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DOI

[10.1099/0022-1317-78-9-2225](https://doi.org/10.1099/0022-1317-78-9-2225)

Publication date

1997

Published in

Journal of general virology

[Link to publication](#)

Citation for published version (APA):

Simonon, A., Mulder-Kampinga, G. A., van de Perre, P., Karita, E., Msellati, P., Kuiken, C. L., & Goudsmit, J. (1997). Evolution of human immunodeficiency virus subtype A in women seroconverting post partum and their offspring post-natally infected by ingestion of breast milk. *Journal of general virology*, 78, 2225-2233. <https://doi.org/10.1099/0022-1317-78-9-2225>

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Evolution of human immunodeficiency virus subtype A in women seroconverting post partum and in their offspring post-natally infected by ingestion of breast milk

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The evolution of genomic RNA of human immunodeficiency virus type 1 (HIV-1), subtype A, was studied in three Rwandan mother–child pairs over a period of 12–30 months. In two pairs a homogeneous subtype A V3 sequence population was observed at seroconversion and the virus populations in the children resembled those in the mothers. One of these mother–child pairs was infected with an A/C recombinant virus (A^{p17}/C^{p24}). In the third pair, a heterogeneous V3 sequence population was observed in the maternal seroconversion sample but the V3 sequence population in the child's sample was homogeneous. In each individual the intra- and intersample variation (between the seroconversion and follow-up samples) increased over time in both the V3 region and p17^{gag}. Independent evolution for 1–2 years did not abolish the epidemiological relationship between virus populations in mother and child.

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Nucleotide sequences reported in this paper have been assigned the accession numbers Z47881–Z47902, Z47951–Z47997, Z478010, Z478011, Z76706–Z76734, Z75958–Z76462, Z76649 and Z76650.

In sub-Saharan Africa human immunodeficiency virus type 1 (HIV-1) infections are caused by a wide variety of HIV-1 subtypes. Infections with the subtypes A, C and D are the most frequently reported (Myers *et al.*, 1995). HIV-1 subtypes co-circulate in several central African countries and as a consequence subtype recombinants are found (Robertson *et al.*, 1995).

The present study focused on the evolution of the V3 region of *env* and p17 region of *gag* of HIV-1 subtype A in mother–child pairs. HIV-1 genomic RNA was isolated from serum samples from three mother–child pairs originating from a prospective cohort study in Kigali, Rwanda. The women seroconverted after childbirth and their children became infected through breast milk. The design of the cohort study and details of serological and diagnostic PCR data are given elsewhere (van de Perre *et al.*, 1991, 1992). The pairs designated 10, 12 and 16 in the reports of van de Perre *et al.* (1991, 1992) correspond to 538, 566 and 564 in this study. Children 538 and 566 seroconverted within the same 3 month period as did their mothers, suggesting vertical transmission during the acute phase of maternal infection (van de Perre *et al.*, 1991). Child 564 seroconverted 18 months after maternal seroconversion (van de Perre *et al.*, 1992). Some of the sequence results of pair 564 have been published previously and are included in the present paper for comparison only (Mulder-Kampinga *et al.*, 1995). None of the mothers and children fulfilled the WHO clinical case definition of AIDS (World Health Organization, 1986, 1994). No information was available about CD4⁺ and CD8⁺ T-cell numbers.

The procedures for RNA isolation, reverse transcription, amplification of cDNA by nested PCR, cloning and sequencing have been published previously (Mulder-Kampinga *et al.*, 1993, 1995). For each of the tested samples from these mother–child pairs, a specific signal was obtained after a single PCR amplification procedure (data not shown). The detection limit of the first PCR is 10–100 copies of DNA for both the V3

