Persistence of Multiple Maternal Genotypes of Human Immunodeficiency Virus Type I in Infants Infected by Vertical Transmission

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Abstract

The extent of nucleotide variation within the HIV-1 *env* hyper-variable domains serves as a marker of virus genotypes within infected individuals and as a means to track transmission of the virus between individuals. We analyzed *env* V1 and V2 sequences in longitudinal samples from two HIV-1–infected mothers, each with three children infected by maternal transmission of the virus. Sequences in samples that were obtained from two infants at 2 d and 4 wk after birth displayed more variation in V1 and V2 than maternal samples obtained at the same times. Multiple HIV-1 genotypes were identified in each mother. In each family, multiple maternal HIV-1 genotypes were transmitted to the infants. Specific amino acid residues in the hypervariable domains were conserved within sequences from each family producing a family-specific amino acid signature pattern in V1 and V2. Viruses that were highly related to maternal viruses in signature pattern persisted for as long as 4 yr in the older children. Results support a model of transmission involving multiple HIV-1 genotypes with development of genetic variation from differential outgrowth and accumulation of genetic changes within each individual. (*J. Clin. Invest.* 1994. 93:380–390.) Key words: human immunodeficiency virus 1 genetic variation • maternal transmission

Introduction

Maternal transmission of human immunodeficiency virus type 1 (HIV-1) occurs in ~ 15 to 35% of infants born to infected women (1–10). Why more than two thirds of the infants born to HIV-1–infected women apparently escape infection by the virus raises important questions about virus–host interactions. Mother-to-child transmission of HIV-1 is undoubtedly multifactorial, although the factors that contribute to, or inhibit, maternal transmission of HIV-1 are not completely defined (11). Because passive maternal antibody is the primary immunity that a fetus acquires, it is likely that maternal humoral immunity is one factor in vertical transmission of HIV-1 (12–15). However, the specificities of maternal antibodies that can protect against HIV-1 transmission are unclear (16–20). It is also likely that factors related to maternal virus titers, virus

tropism for different cell types, and virus variability contribute to vertical transmission.

Genetic variation within HIV-1 in vivo can have a significant impact on the biological characteristics and pathogenesis of the virus (21–25). Phenotypic changes of the virus can be related directly to genetic changes that result in a high frequency of amino acid substitutions, deletions, and insertions clustered in five hypervariable domains, V1 to V5, of the *env*-encoded gp120 molecule (26, 27). Multiple functional determinants for virus cell tropism and host immune response map to the *env* hypervariable domains (28–36).

Nucleotide diversification in HIV-1 *env* hypervariable domains results in viruses that can be distinguished by genetic analyses (37–41). Analyses of genetic variation primarily in the V3 domain of *env* within infected mother–infant pairs indicated that HIV-1 sequence diversity during the first 4 mo after birth was greater in samples from the mothers than from their infants (42, 43). Because there was no evidence in either study for transmission of multiple maternal HIV-1 genotypes to the infants, it was proposed that vertical HIV-1 infection involves selective transmission of a particular maternal variant of the virus.

Our studies have focused on the genetic variability of HIV-1 over time in two different families, where neonates and their older siblings were infected by maternal transmission of the virus. The V1 and V2 hypervariable domains of *env* were analyzed because previous studies demonstrated that extensive genetic variability in V1 and V2 could be used to distinguish among strains of HIV-1 (37). The goals were to determine whether or not multiple HIV-1 genotypes were present in the mothers, to assess the possibility that multiple genotypes could be transmitted, and to evaluate the extent of genetic divergence over time in epidemiologically related viruses infecting different individuals.

