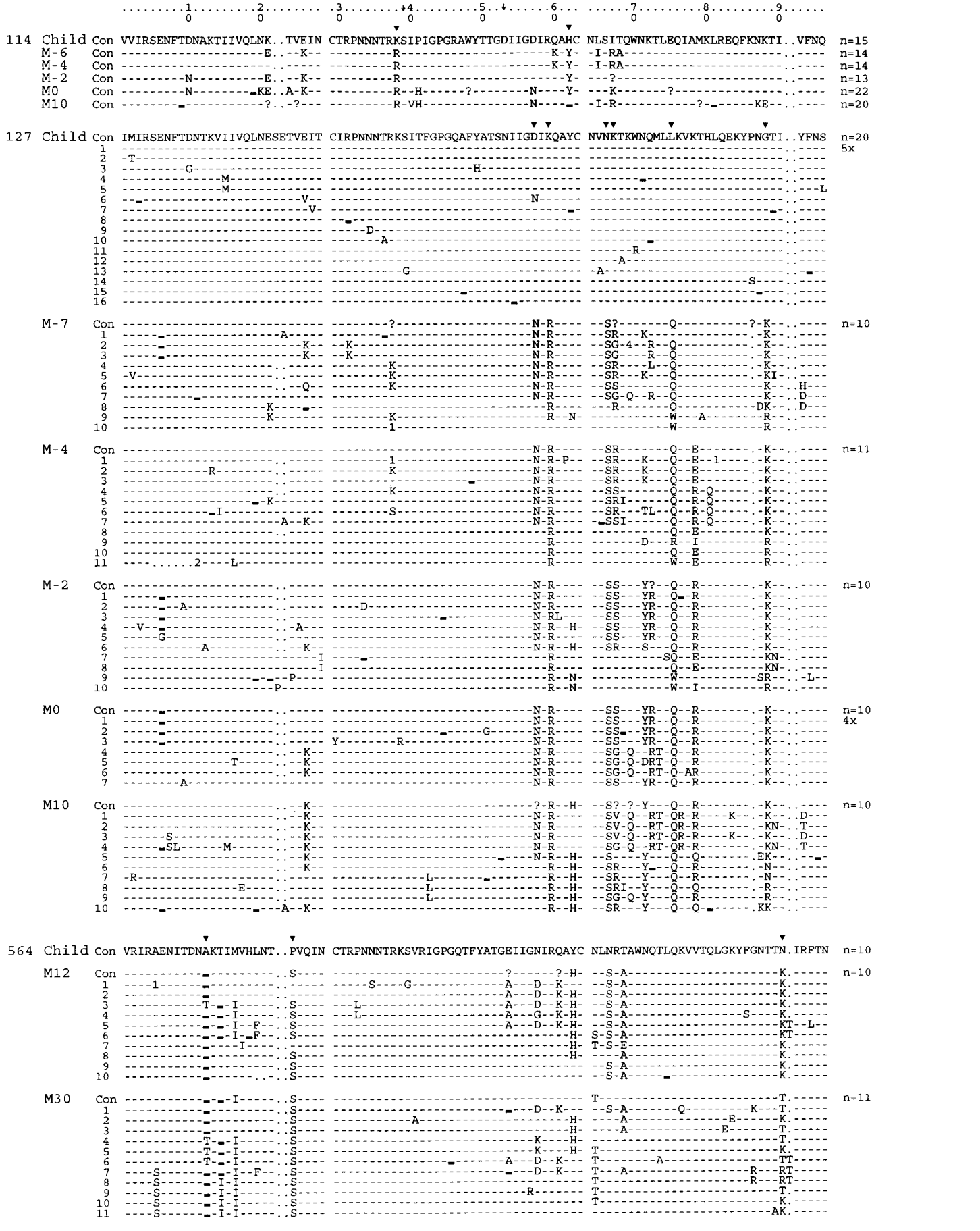
Nucleotide sequence accession numbers. The V3 sequences of mother-child pair 114 have been assigned the accession numbers L21028 to L21153 in the GenBank database. The other sequences have been assigned GenBank accession numbers Z47817 to Z47972 and Z48010 to Z48011.

RESULTS

As previously reported, child 114 was infected in utero, since cord blood was positive for viral RNA and comparison of V3 sequences excluded the possibility of contamination with maternal blood during delivery (31). In cord blood of child 127, no viral RNA was detectable in two separately tested aliquots of serum. Additional testing of peripheral blood mononuclear cells for the presence of viral DNA by PCR or virus by culture also gave negative results. At the age of 6 weeks, serum was positive for viral RNA. These results suggest intrapartum or late in utero transmission. No viral RNA was detectable in the last seronegative sample of child 564, collected 24 months after birth. While apparently no transmission had occurred during a period of 12 months of breast-feeding following seroconversion of the mother, infection of the child was documented 2 months after the mother had developed a severe breast abscess. These data strongly suggest that transmission to the child was related to the presence of the breast abscess.

With the exception of the two samples mentioned above, all the other samples selected for the study were positive for viral RNA. An overview of the time of collection of these samples is given in Table 1. In all these samples, except for one of two tested aliquots of sample 114ME (31), a specific signal was obtained after a single PCR amplification (data not shown), suggesting an input of more than 10 cDNA molecules, since the detection limit of the first PCR was between 10 and 100 copies. For the samples of mother-child pairs 564 and 127, the intensity of the PCR product after a single PCR amplification was comparable and mostly higher than that obtained with 50 copies of a molecular clone amplified in parallel with the patient samples.