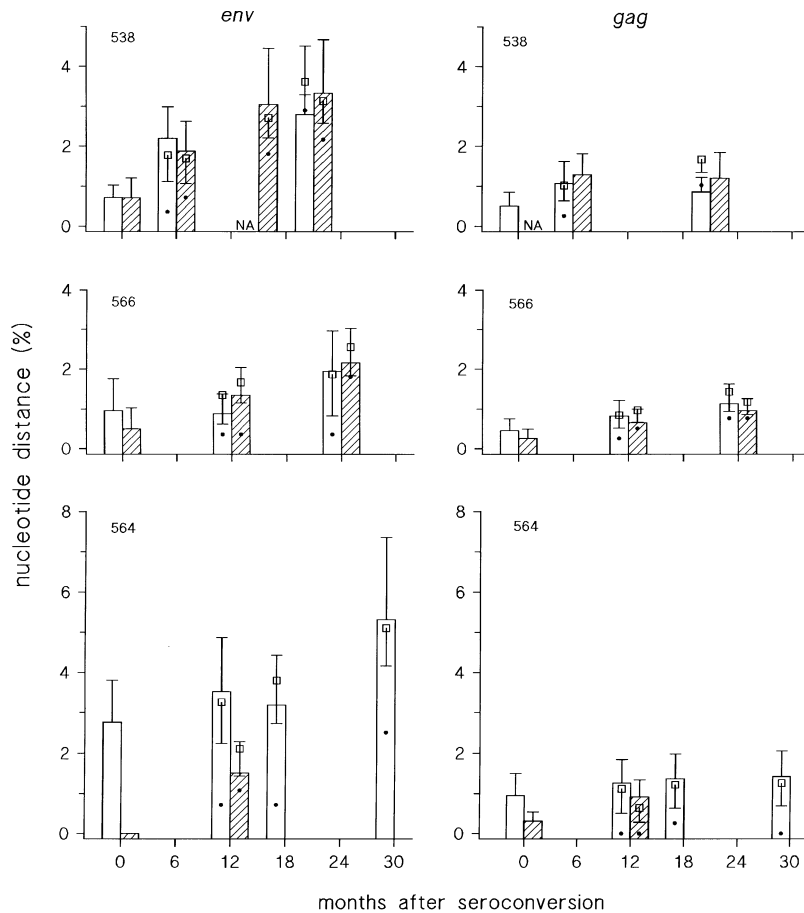


Fig. 1. Intra- and intersample variation (between sequences of the seroconversion sample and follow-up samples). Proportional nucleotide distances were calculated. The fragments analysed consisted of 276–282 bp for the V3 region of *env* (HXB2 positions 7031–7312) and of 390 bp (HXB2 positions 858–1247) for the p17 region of *gag*. Bars, mean intrasample variation (T one standard deviation); open bars, mother; hatched bars, child; squares, mean intersample variation (⊥, one standard deviation); dots, minimum intersample variation.

and p17 regions (Mulder-Kampinga *et al.*, 1993, 1995). The rate of misincorporation was 0.22–0.26% for the V3 region and 0.03–0.11% for the p17 region (Mulder-Kampinga *et al.*, 1995). All calculations were carried out with nucleotide sequences. Pairwise comparisons were performed to establish proportional nucleotide distances (*p*-distances) with pairwise gap deletion in the computer program MEGA (Kumar *et al.*, 1993). The intra- and intersample variation (between the seroconversion sample and follow-up samples) was expressed as the mean of the *p*-distances between clonal sequences. The increase in variation due to *Taq* errors is expected to be about twice the misincorporation rate, taking into account that misincorporations have been found to be randomly distributed (Mulder-Kampinga *et al.*, 1995). Calculation of synonymous (K_s) and nonsynonymous (K_a) *p*-distances was performed according to the method of Nei & Gojobori (1986) using the program MEGA. For analysis of the K_s and K_a between seroconversion and follow-up samples, the clonal sequences of the seroconversion samples were compared with the clonal sequences of the follow-up samples. Some seroconversion samples were initially analysed only for the V3 region, then later for p17^{gag}. However, the seroconversion sample from child 538 was no longer available for analysis of p17^{gag}.

Mother-child pairs 538 and 564 were infected with HIV-1

subtype A based on phylogenetic analysis of the V3 region and p17^{gag} (Kampinga *et al.*, 1997). Mother-child pair 566 was infected with an A/C recombinant virus. The V1–V3 region of *env* and the p17^{gag} region clustered with HIV-1 subtype A, but the first two-thirds of p24 clustered with subtype C (Kampinga *et al.*, 1997). Moreover, the mother was infected with two highly divergent virus populations: one with a subtype A V1–V3 region and one with a subtype C V1–V3 region (Kampinga *et al.*, 1997). In this study we focused on the evolution of the V3 region of HIV-1 subtype A.