Methods

HIV-1 infected mothers and children. Samples of peripheral blood were collected over a period of almost 2 yr from members of two families, Family A and Family B (Fig. 1). The mothers, MA and MB, each had three infected children. A1 and B1 indicate the eldest child in each family; A2 and B2, the second child; A3 and B3, the youngest child. Samples are coded with numbers to represent the dates collected. In all cases, the earliest available samples were studied. In several instances, samples were collected from the mothers near the time of conception (Fig. 1). Samples were obtained through the Pediatric Immunology Clinic with maternal informed consent under a protocol approved by the Institutional Review Board of the University of Florida College of Medicine.

Peripheral blood T-cell subsets were analyzed by two-color flow cytometry (FACSCAN®; Becton Dickinson & Co., Mountain View, CA). Sera HIV-1 p24 antigen was determined by antigen capture (Abbott Laboratories, North Chicago, IL). Assays were carried out in the

Clinical Immunology Laboratory of Shands Hospital at the University of Florida.

MA was identified as HIV-1 infected when A1, her 21-mo-old child, was found to have lymphoid interstitial pneumonitis, lymphadenopathy, and positive HIV-1 serology. MA had no history of intravenous drug use by toxicology profile or evidence of other sexually transmitted diseases. Her risk factor was sexual contact with an intravenous drug user. MA's CD4 T-cell count fell from 587 cells/mm³ in October 1989, when she was enrolled in the study, to 38 cells/mm³ in January 1991, when zidovudine therapy was initiated. MA developed toxoplasmosis as her AIDS-defining illness in June 1991.

The mother in Family B was identified as infected by HIV-1 when her oldest child, B1, was evaluated at 9 mo of age for lymphadenopathy and interstitial pneumonitis. Similar to mother A, MB's risk factor was a sexual partner who was an intravenous drug abuser. MB was asymptomatic with 781 CD4 cells/mm³ in January 1988. Her CD4 T-cell count dropped to 296 cells/mm³ in December 1990 and to 39 cells/mm³ in October 1991. During that time she developed HIV-associated wasting syndrome but had no opportunistic infections. Antiviral therapy was initiated after October 1991.

Each of the six children were full-term infants born by spontaneous vaginal delivery with normal birth histories and neonatal physical examinations. There was no clinical evidence for fetomaternal hemorrhage. Clinical profiles of the children are summarized in Table I. Two infants in Family A were breast fed; A1, until 11 mo of age, and A2 until 7 wk of age. Child A1 developed generalized lymphadenopathy at 14 mo and interstitial pneumonitis at 21 mo. Infant A2 developed pneumococcal meningitis at 18 wk of age. Infant A3 was evaluated at 9 wk of age for fever of unknown origin, oral candidiasis, and lymphadenopathy, and developed *Pneumocystis carinii* and cytomegalovirus pneumonia at 14 wk of age. Infant B2 was positive for p24 antigen at 3 mo, but clinically asymptomatic until February 1991, when he developed *Pneumocystis carinii* pneumonia. Infant B3 showed evidence of oral candidiasis and lymphadenopathy at 4 wk of age.

Nucleic acid extraction, amplification, and sequencing. Separation of peripheral blood mononuclear cells (PBMC), DNA purification, and amplification procedures were carried out in a laboratory free of cultured HIV-1 isolates or cloned HIV-1 sequences. DNA was prepared from Ficoll-Hypaque separated PBMC according to standard procedures (45).

Sequences of HIV-1 *env* V1-V2-C2 region were amplified using the PCR and nested oligonucleotide primers prepared in the DNA synthesis core facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. The outer primers were ENV5 (5′-GGTAGAACAGATGCATGAGGAT-3′) and ENV6 (5′-CCATGTGTACATTGTACTGTGCT-3′), located at nucleotides 6102–6123 and 6544–6566 in the genome of HIV$_{LAI}$ (27). The inner primers were SK122 and SK123 (37). HIV-1 *env* sequences were amplified from 1 µg of PBMC DNA in 100 µl reactions with PCR buffer (1.75 mM MgCl$_2$, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.1% BSA), 100 pmol of each outer primer, 200 µmol of each dNTP, and 2.5 units Amplitaq (Perkin Elmer Cetus Corp., Norwalk, CT) overlayed with 25 µl mineral oil. Reagent controls and DNA from uninfected cells were included in every experiment. Amplification reactions were carried out in an automated, 48-well thermal cycler (Perkin Elmer Cetus Corp.) programmed for one cycle of denaturation (94°C for 10 min), 25 cycles of amplification (each cycle included 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C), followed by one cycle at 72°C for 10 min. After passage through a Centricon 100 filter (Amicon/W. R. Grace & Co., Beverly, MA) one tenth of the product was used as template in a second round of amplification for 20 cycles with inner primers and the same profile as above.