Sequence variability in the V3 region. The mother-child pairs were infected by different HIV-1 subtypes as determined by phylogenetic analyses of the V3 sequences (data not shown): the B subtype for pair 114, the G subtype for pair 127 (22a), and the A subtype for pair 564 (1, 19, 32). The sequence populations in the maternal samples were clearly heterogeneous with a mean nucleotide variation of 1.8 to 4.2% (Table 1). The deduced amino acid sequences are shown in Fig. 1. Only consensus sequences are shown for pair 114. Clonal sequences of this mother-child pair have been published previously (31). In the maternal samples, identical substitutions were observed in different clones. In addition to nucleotide substitutions, sequences from mother 564 and particularly from mother 127 showed length polymorphism outside the V3 loop. Surprisingly, the first seropositive sample of mother 564 was rather heterogeneous. The mean variation was comparable to that found in sample M30, collected 18 months after seroconversion (2.8 and 3.3%, respectively; Table 1).


114 Child Con VVIRSENFTDNAKTIIVQLNK..TVEIN CTRPNNNTRKSIPIGPGRAWYTTGDIIGDIRQAHC NLSITQWNKTLEQIAMKLREQFKNKTI..VFNQ n=15
M-6 Con n=14
M-4 Con n=14
M-2 Con n=13
M0 Con n=22
M10 Con n=20
127 Child Con IMIRSENFTDNTKVIIVQLNESETVEIT CIRPNNNTRKSITFGPGQAFYATSNIIGDIKQAYC NVNKTKWNQMLLKVKTHLQEKYPNGTI..YFNS n=20
1 5x
M-7 Con n=10
M-4 Con n=11
M-2 Con n=10
M0 Con n=10
1 4x
M10 Con n=10
564 Child Con VRIRAENITDNAKTIMVHLNT..PVQIN CTRPNNNTRKSVRIGPGQTFYATGEIIGNIRQAYC NLNRTAWNQTLQKVVTQLGKYFGNTTN.IRFTN n=10
M12 Con n=10
M30 Con n=11
2288

A completely homogeneous V3 sequence population was observed in the sample of child 564 which was collected within 2 months of the presumed time of infection. It is highly unlikely that we have sequenced identical copies, because a very strong signal which clearly exceeded that obtained with 50 copies of a fragment of *env* of HXB2 (data not shown) was obtained after a single PCR amplification. The sequence variability in the samples of the other two children was much lower than in the maternal samples (mean variation of 0.7 to 1.1%; Table 1) and was mainly caused by apparently random nucleotide substitutions, since most of the substitutions were seen only once in the sequence set of the child and were also not observed in the maternal samples (Fig. 1). The proportion of polymorphic sites in the V3 region was 0.29% in the sample from child 114 and 0.35 and 0.57% in two separately tested aliquots of serum from child 127. This is just above to two times higher than the observed experimental misincorporation rates (0.22 to 0.26%). The observation of an identical mutation in sequences obtained from two separately tested aliquots of serum (child 127, M at position 15; Fig. 1) or from follow-up samples (child 114, D at position 20; Fig. 1 in reference 31) makes it likely that these mutations represent true variation.

On the basis of the absence of a positively charged amino acid at positions 39 and 53 (corresponding to positions 11 and 25 of the V3 loop), the viruses of all mother-child pairs are predicted to be non-syncytium-inducing viruses (7, 9, 12). Virus cultures of samples of mother-child pairs 114 and 127 confirmed the predicted non-syncytium-inducing phenotype (47).

The proportion of synonymous (K_s) and nonsynonymous substitutions (K_a) is given in Table 2. The substitutions in most of the maternal samples were characterized by a predominance of nonsynonymous substitutions resulting in K_s/K_a ratios lower than 1. In a minority of the maternal samples the K_s/K_a ratio was just above 1. However, in the children's samples (114 and 127) K_s/K_a ratios were higher than 2, because of a relatively low proportion of nonsynonymous substitutions compared to that in the maternal samples.

TABLE 2. Proportion of synonymous and nonsynonymous substitutions

Pair	Sample	Avg value[a] in:					
		V3 region of *env*			p17 region of *gag*		
		K_s	K_a	K_s/K_a	K_s	K_a	K_s/K_a
114	Mother						
	M−6	0.0133	0.0269	0.49	0.0248	0.0021	11.81
	M−4	0.0163	0.0305	0.53	ND	ND	ND
	M−2	0.0227	0.0434	0.52	ND	ND	ND
	M0	0.0175	0.0485	0.36	0.0292	0.0039	7.49
	M10	0.0394	0.0352	1.19	ND	ND	ND
	Child	0.0114	0.0052	2.19	0.0052	0.0034	1.53
127	Mother						
	M−7	0.0230	0.0356	0.65	0.0123	0.0037	3.32
	M−4	0.0189	0.0347	0.51	ND	ND	ND
	M−2	0.0327	0.0429	0.76	ND	ND	ND
	M0	0.0199	0.0178	1.12	0.0084	0.0070	1.20
	M10	0.0347	0.0506	0.69	ND	ND	ND
	Child	0.0168	0.0071	2.37	0.0025	0.0039	0.64
564	Mother						
	M12	0.0223	0.0292	0.76	0.0258	0.0052	4.96
	M30	0.0156	0.0368	0.42	0.0375	0.0072	5.21
	Child	0.0000	0.0000	NA	0.0121	0.0007	17.3

[a] Calculation of the proportion of synonymous (K_s) and nonsynonymous (K_a) substitutions was performed according to the method described by Nei and Gojobori (33). Every sequence within a sample was compared to every other. NA, not applicable; ND, not done.