At seroconversion, the V3 sequence populations from mother-child pairs 538 and 566 (when excluding the subtype C sequences) were highly homogeneous with a mean intra-sample variation of 0.5–1.0% for the V3 region and 0.3–0.5% for p17^{gag} (Fig. 1). This is in the range of, or only slightly higher than, the expected artificially introduced variation due to *Taq* errors. Most of the substitutions observed in these samples represented unique substitutions, i.e. those which were found only once in the total sequence set of an individual, and part of them may represent *Taq* misincorporation errors (Fig. 2A). In each of these two pairs, the major V3 variant in the child's sample was identical to the major variant in the maternal sample. The major p17 variant in the sample of child 566 was identical to one of the two major variants in the maternal

sample (with a Q or R at position 7; Fig. 2B). A much more heterogeneous V3 and p17^{gag} sequence population was found in the seroconversion sample of mother 564 (mean intrasample variation of 2.8 and 1.0%, respectively). Most of the heterogeneous positions, particularly those where identical amino acids or silent substitutions were found in at least two sequences of the seroconversion sample, remained heterogeneous for the same amino acids or silent substitutions in the follow-up samples (Fig. 2A). No V3 variation was observed in the seroconversion sample of child 564, but several substitutions, mainly synonymous, were seen in p17^{gag} (mean variation 0.3%). The child's V3 sequence was not detected in the maternal samples, but the major p17 variant in the child's sample was observed in three of the four maternal samples (Fig. 2B, M0-Con, M12-7, M30-8). The results from the V3 region

suggest that the virus transmitted to the child represented a minority in the maternal virus population.

For each individual, the intrasample variations of the follow-up samples were significantly higher than those of the seroconversion samples (Student's *t*-test: $P < 0.000-0.004$ for the V3 region and $P < 0.012-0.000$ for p17^{gag}), except for the V3 variation in the 12 month sample of mother 566 and the 18 month sample of mother 564. The intersample variation, between the seroconversion and the follow-up samples, increased over time (Fig. 1). The intra- and intersample variations in the V3 region and p17^{gag} after 1-2 years of infection were in the range of those found in adults and children infected with HIV-1 subtype B (Mulder-Kampinga *et al.*, 1993; Ahmad *et al.*, 1995; Wolfs *et al.*, 1991; Kasper *et al.*, 1995). The intersample K_s/K_a ratios for the V3 region ranged