Amplified products of approximately 400 to 450 basepairs (bp) were isolated from agarose gels and blunt-end ligated into pGEM-3Z plasmid vectors (Promega Corp., Madison, WI). Multiple recombinant plasmids from each sample were sequenced by the dideoxy chain termination method with SP6 and T7 primers, ^{35}S-labeled dATP, and Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH).

Analyses of nucleotide sequences. Sequences were aligned to give minimum evolutionary distances using the multiple aligned sequence editor program (kindly provided by D. Faulkner and Y. Jerka, Molecular Biology Computer Research Resource, Harvard University, Cambridge, MA). After translation of nucleotide sequences using Microgenie (IntelliGenetics, Inc., Mountain View, CA), alignments were subsequently edited by hand due to extensive length variation in the hypervariable domains. The amplified region included 70 to 100 bp of

Table I. Clinical Profiles of HIV-1–Infected Siblings in Two Families

Patient	Date of birth	Birth weight	Sample code*	Age	CDC stage[‡]	CD4 count	CD4/CD8 ratio	HIV p24 Ag[§]
		kg	*mm/yy*			*cells/mm³*		
A3	9/16/90	2.86	0990	2 d	P1-A	ND	ND	+
			1190	9 wk	P2-A	1170	0.9	+
			0191	14 wk	P2-A, B, D1	1154	0.4	+
A2	8/30/89	2.75	1089	7 wk	P1-B	ND	ND	+
			0190	18 wk	P2-A, D2	1402	2.4	+
A1	1/05/88	3.43	1089	21 mo	P2-A, C	1342	0.3	–
			0590	29 mo	P2-A, C	925	0.5	ND
			0191ǁ	36 mo	P2-A, C	1012	0.8	–
B3	9/03/91	3.23	1091	4 wk	P2-A	2450	2.7	–
B2	2/04/90	3.54	0291	12 mo	P2-A, D1	257	0.9	+
			0491	14 mo	P2-A, D1	1210	1.1	+
B1	2/21/87	3.10	0789	29 mo	P2-A, C	200	0.2	+
			0989	31 mo	P2-A, C	224	0.2	ND
			0890	42 mo	P2-A, C	287	0.3	–
			1290ǁ	45 mo	P2-A, C	724	0.4	–

* Sample code represents date (month and year) of sample collection and is the same as indicated in Fig. 1. In all cases the earliest sample indicated was the earliest one obtained. ‡ Centers for Disease Control classification system for staging of pediatric HIV-1 infection (44). § Determined by antigen capture test (Abbott Laboratories, North Chicago, IL). ǁ Zidovudine therapy was initiated before these samples.

V1 sequences, 100 to 150 bp of V2, and 140 bp of conserved region C2. The V1 and V2 domains were defined by the cysteine residues proposed to form disulfide linkages in the region (46).

The program SYNO (kindly provided by M. Nei, Pennsylvania State University, University Park, PA) was used to calculate nucleotide distances transformed by the Jukes–Cantor formula (47, 48). The Student's t test was used to evaluate the significance of differences in nucleotide distances within an individual and between individuals (47). Phylogenetic trees resulted from analyses of selected sequences using Fitch–Margolish version 3.2.