Comparison of V3 sequence populations in mother and infant. Figure 2 shows the distribution of nucleotide distances between the V3 sequences of mother and child for each maternal sample separately. A maternal sequence identical to one of the child's sequences was observed only in mother-child pair 114 (sample M0), representing 1 of 83 clones sequenced. In this mother-child pair, sequences very similar to those of the child with a nucleotide distance of ≤1% were observed in all maternal samples collected until delivery. Overall, the child's sequences showed more resemblance to the sequence populations from the maternal samples collected during pregnancy than to those from the samples collected at delivery and thereafter (Fig. 2).

For mother-child pair 127, the minimum distance between the V3 sequences of mother and child was 1.4% (sample M−7, sequence 10). In this pair too, sequences from the samples collected during pregnancy were more closely related to the child's sequence population than were those from the samples collected at delivery or thereafter (Fig. 2). Moreover, variants containing the same insertions as the infant's sequences were observed only in the samples collected during pregnancy (Fig. 1). We have done another RNA isolation of the sample collected at delivery. A direct sequence of this aliquot was identical to the consensus sequence of the first aliquot. This confirms that the sequences obtained from the first sample were characteristic for the major population of cell-free virus present in the serum at the time of delivery.

In mother-child pair 564, the distributions of nucleotide distances between the maternal and the child's V3 sequences were more or less comparable for the two samples from this mother studied. Variants with the smallest distance (1.8%) to the child's viral sequence were observed in the sample collected 16 months before the presumed time of transmission (sample M12, sequences 8 and 9). However, when excluding mutations which were seen only once in the sequence set of the mother and could represent in vitro introduced misincorporations, variants with equal distance were also observed in the sample collected close to the presumed time of transmission (sample M30, sequences 2 and 3).

We have studied sequentially collected maternal samples to control for the possibility that differences observed between the maternal and the infants' sequence population represented

FIG. 1. Deduced amino acid sequences of the V3 region. Position 1 corresponds to amino acid 269 of the HXB2 envelope protein. Only consensus sequences are shown for mother-child pair 114. Clonal V3 sequences of this mother-child pair have been published previously (Fig. 1 in reference 31). The sequences of each mother-child pair are aligned against the consensus (Con) sequence of the child's sample. The number of clones sequenced from each sample is shown at the end of the consensus sequence. The frequency of clones with identical sequences is given at the end of the clonal sequence. For child 564, all clonal sequences were found to be identical. Amino acid positions involved in syncytium-inducing capacity are marked (↓) (7, 12). Dashes indicate identity with the reference sequence; dots are introduced to maximize alignment. ▬, silent mutation compared with reference sequence; ×, stop codon; 1, 2, or 4 deletion of 1 or 2 nucleotides or insertion of 1 nucleotide, respectively; ?, no 50% consensus sequence could be ascertained; ▼, the majority of the maternal sequences contains a different codon at this position compared with the child's sequences.