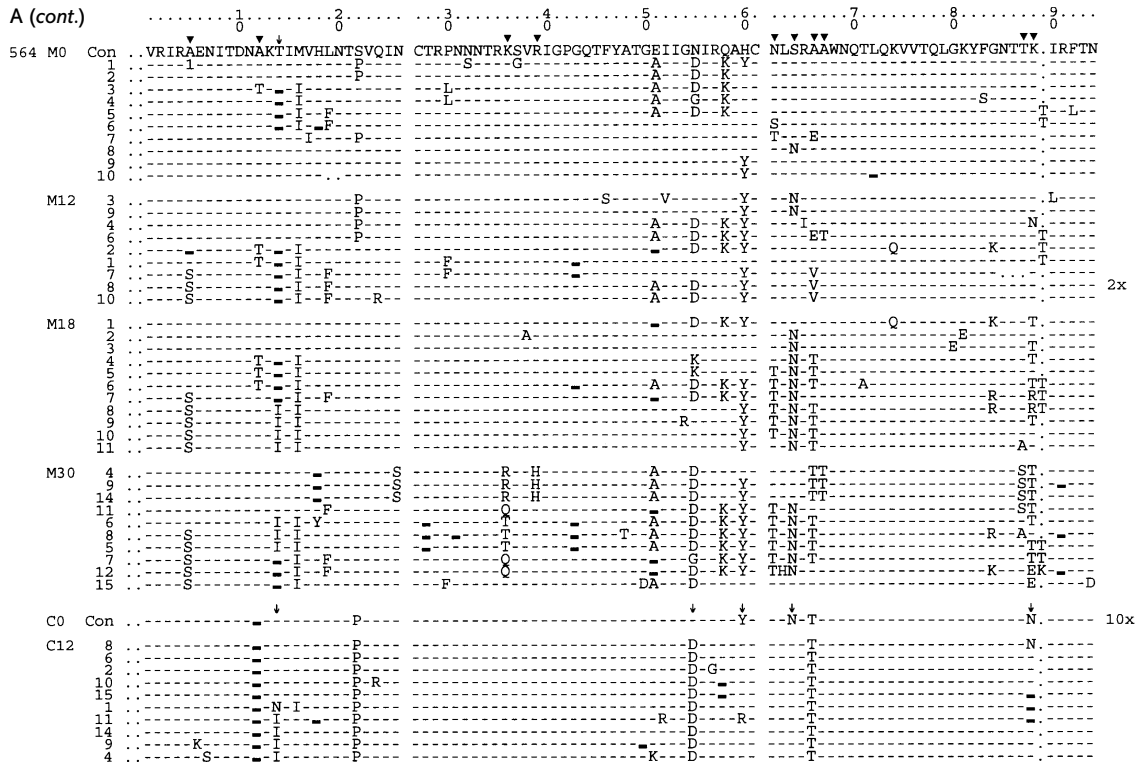


Fig. 2. Deduced amino acid sequences of the V3 region (A) and the p17 encoding region (B). Position 1 of the V3 region corresponds to amino acid 269 of the HXB2 envelope protein; position 1 of the p17 region corresponds to amino acid 24 of the HXB2 p17^{gag} protein. The start of the p24 encoding region is marked. Sequences of each mother-child pair are aligned against the consensus sequence (Con) of the maternal seroconversion sample. Dashes indicate identity with the reference sequence; dots are gaps introduced to optimize alignment. The frequency of a particular sequence is given at the end of the sequence. From clonal sequences that were identical to the consensus sequence except for one to three unique substitutions (substitutions observed only once in the total sequence set of the individual), only the unique substitutions are shown. For example, 10 nucleotide substitutions and one nucleotide deletion were observed in a total of eight clonal V3 sequences of the seroconversion sample from mother 538. Mother 566 contained V3 sequences of subtypes A and C (see text). The subtype A V3 sequences from mother 566 were obtained from two separately tested aliquots of serum (numbers below and above 20). For each time-point, identical sequences were obtained from both tested aliquots of serum (including the sequences M0-Con, M12-2, M24-1 and M24-9). ▼, Position where amino acid substitutions were observed (compared to the seroconversion sample) in at least 25% of the sequences of follow-up samples and which were not seen (or only seen once) in the seroconversion sample; ↓, position where identical substitutions were observed in the mother's and child's samples in at least 25% of the sequences of follow-up samples, or position with a substitution to an amino acid which was common in the maternal sequence set (child 564); -, silent mutation compared with reference sequence; *, stop codon; 1, 2, deletion of 1 or 2 nucleotides, respectively.