Nucleotide sequence accession numbers. Sequences have been submitted to the GenBank database (accession numbers L15085 to L15187), and alignments of sequences have been provided to the Human Retroviruses and AIDS Database. Both data resources are maintained at Los Alamos National Laboratory, Los Alamos, NM.

Results

HIV-1 genetic variation within mothers and their infants. The extent of nucleic acid variation in *env* V1-V2-C region was analyzed within samples from each of the two mothers and three of their infants, A2, A3, and B3, shortly after birth (Fig. 1). In infant A2, *env* sequences were essentially homogeneous in sample A2.1089 obtained at 7 wk of age and in a second sample set, A2.0190, obtained at 18 wk of age (Table II). Yet, variation within maternal sample MA.1089, obtained at the same time as infant sample A2.1089, was 8.2% across the V1-V2-C region, reflecting variation of 22.4% in V1 and almost 12% in V2. It is unlikely that the sequence homogeneity in the infant was due to nonreplicating virus because A2 was positive for p24 antigen at both time points (see Table I).

In contrast, samples from two infants, A3 and B3, displayed greater nucleotide sequence variation than samples obtained from their mothers at the same time (Table II). Variation in sequences from infant A3 at 2 d of age (A3.0990) was 1.3% compared with variation of 0.2% in maternal sample MA.0990. Infant samples A3.1190 and A3.0191 increased in variation at 9 and 14 wk after birth, relative to variation in

Figure 1. Samples from two families. Family A is on the left; Family B is on the right. Line along the top indicates time in years. Lines beneath date line represent individuals. MA and MB, mothers; A1 and B1, oldest child; A2 and B2, second child; A3 and B3, youngest child. For infant A2, the horizontal line begins at the time of birth. For infants A3, B2, and B3, dotted lines indicate gestation. Vertical lines designate the death of infants A2 and A3. Black boxes represent samples. Numbers underneath each box designate the sample by the month and year of its collection. Samples with the same number were collected at the same time.

Table II. Nucleic Acid Variation in HIV-1 env within Samples from Mothers and Infants near Birth

Sample set*	Regions of HIV-1 env		
	V1/V2/C	V1	V2
MA.1089	8.2 (1.1)†	22.4 (3.7)	11.9 (2.2)
A2.1089	0	0	0
A2.0190	0	0	0
MA.0990	0.2 (0.1)	<0.2 (0.2)	<0.1 (0.6)
A3.0990	1.3 (0.3)	1.0 (0.6)	1.5 (0.6)
MA.1190	1.6 (.02)	2.3 (1.1)	2.7 (0.9)
A3.1190	▓▓§ (0.4)	1.3 (0.5)	▓▓ (1.2)
A3.0191	▓▓ (1.1)	▓▓▓ (2.3)	▓▓ (1.7)
MB.1091	4.5 (0.7)	6.3 (1.7)	6.8 (1.7)
B3.1091	▓▓ (1.1)	▓▓▓ (3.5)	10.5 (2.3)

*Each sample set contains five to eight sequences. † Percent mean variation (± SEM). § Shaded values indicate infant samples that are significantly more variable than maternal samples by Student's t test (53).

maternal *env* sequences ($P < 0.01$). In general, both the V1 and V2 domains in sequences from infant A3 were significantly more variable than maternal MA.1190 sequences ($P < 0.001$) (Table II). Likewise in Family B, the population of *env* sequences from infant sample B3.1091 was more variable across the entire V1-V2-C region than maternal *env* sequences MB.1091 ($P < 0.01$). These maternal–infant samples were obtained at the same time when B3 was 4 wk of age. Each hypervariable domain varied independently. V1 sequences were significantly more variable in infant sample B3.1091 than in maternal sample MB.1091 ($P < 0.001$), whereas V2 sequences were not ($P = 0.1$). Results of these analyses indicated that HIV-1 sequences in neonates can be significantly more variable than maternal virus sequences at the same point in time.