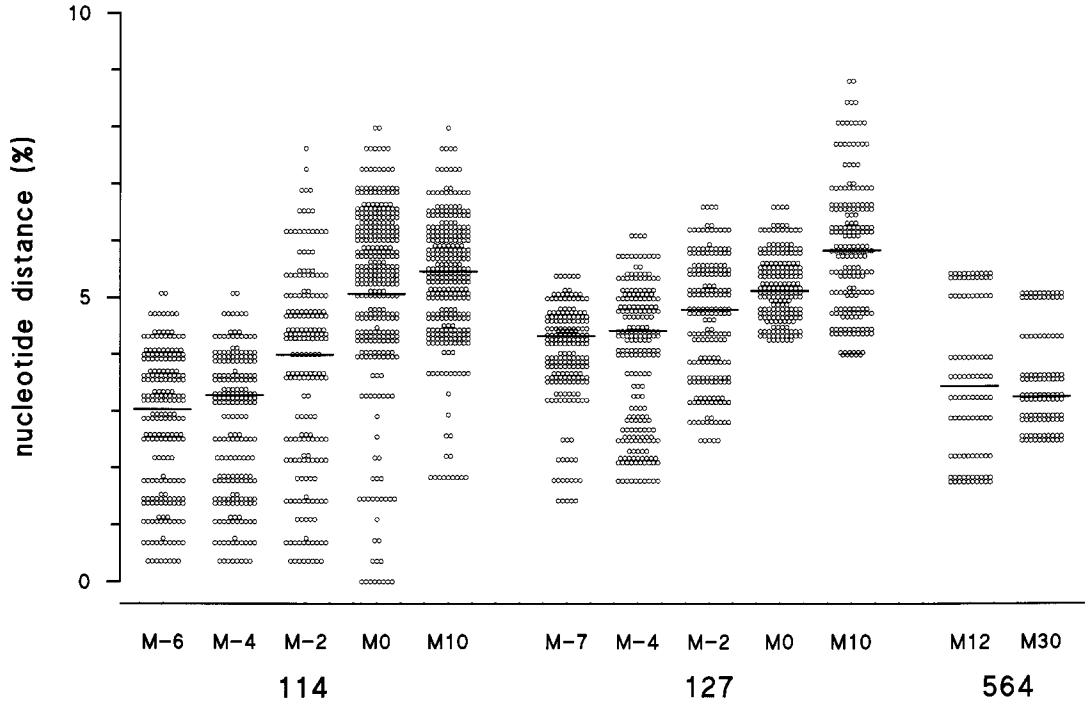


FIG. 2. Distribution of nucleotide distances between the V3 sequences of mother and child. The time of collection of the maternal samples is given in months with respect to the time of childbirth. Each dot represents the nucleotide distance between a maternal sequence and a child's sequence. The horizontal line shows the median distance.

only a transient characteristic of the maternal population. In all three mother-child pairs, certain positions that were different between the child's and the maternal V3 sequences were found in the majority or all of the clonal sequences of the maternal samples. For pair 114 this was true for K-38 and H-62 (third nucleotide of the codon); for pair 127 it was true for D-57, K-59, N-66, K-67 (second nucleotide of the codon), L-75 (second nucleotide of the codon), and G-88 (the first and second nucleotides of the codon); and for pair 564 it was true for A-12 (third nucleotide of codon), P-24, and N-90 (third nucleotide of the codon). So, the virus initiating infection in the child had characteristics which were observed only in a minority or even in none of the sequences obtained from cell-free virus of the maternal samples.

The results of phylogenetic analysis are shown in Fig. 3. The clonal sequences of the samples of children 114 and 127 formed a cluster with the same maternal sequences as the consensus sequences of these samples (Fig. 4 in reference 31 and data not shown). Since the sequence sets were rather large, for reasons of clarity we included only the consensus sequences of the samples of children 114 and 127 and a subset of the maternal sequences. Sequences which differed at only one or two positions from another maternal sequence, particularly if this included nucleotides observed in only one sequence, were excluded. Sequences of each mother-child pair clustered together and were clearly separated from those of the other mother-child pairs. This was to be expected since the V3 sequences of these mother-child pairs belonged to different HIV-1 subtypes. For pair 114, one to three sequences from each maternal sample collected until delivery clustered with the child's sequence (bootstrap value of 73%). In mother-child pair 127, only sequences obtained from the samples collected during pregnancy clustered with the child's sequence (bootstrap value of 77%). Thus, in this mother-child pair the result of sequence analysis of the V3 region suggested that transmission at the time of delivery was less likely than, for example, transmission early in utero. For pair 564, some sequences of both maternal samples clustered with the child's sequence. However, bootstrap values of this cluster were low (22%).

The viral envelope, and in particular the V3 region, is involved in tropism and replication efficiency of the virus and contains an important epitope for neutralizing antibodies (7, 8, 12, 15, 18, 20, 43). These factors could be involved in selection for particular variants during transmission or replication in the infant. Therefore, in addition to the V3 region we have studied a part of the genome of HIV-1 assumed to be under less selective pressure, the p17 encoding region of *gag*.

p17 encoding region of *gag*. Initially we studied for mother-child pair 114 a fragment of 242 bp, corresponding to positions 1005 to 1247 of HXB2 (31a) and encoding amino acid 74 of p17 to amino acid 21 of p24. The variation was found to be rather low, and we decided to examine a larger fragment of 390 bp, corresponding to positions 858 to 1247 of HXB2, starting at amino acid 24 of p17. Since direct sequences of this larger fragment from mother-child pair 114 did not show any differences between the two maternal samples and the child's sample, no additional clonal sequences of the larger fragment were obtained for this mother-child pair.

For the p17 encoding region, too, the variation in the maternal samples was larger than in the children's samples (mean variations of 0.6 to 1.4% and 0.3 to 0.4%, respectively; Table 1). Figure 4 shows the deduced amino acid sequences. As in the V3 region, most of the mutations in the children's sequence sets seemed to be random. However, all contained a substitution identical to one seen in the maternal sequence set (position 65 [silent mutation], R-53 and position 22 [silent mutation] for children 114, 127, and 564, respectively). The proportion of polymorphic sites (0.16, 0.18, and 0.15% for