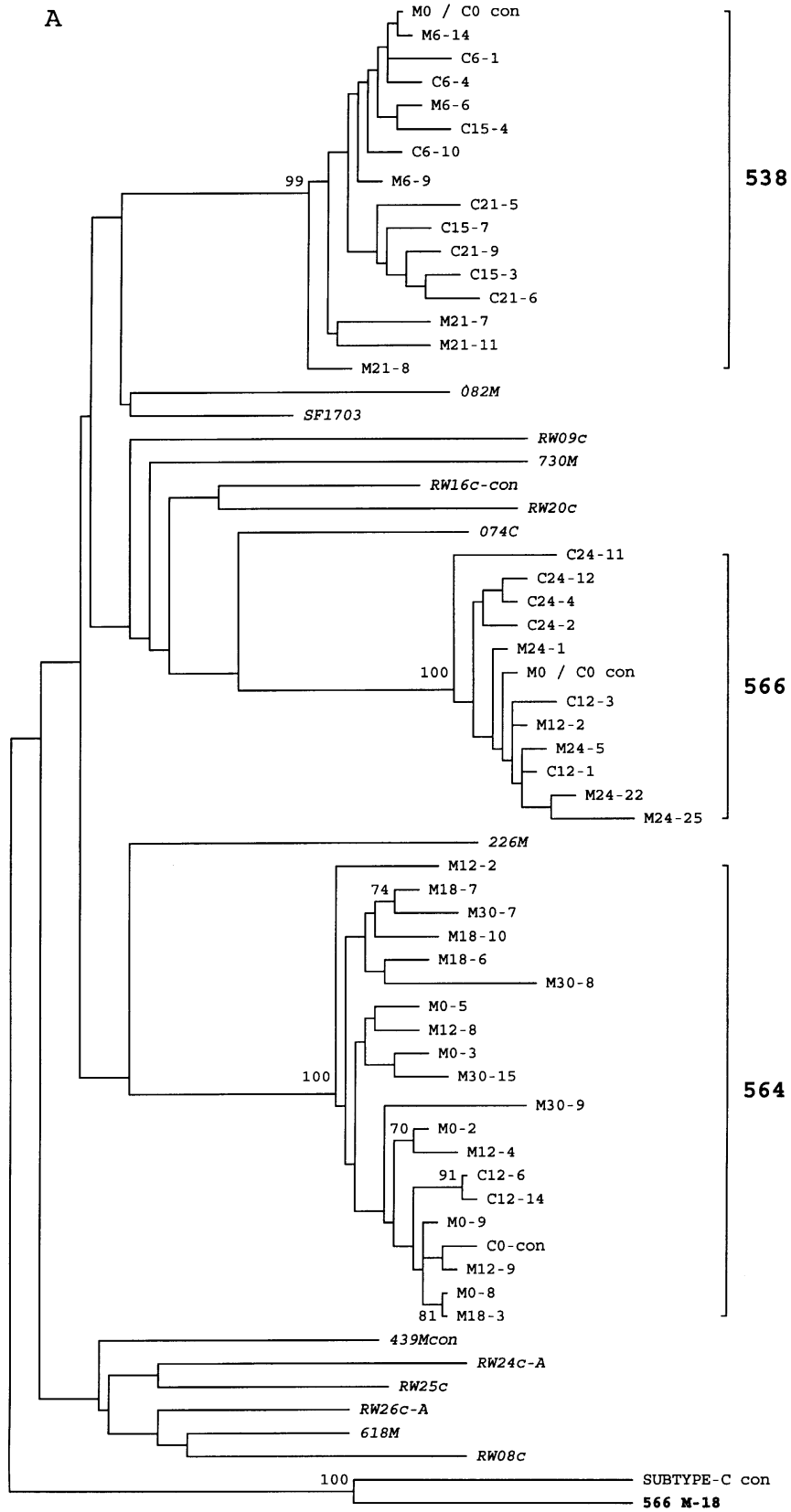


Fig. 3. For legend see facing page.