Genetic variation in V1 and V2 sequences in epidemiologically unrelated viruses. The extent of variation within virus populations that were epidemiologically related in a mother and her infants led us to define the distance between epidemiologically unlinked virus populations. All V1 and V2 amino acid sequences from the two mothers were aligned, guided by the 8 cysteine residues dispersed across the region, and the 12 conserved amino acid residues between V1 and V2 (Table III). Because of length variation in each of the hypervariable domains, spacing was introduced to maximize alignments between the two sets of sequences.

In contrast to the C2 region, where nucleotide variability between MB and MA sequences was 9.4±2.5%, distance in the V1 hypervariable domain was 30.5±4.2% and in V2, 15.9±2.7% (Table III). Variability in the HIV-1 sequences between the two mothers was not significantly different in some cases from the variation in HIV-1 sequences between infected members of the same family (data not shown). Yet, there was no evidence from the clinical histories of the mothers to indicate that their viruses were epidemiologically linked. The results raised the possibility that genetic variation, which evolved in V1 and V2 over a period of several years, may be too extensive to distinguish between populations of epidemiologically related and unrelated viruses. Alternatively, there can be fea-
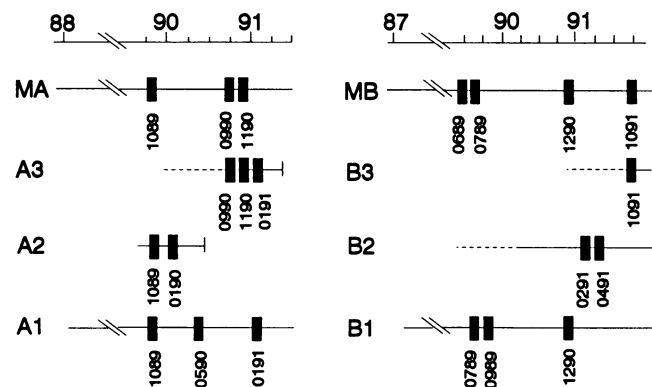
Table III. Alignment of V1-V2-C2 Peptides Encoded in Representative HIV-1 env Sequences from Two Mothers Infected by Epidemiologically Unrelated Viruses

Clones of maternal samples[†]	Amino acid sequence of HIV-1 env*		
	V1	V2	C2
MB.1091.5	CVLNCSNVSNQSRN DVNATEGNWKKEEE GEIKNCSFNITTS	IRDKVKKEYALFYKLDIVPINDNENNSHSRNNDGSYRLINCNTSVITQACPKVSFEPIPIH	CTPAGFAILKCNVKYKGSGPC
.1	---K----T----R--	N-KK-NN--R-	--FN
.2	------TR-----N--	R----YNY----N-N-	-M---FN
MB.1290.1	I-A-GT-V--T----NTS-	R----H-S--K-	-M---FN
.2	-------T----N-SY-R-	R-S---	---RFN
.3	-------T-------R--	-NQ--T-	---FN
MB.0789.3	-T-GVTG-GV----E-R	-K---SS--N-	-T---FN
.4	-T-A-GIMV-GT-V----STNVT-DMK-		---RFN
.5	-------T----R--	-D-NQ--T-	---FN
MB.0689.3	-T-A-GVTG-G-V---E-R	-K---S-	---FN
.4	-T-A-GVTG-G-V---E-R	-R---N-	---FN
.5	-IA---ST-V---D-K	-T--K-TNNK---MWT	--N---FN
MA.1190.1	-S-T-TDVG-NTG-S--E--G	RRE-RI-N-EG-K-FI-M	-M---QFN
.5	-G-T-TDVG-STNSTG-S-WE--G	RRE-RF-N-EG-K--I-M	-M---QFN
MA.0990.6	-S-T-TDIG-NTG-S--E--G	RRE-F-N-EG-K--I-M	-M-S---QFN
MA.1089.1	-S-T-TDIG-NTG-S--E--G	RRE-HF-N-EG-K--I-M	-M---QFN
.2	-S-H-I-S---SS WD-Q--I	R-G-IQID-F-NA--SM	-KD---QFN
.3	-T-D-K-LT-N-VG-M--R	RRE-F-N-EG-K--I-M	-M---QFN
Mean variation (±SEM)[§]	30.0(±4.2)%	15.9(±3.7)%	9.4(±2.5)%