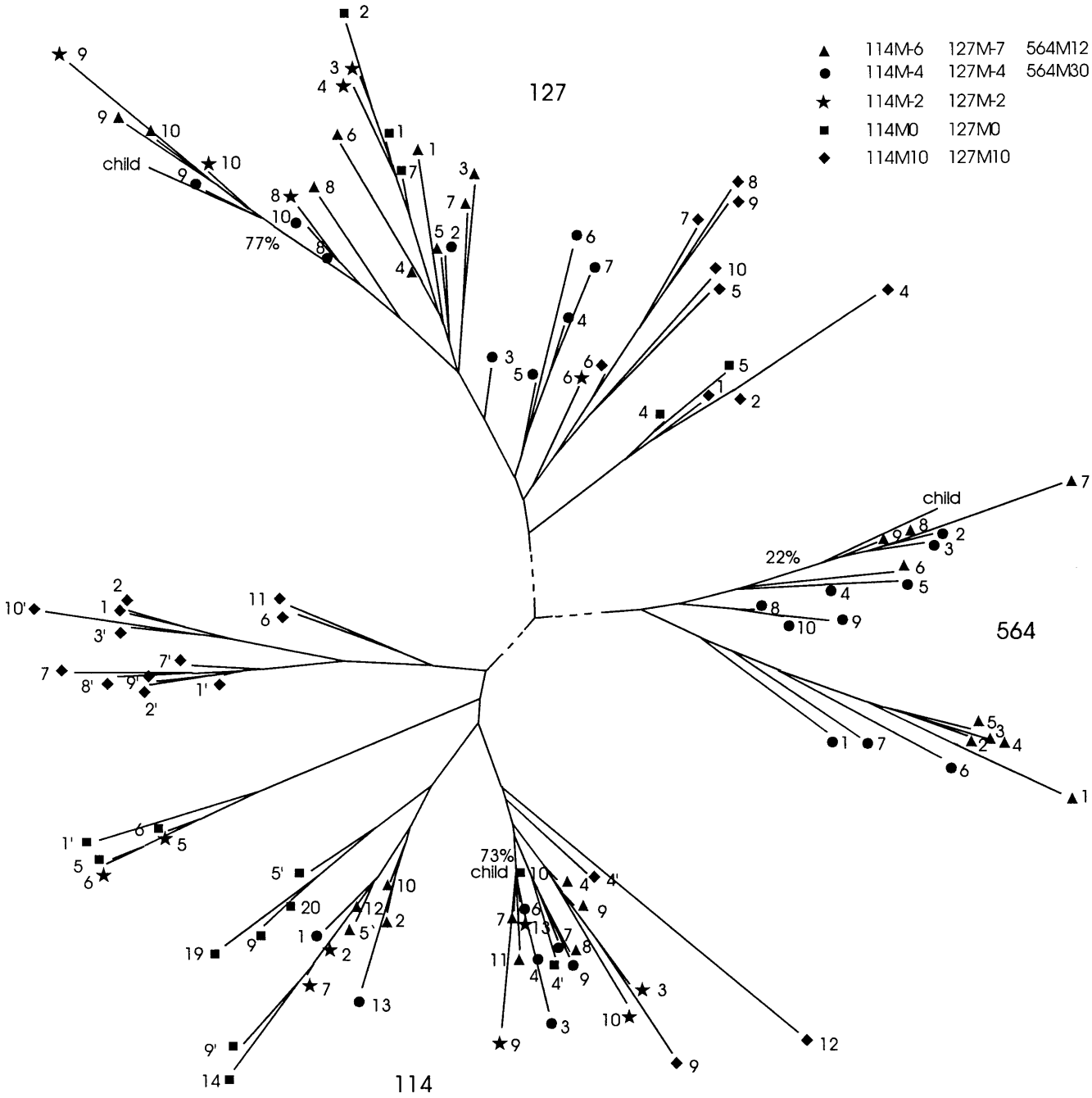


FIG. 3. Results of phylogenetic analysis of the V3 region (neighbor-joining method). The analysis was performed with a subset of the sequence sets (see text). The V3 sequences of these mother-child pairs belonged to different HIV-1 subtypes (data not shown): type B (pair 114), type A (pair 564), and type G (pair 127). For reasons of legibility of the figure, the branches connecting the different mother-child pairs have been shortened. The lengths of the branches connecting the clusters of pairs 114 and 127, of pairs 114 and 564, and of pairs 127 and 564 were approximately 22, 20, and 22%, respectively. The mean of bootstrap values obtained for the cluster including the child's sequence is given at the root of the cluster. Numbers at the symbols refer to the numbering of the clonal sequences in Fig. 1 of this paper and Fig. 1 in reference 31.

children 114, 127, and 564, respectively) was higher than the observed experimental misincorporation rates (0.03 to 0.11%). In both the maternal and the children's samples, the substitutions in the p17 region were characterized by a predominance of synonymous substitutions, resulting in K_s/K_a ratios higher than 1.0, except for child 127.

A maternal sequence identical to the major sequence population of the child was observed only in mother-child pair 564 (Fig. 4, sample M12, sequence 9). In child 114, a minority (14%) of the sequence set was identical to the major population in the maternal samples, characterized by a silent mutation at position 65 (Fig. 4, sequence 9). The distribution of nucleotide distances between the maternal and child's sequences was comparable for the two samples studied of mothers 114 and 564 (Fig. 5). In mother-child pair 127, sequences obtained from the sample collected at the time of childbirth