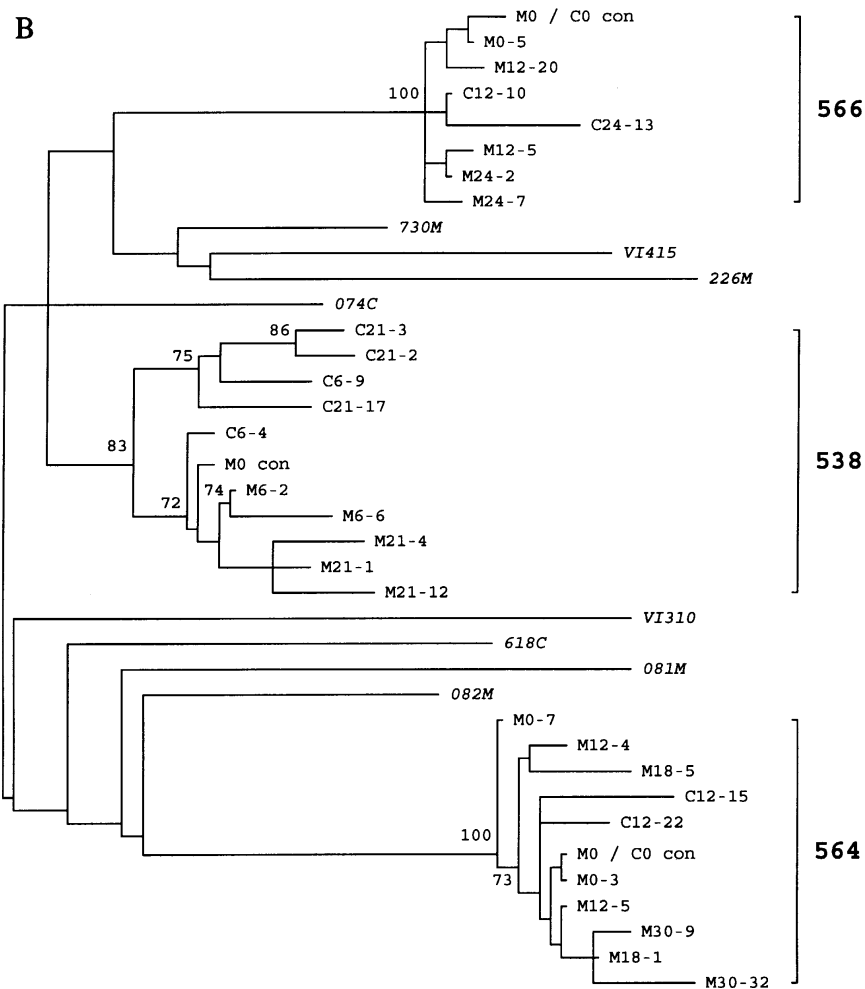


Fig. 3. Phylogenetic trees using consensus (con) and representative clonal sequences of the V3 region (A) and p17^{gag} ↓ (B). HIV-1 subtype A sequences (*in italics*) obtained from additional participants of the Rwandan mother–child cohort, 618M, 074C, 439M, 730M, 081M and 082M (Kampinga *et al.*, 1997) or from other persons from Rwanda (Myers *et al.*, 1995; de Wolf *et al.*, 1994), and the consensus sequence of HIV-1 subtype C (Myers *et al.*, 1995), were included in the analysis. A consensus tree generated with the neighbour-joining method is shown. Branch lengths were calculated by using the Kimura two-parameter distance. Bootstrap values $\geq 70\%$ (200 data sets) are given at the branch nodes.

from 0.46–1.13, comparable to what is observed for HIV-1 subtype B (Lukashov *et al.*, 1995; Strunnikova *et al.*, 1995). It is noteworthy that intersample K_s/K_a ratios for p17^{gag} were always higher in both mother and child from pair 564 (4.07–8.00) than in mother 538 (1.14 and 3.00) and pair 566 (0.68–1.50). Complete or nearly complete amino acid replacements were not observed in the follow-up samples of pair 564, but they were seen in the other two pairs (Fig. 2B). This is similar to results obtained by Kasper *et al.* (1995).

In each individual the changes in the V3 region and p17^{gag} over time had unique features. However, 1–2 years of independent evolution in mother and child did not obscure the epidemiological relationship between the two virus populations (bootstrap values 99–100% for the V3 region and 83–100% for p17^{gag}, Fig. 3), in accordance with results obtained in donor–recipient pairs infected with HIV-1 subtype B (Kuiken *et al.*, 1996; Ahmad *et al.*, 1995; Kasper *et al.*, 1995).

It has been suggested that it is more appropriate to determine epidemiological relationships between individuals after a period of independent evolution using p17^{gag} rather than the V3 region (Holmes *et al.*, 1995). However, we observed the reverse in pair 538. The p17 sequences of the 21 month samples of this pair were placed into separate clusters (Fig. 3B), and the bootstrap value of the mother–child cluster was markedly reduced (from 83 to 58%) when only clonal sequences from the mother's and child's 21 month samples were included in the analysis. This shows that analysis of multiple genes of HIV-1 is desirable in order to assess epidemiological relationships.