* Single-letter amino acid code is used. Dash indicates identity with amino acid in reference sequence. MB sequences were used as the reference sequence. Conserved amino acids located between V1 and V2 are bold. Shaded residues indicate signature sequences. [†] Individual clones are indicated by and extension following the sample number. [§] Mean variation was calculated between all MB and all MA nucleotide sequences without gaps.

tures of the *env* sequence, in addition to nucleotide variation, that characterize epidemiologically related viruses. For example, conserved amino acid signature patterns in *env* contributed to establishing the epidemiological link among HIV-1 variants in a group of infected individuals (41).

Amino acid signature pattern. Based on the alignment of maternal sequences, it was possible to identify a pattern in the V1-V2-C2 region where particular amino acids appeared in nearly all sequences from one mother but not in the sequences from the second mother. This array of amino acids comprises the signature pattern for an individual's sequences. A unique signature pattern was identified in 16 amino-acid positions dispersed along the V1-V2-C region (Table III, *shaded positions*). Only 4 signature positions were located in the conserved region C2, whereas 12 signature residues were located in the hypervariable domains, 7 in V1 and 5 in V2. The pattern of amino acids in these positions did not predominate when V1-V2-C2 sequences from molecular clones of HIV-1 (27) or in HIV-1 isolates (37) were analyzed (data not shown), suggesting that these residues were conserved among epidemiologically related viruses. If this were the case, then V1-V2-C2 sequences within the infants and children in each family should contain their respective maternal signature patterns.

To examine this possibility, representative sequences from each mother were used as reference for the alignment of all sequences from her children. Significantly, the amino acid signature pattern identified in the maternal sequences was present in most sequences from all members of each family (Tables IV

and V, *shaded residues*). The amino acid signature positions in the V1 domain were designated 1 through 7 and those in V2, 8 through 12. Positions in C2, designated 13 through 16, were also conserved in a family-specific pattern (data not shown).

Analyses of 65 clones from Family A and 57 clones from Family B revealed in general two predominant amino acids at any signature position (Table VI). There was essentially no overlap in amino acid usage at any signature position between sequences from the two families. The family-specific amino acid signature pattern was apparent within epidemiologically related viruses in the face of considerable overall sequence variation. Moreover, the signature patterns persisted for as long as 3 to 4 yr not only in maternal HIV-1 sequences, but in HIV-1 sequences of their children as well.

Transmission of multiple genotypes. It became clear from the alignment of V1 and V2 sequences that a number of distinct HIV-1 genotypes could be identified within each mother. Likewise, more than one genotype appeared in most of the children. The relationship between representative maternal virus genotypes and sequences found in the infants or their older siblings was assessed by phylogenetic tree analyses.

In Family A, three major V1 genotypes were found in the maternal sequences, represented by MA.1089.1, MA.1089.2, and MA.1089.3 (Fig. 2 *A, upper diagram*). Sequences closely related to two maternal genotypes were found in infant A3. Variant A3.0191.3 clustered with maternal genotype MA.1089.2, whereas several additional A3 variants clustered with maternal genotype MA.1089.1. In the V2 domain, two

Table IV. *HIV-1 Amino Acid Signature Sequences in Mother–Child Samples of Family A*