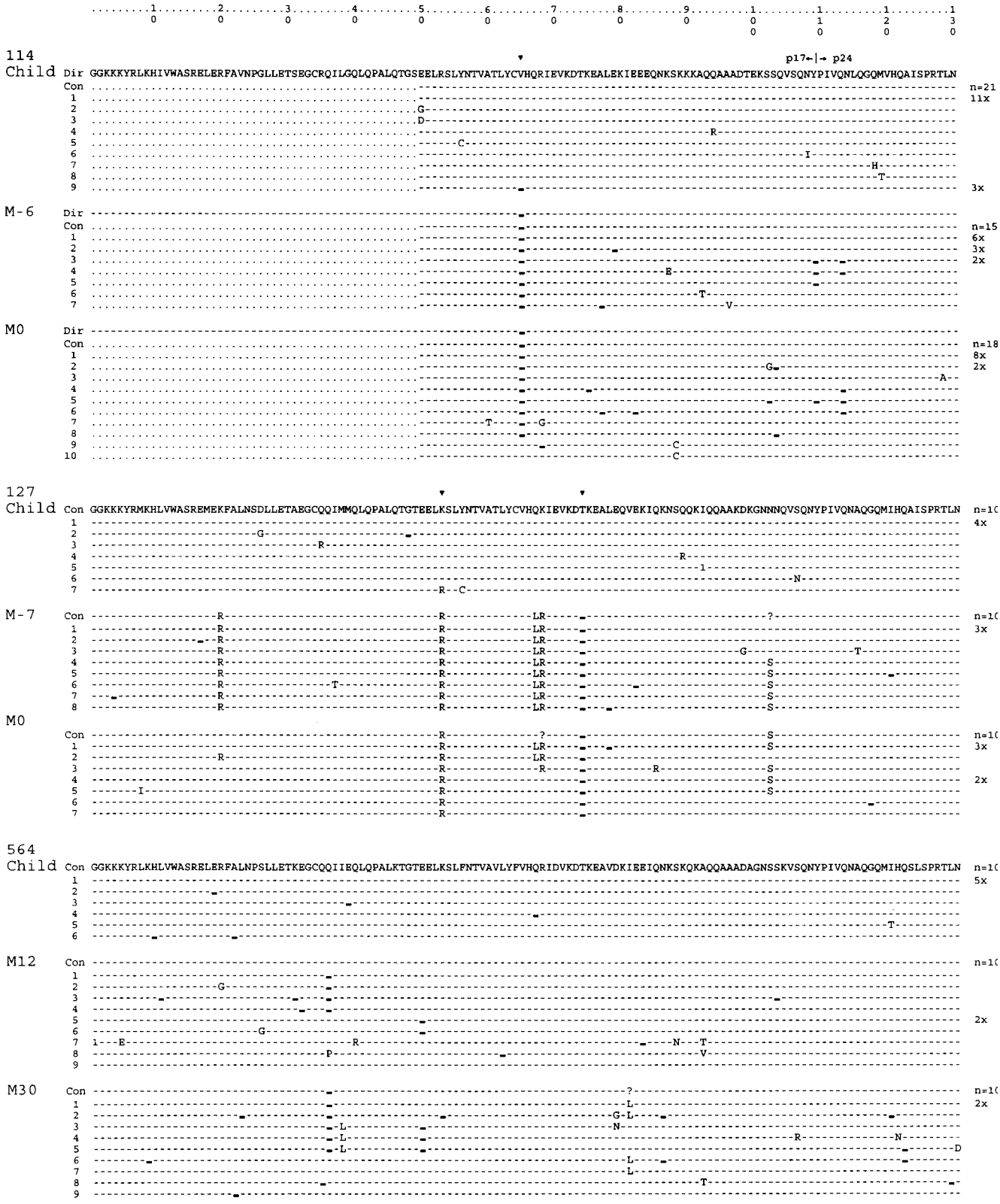


FIG. 4. Deduced amino acid sequences of the p17 encoding region. Position 1 corresponds to amino acid 24 of the HXB2 Gag protein. The beginning of the p24 encoding region is marked. From mother-child pair 114, clonal sequences were obtained from a fragment of 242 bp and a direct sequence (Dir) of a fragment of 390 bp (see text). For an explanation of the symbols, see the legend to Fig. 1.

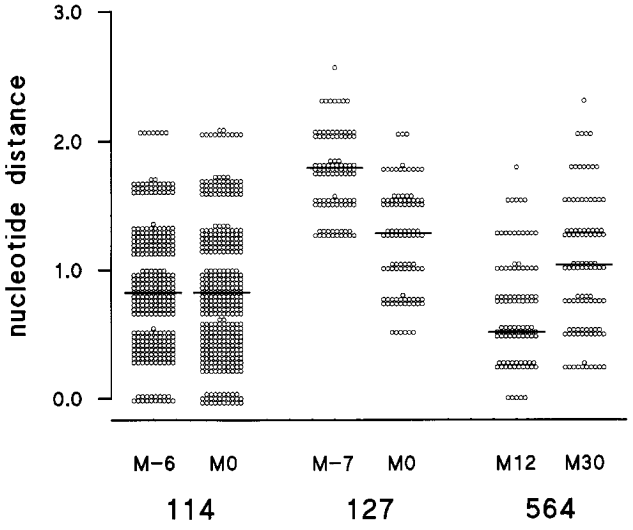


FIG. 5. Distribution of nucleotide distances between the p17 sequences of mother and child. For an explanation of the symbols, see the legend to Fig. 2.

showed more similarity to the child's sequences than those from the sample collected 7 months before delivery, in contrast to what was observed in the V3 region. The majority of the child's sequences differed at two positions from sequences from the maternal samples (positions marked in Fig. 4).