As in infections with HIV-1 subtype B, the position corresponding to position 308 of HXB2 (39 in Fig. 2A) seems under particular pressure for change (Mulder-Kampinga *et al.*, 1993; Wolfs *et al.*, 1991; Kasper *et al.*, 1994; Lukashov *et al.*, 1995). All five individuals with a follow-up of more than 12

months showed substitutions at this position. A complete replacement was observed in only one individual (child 538), but this may be related to the relatively short follow-up time. Position 308 plays a crucial role in the binding of V3-antibodies (Zwart *et al.*, 1992; Wolfs *et al.*, 1992). While H to R substitutions or vice versa are rare in HIV-1 subtype B, these substitutions are apparently preferred in the genetic background of HIV-1 subtype A. This could explain the predominance of H or R at this position in HIV-1 subtype A isolates (Myers *et al.*, 1995).

The changes in virus populations in samples from a mother-child pair were more similar to each other than changes in populations from unrelated individuals. Amino acid changes at identical positions and replacements by identical amino acids were seen more often in the sequence populations from a mother and her child than in those of unrelated individuals. In child 564, the original V3 sequence population was replaced by a population showing four or five substitutions which were also typically seen in the maternal samples, although the isoleucine at position 14 was encoded by different codons in the mother's and the child's samples (ATC and ATA, respectively). It is possible that the new population represented the progeny of other transmitted maternal variants which showed only after suppression of the initial virus population (Cornelissen *et al.*, 1995). Previously, we described a prenatally infected infant who within 9 months showed an almost complete replacement of the initially homogeneous virus population by a population sharing three amino acid substitutions (Mulder-Kampinga *et al.*, 1993). The observation of intermediate variants in an earlier sample from this child suggested that the new variants have arisen in the child. In the case of child 564, it is noteworthy that the silent mutation at position 12 remained perfectly conserved.

In both mother and child of pair 564, substitutions in p17^{gag} were mainly synonymous. However, complete or nearly complete amino acid replacements within p17^{gag} were observed in the other two pairs, including two combined identical amino acid changes in the mother and child of pair 566.

Identical amino acid changes in the V3 region (particularly in the V3-loop) and in p17^{gag} have also been observed in genetically unrelated persons following infection with genetically related viruses (Kasper *et al.*, 1994, 1995).

In samples from mother 566, two distinct populations of V3 sequences were observed which clustered with sequences of the HIV-1 subtypes A and C (Fig. 3A). The subtype C V3 sequences were detected after direct sequencing of PCR products generated with an alternative nested primer set (Kampinga *et al.*, 1997). As the antisense inner primer used for the generation of PCR product for cloning contained mismatches with these sequences, additional clones were generated from semi-nested PCR products. V3 sequences of both subtypes were detectable in all of the tested samples from this mother. The relative frequency of the two virus popu-

lations fluctuated over time. At the time of seroconversion and 24 months after seroconversion, subtype A was found to be predominant, representing eight and nine, respectively, out of 10 clones generated from semi-nested PCR products. In contrast, all clones of the 12-month sample represented subtype C V3 sequences, including two sequences which showed evidence for recombination between subtype A and C sequences (clones 21 and 28, Fig. 2A). Subtype C sequences were apparently also predominant in the 6-month sample, according to a direct sequence. For direct sequences derived from two to three separately tested aliquots from the seroconversion, the 12 and 24 month samples were in accordance with the results of the clonal sequences.

All 10 clones of semi-nested PCR products from the child's seroconversion sample belonged to subtype A.

In conclusion, the virus-host interaction in HIV-1 subtype A infections appears to result in evolutionary patterns similar to those observed in HIV-1 subtype B infections. Evidence was found for parallel evolution in genetically linked virus populations replicating in genetically linked hosts. This suggests that changes in the virus population are not random, but are influenced by selective processes which may be determined by intrinsic characteristics of the virus and/or the immunological response of the host.

A. Simonon and G. A. Kampinga contributed equally to this work. This study was funded by grant 28-2413 of the Dutch Foundation of Preventive Medicine, grant 962 of the Dutch AIDS Foundation, the AIDS Task Force of the European Economic Community, the Rwandan-Belgian Medical Cooperation, and the Agence Nationale de Recherches sur le SIDA (France). We would like to thank the physicians, nurses, social assistants and the mothers and children of the Mother-to-Child Transmission Study (ETME) for their cooperation, and John Dekker and Fokla Zorgdrager for technical support.

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Received 6 February 1997; Accepted 28 April 1997