Clones of samples[†]	Sequences*		
	V1	**V2**	
MA.1190.1	CVSLTCTDYVG NNTGGNSTGEKIEGGEIKNCSFNITTS	RRDKMREEYRIFNKLDLVPI NDE	GNKSFILIHC
MA.1089.1*	--S------V- ----G------E------------	----M----HF-K--D--- -N-	----Y--T--
.2*	---S-H-IN L -SSR--SWDQ--I------------	--G-MQID-AF--A-L---D--S	M-YR----
.3*	--S--- SNENK-L- -N-VGMT-R------------	----M----AF-K--D--- K--	----Y--T--
A3.0191.1*	--S------V- ----G------E------------	----M----AF-K--D--- K--	--------S-
.3*	--S-H-MN L -SS K--SWGQ--I-----S------M	--R-IEID--F--A-L---D--S	I-YR----
A3.1190.2	--S------V- ----G------E------------	----M----AF-K--G--- K--	--------S-
.7*	--S------V- ----G------E------------	--G-MQID-AF--A-N-I--N--D	I-YR----
A3.0990.4*	--S------V- ----G-------V-----------A	----M----AF-K--D--- K--	----Y--T--
A2.1089.1	---S-H-IN L -SSR--SWDQ--R------------	--G-MQID-AF--A---L--D--S	M-YR----
A2.0190.1*	--S-H-IN L -SSR--SWDQ--R------------	--G-MQID-AF--A---L--D--S	M-YR----
A1.0191.1*	--S--N--IL- -D-S--NSWGQ-QK--M---------	IG-R-KKD-AY--A-L---DD-S	R-D-YR----
.2*	--S--N--IL- ---S--K-WDQV-K--M---------STS--GR-L D--F--T--L---D--S	RNDSRNSNYS-YR----
.4	--S--N--IL- -D-L--NSWGQ-Q---M---------	IGVR-KKD-A---A-L---DD-K	Y-S-YR----
A1.0590.6	--S--N--IL- ---S--K-WDQV-K--M---------	IGGR-KKA-AF--A-L---D--S	RNDSR-D-YR----
.7*	--S--N--IL- ---S--NNWGQ-QK--M---------	TGGRLKKD-A---A-L---N--SDLVPI DNDSR-S-YR----
.9	--S--N--IL- ---S--NNWGQ-QK--M---------	IGGRLKKD-A---A-L---N--SDLVPIDNDSRNDSR-S-YR----
A1.1089.1*	--S--N--N L -T--S-- WG--K------------	--G-FQD -AF--A-L---D--N	DSS-YR----
.2*	--S--N--IL- ---S--K-WDQV-K--M---------	IGGR-KQD-AF--A-L---D--S	RNDSR-S-YR----
.5	--S--N--IL- ---S-HK-WDQV-K--M---------STS--GR-KQD-AF--A-L---D--G	R-D-YR----
Signature residues	1 2-4 5 6 7	8 9 10 11	12

* Alignment of constant region is not included because sequences in children are highly related to maternal sequences. † Sequences of representative clones from a sample are included. Stars indicate sequences included in the phylogenetic tree analyses in Fig. 2.

Table V. HIV-1 Amino Acid Signature Sequences in Mother–Child Samples of Family B