DISCUSSION

We have compared V3 and p17 encoding sequences of genomic HIV-1 RNA in three mother-child pairs. One child was infected in utero (child 114), one child was infected probably intrapartum or close to the time of delivery since no viral markers were detectable at birth (child 127), and one child was infected postnatally by breast-feeding (child 564). The maternal samples were collected over a period of 16 to 18 months, encompassing the time of transmission to the child. From the children, the first RNA-positive sample was studied.

In both regions of the HIV-1 genome, the viral population in the mothers showed considerable heterogeneity and subgroups of variants sharing identical substitutions were observed. The sequence variability in the maternal samples was much higher in the V3 region than in the p17 region. The ratios of synonymous to nonsynonymous substitutions (K_s/K_a) were mostly below 1 for the V3 region but always above 1 for $p17^{gag}$. These characteristics are typical for the virus populations in chronically infected persons and show that this part of the envelope is under greater pressure for change than $p17^{gag}$ (55, 56). In contrast to the maternal samples, the children's samples contained both in the V3 region and in the p17 region a highly or even completely (V3 region of child 564) homogeneous sequence population. The variability consisted mainly of apparently random mutations unique to each sequence. The proportion of polymorphic sites was just above to two times higher than the observed experimental misincorporation rates. Part of the variation, therefore, may have been caused by in vitro-introduced misincorporations. However, the observation of identical substitutions in V3 sequences obtained from two separately tested aliquots of serum (child 127) or from follow-up samples (child 114) suggests that these substitutions represent true variation. Also, the predominance of silent mutations in the *gag* sequences of child 564 (five of the six mutations) is rather typical for this region (55), while introduction of random mutations by experimental misincorporations would result in a fraction of silent mutations of roughly only one in four. A study of the V4 region during the acute phase of infection suggested that the occurrence of random substitutions is characteristic for the development of variation early in infection (34). The K_s/K_a ratio of V3 sequences in the children's samples was above 2 (2.19 to 2.37). Interestingly, in another data set consisting of seroconversion samples following sexual transmission, in five of the six samples the K_s/K_a ratios were also above 2 (2.07 to 3.66) (51). This suggests that early in infection the pressure to change is less than in the chronic stage of infection, possibly because the influence of selecting forces for change like neutralizing antibodies is still limited in this period (23). Introduction of random substitutions by RT during replication is expected to result in predominance of synonymous substitutions, since nonsynonymous substitutions are more likely to be deleterious and are selected against. These numbers are probably no artifact of experimental misincorporations. Results of two reconstruction experiments showed K_s/K_a ratios of 0.52 and 1.08. The ratios found in p17 sequences were above 1, except in child 127. However, the p17 ratios are based on a small number of substitutions (6 to 10) and are therefore rather unstable.

There was no evidence that infection in the children was initiated by multiple maternal V3 variants, which is in agreement with the observations by others (38, 53). A study of the V1-V2 region of *env* by Lamers et al. (26) and a recent study of sequences of the V3 loop of biological clones of mother-child pairs (47) have shown that infection by multiple *env* genotypes is possible. The observation of a heterogeneous V3 and p17 sequence population in the seroconversion sample of mother 564 suggests that also in this case infection was established by multiple virus variants. We have recently examined the V1-V2 and V3 regions in two donor-recipient pairs and observed that biologically as well as genotypically distinct variants were detectable in a recipient injected with several milliliters of blood of an AIDS patient but not in a recipient injected with only a small volume (100 to 200 μl) of blood (6, 47). This suggests that besides the composition, the size of the inoculum may influence the likelihood of successful infection with multiple *env* variants.

An explanation for the homogeneous V3 sequence population in the children's samples could be that the inoculum was small and the viral RNA population represents the progeny of only one infectious unit. Interestingly, results of the p17 region suggest that infection was established by more than one infectious unit. In each child we observed substitutions in $p17^{gag}$ that were identical to substitutions seen in the maternal sequence sets; in two cases (pairs 114 and 127) these substitutions were characteristic for the majority of the maternal sequence population. Recent studies of sexual and parenteral transmission of HIV-1 provide evidence that the observed homogeneity in the *env* gene early in infection is the result of a strong selection for specific *env* sequences upon transmission or during replication in a new host, since sequence variability was found to be higher in the normally conserved $p17^{gag}$ gene (55, 56). In the postnatally infected child, variability was indeed higher in $p17^{gag}$ (0.3%) than in the V3 region (0%), but the reverse was found in the other two children. However, it was noted that under the experimental conditions used, the rate of in vitro-introduced misincorporations was larger for the V3 region (0.22 to 0.26%) than the p17 region (0.03 to 0.11%). Therefore, we cannot exclude the possibility that this obscured subtle differences between variability in $p17^{gag}$ and V3.

Evidence that selection mechanisms may be involved upon transmission from mother-to-child is provided by the fact that

the V3 region in the children's samples had characteristics which were observed in only a minority (mother-child pair 114) or even in none (mother-child pairs 127 and 564) of the maternal sequences. The viral envelope, and in particular the V3 region, is involved in tropism and replication efficiency of the virus and contains an important epitope for neutralizing antibodies (7, 8, 12, 15, 18, 20, 43). These determinants may modify the virus population of the inoculum upon transmission to a new host. Differences between viruses in tropism for cells lining the place of entry, like the gastroenteral mucosa and cytotrophoblast of the placenta, may give certain viral variants a selective advantage in penetrating this first barrier (10, 54). A characteristic of the virus population found early in infection is tropism for macrophages (47, 56). Following experimental infection of intestinal and ectocervical explants, HIV was detected principally in macrophages and only rarely in lymphocytes below the epithelium (reviewed in reference 30). Migration of infected macrophages or virus produced by these cells to the draining lymph nodes may subsequently cause massive infection of lymphocytes and dissemination of the virus through the body (35). The detection of HIV in macrophages in the placenta (Hofbauer cells) indicates that these cells may play a role in transplacental transmission of HIV to the infant (27, 29). Viral isolates obtained from children 114 and 127 were able to replicate in macrophages (47).

Apart from modulating factors during entry, results of studies in parenterally infected patients support the hypothesis that following entry, certain virus variants may be selectively amplified, resulting in a homogeneous virus population for the *env* gene but not necessarily for the p17 region of *gag* (6, 55). The importance of selective amplification became particularly clear in a study with rhesus macaques following intrarectal or intravenous challenge with a swarm of simian immunodeficiency virus variants. The virus population obtained from animals infected by different routes of transmission showed a high degree of similarity in the *env* gene, while these variants represented a minority (<1%) in the original inoculum (Pauza et al. in reference 30). A recent study described differences in susceptibility for neutralization or enhancement by maternal antibodies between viral isolates obtained from the mother and the infant, suggesting that the presence of neutralizing or enhancing maternal antibodies in the child could play a role in selective amplification of certain variants (22).

Transmission of a minor variant was also observed in the p17 region in mother-child pairs 114 and 127. Our analysis of the p17 sequences of the donor-recipient pairs examined by Zhu et al. (56) showed that in these pairs too, the virus of the recipients had characteristics which were rare or absent in the sequence population of the donor. This could mean that determinants within or close to the p17 region are also involved in selection. A more likely possibility is that we are dealing with a phenomenon known as genetic hitchhiking (42): selection for a particular *env* sequence will also result in selection for the *gag* gene associated with this *env* sequence. The existence of this phenomenon is supported by an observation in the study of Zhang et al: of the four hemophiliacs infected by the same batch, the only patient showing a difference in the V3 loop (p77) also contained a characteristic silent mutation in the p17 sequences which was not observed in the other patients (55).

Apart from selection, compartmentalization of viral variants may be another reason that infection in the children seems to have been established by a minor maternal variant. Differences in quasispecies have been described between proviral DNA in peripheral blood cells and cell-free virus in plasma and between viral sequences obtained from samples of blood and cervical lavage (30, 41). The variant transmitted to the infant could have been predominant in compartments other than serum which may be involved in mother-to-child transmission, like mononuclear cells in blood or virus in cervical secretions and breast milk. Scarlatti et al. (38) observed that in some mother-child pairs the virus transmitted to the child had more resemblance to maternal sequences derived from viral RNA, while in others it was more similar to proviral DNA sequences. However, preliminary results of V3 sequences of proviral DNA of samples collected in the first trimester of pregnancy and at the time of delivery of mother 127 were comparable to the ones observed for viral RNA and do not indicate that the virus transmitted to the infant was a predominant variant within the cell-associated virus population.

In the cases studied here, analysis of genomic RNA-derived sequences did not point to a particular time of transmission to the child. In mother-child pair 114, sequence analysis excluded the possibility of viral RNA detection in cord blood due to contamination with maternal blood during delivery, confirming that the child was infected in utero. However, a more accurate timing of transmission during pregnancy does not seem possible, since V3 and p17 sequences very similar to the virus transmitted to the child were found in all maternal samples collected until delivery. In pair 564, V3 and $p17^{gag}$ sequences from the maternal sample collected 18 months prior to the first RNA-positive sample of the child resembled the infant's sequences as much as the sample collected close to the presumed time of infection. The results of mother-child pair 127 were ambiguous. The V3 sequences obtained from samples collected during pregnancy showed more similarity to the child's virus than did those obtained from the sample collected at the time of delivery. However, for the p17 encoding region we observed the reverse. Although no clonal sequences of samples from the second and third trimesters of pregnancy were examined, direct sequences from these samples were identical to the consensus sequence of the first-trimester sample (data not shown), suggesting that sequences of these samples also had less similarity to the child's virus than did that of the sample collected at delivery. The results of the p17 region are more in accordance with the predicted time of transmission based on viral diagnostic tests (intrapartum or close to delivery) than those of the V3 region. For the reasons mentioned above, it is possible that examination of other compartments would be more informative. In addition, local and systemic selection mechanisms may be involved in mother-to child transmission, and this may interfere with the possibility to relate the composition of sequence populations to the moment of transmission.

In conclusion, infection in the children was established by variants representing a minority of the maternal cell-free virus population during the period of transmission. Selection mechanisms may be involved in mother-to-child transmission, but studies of viral populations in other compartments that may contribute to transmission (cells, cervical secretions, and breast milk) are necessary to confirm this. So far, the evolutionary characteristics for both genomic RNA *env* and *gag* genes did not point to a particular time of mother-to-child HIV-1 transmission.

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