Clones of samples[†]	Sequences*	
	V1	V2
MB.1091.5	CV LNCS V SNQSRND NATEGNWKK E	GEIKNCSFNITTSIRDK KKEYALF KLD VPI DNENNSHSRNNDSGSYRLI C
.1*	-- K-- -T----- ------R-	--------------- ------- --- ---- N-KK-NN- -R---
MB.1290.1*	-- ---I A GT-V--G ----NTS--	-----------I--- ------- R-- ---- ---H--S -K-------
.3*	-- ---- -T----- ------R-	--------------- ------- --- ---- -----NQ-----
MB.0789.3*	-- ---T GVTG G -V-- E R	--------------- ------- --- ---- --K---- SS---N---
.4*	-- ---T GIMV--G -V----STN T DMK	--------------- ------- --- ---- --------
.5*	-- ---- -T----- ------R-	--------------- ------- --- ---- ---D-NQ------
MB.0689.5*	-- ---I A --ST -V-- D K	--------------- ------- --- --- --K--IDM----MWT---
B3.1091.2*	-- ---- -T----- K--------	--------------- ------- R-- ---- --K--N ----------
.3	-- ---- -T-P-- H----Y--- K	--------------- ------- R-- ---- ---SSNY-M--N--R----
.4*	-- ---T A GTKV- -----SSLG- W K	--------------- W------ --- ---- -KK--N--S- S------L
B2.0491.1*	-- ---T G- VTGG -V-- E R	--------------- ------- R-- ---- --RIIVNNK---------
.7	-- ---- -T---- ------R-	-----------R--- --G---- --- ---- --K--I ------R---
B2.0291.1*	-- ---- -T---- ------R-	--------------- ------- --- ---- ----- ----------
.4	-- ---- T-W-K- ------R-	
B1.1290.1*	-- ---T GTTV--- -T--SKLE-	--------------- ------- --- -T-- --K-H- -N -N--N---
.5	-- ---T GTTV--G ----S--G-	--------------- ----G-- --- ---- ------------
.6	-- ---I VTMVK-- -T-DSKLE-	--------------- ----G-- --- ---- --HN-NSS-N-S----
.7	-- ---T GTTVS-- T-- GG-	--------------- ----A-- --- ---- -----N--S--N------
.9	-- ---T GT ----SKG--	--------------- ------- --- ---- ----- -N--N------
B1.0890.2	-- ---T GTTVS-S TT--SSLE-	--------------- ------- --- ---- -----N--S -N--N---
B1.0989.1	-- ---T A GTMVS-G T--DSKLE-	-----------A--- ------- --- ---- ---K-NSHSS-N-DN---
.3*	-- ---T A GT H--SDGG-	--------------- ------- --- ---- ---K-NNHSS-N--N---
B1.0789.4*	-- ---T A GT-V-TN T -S LG-	--------------- ------- --- ---- ------ S -N--N---
.5	-- ---T A GTTV--G ----S--G-	--------------- ------- --- ---- --------------
.8	-I ---T GTTVS-- T-- GG-	--------------- ------- --- ---- -----N--S- S------
Signature residues	6 7	

* Alignment of constant region is not included because sequences in children are highly related to maternal sequences. † Sequences of representative clones from a sample are included. Stars indicate sequences included in the phylogenetic tree analyses in Fig. 2.

major genotypes were identified in both the mother and her infant A3 (Fig. 2 A, lower diagram). Infant sequences A3.0990.4 and A3.0191.1 clustered closely with one maternal genotype, represented by MA.1089.3 or MA.1089.1. A second maternal variant, MA.1089.2, was related to infant sequences A3.1190.7 and A3.0191.3.

Multiple HIV-1 genotypes, particularly in V1, were also found in the mother in Family B (Fig. 2 B, upper diagram). Sequences related to at least two maternal genotypes were identified in infant B3. One infant genotype, B3.1091.2, was closely related to a cluster of maternal variants MB.0789.5, MB.1290.3, and MB.1091.1. Another infant genotype, B3.1091.4, was more related to maternal variant MB.1290.1. There was evidence for multiple maternal HIV-1 genotypes in the second infant, B2, as well. One variant, B2.0291.1, was highly related to the cluster that included maternal and B3 sequences, while a second variant, B2.0491.1, was closely related to maternal variant MB.0789.3.

Persistence of genotypes. Although multiple HIV-1 variants could be transmitted, all variants were not necessarily apparent in either maternal or infant samples near the time of birth. For example, infant variant A3.0191.3 was first identi-

fied in a sample obtained when A3 was 14 wk of age (Table I). The maternal progenitor sequence, MA.1089.2, was not found in the mother's samples obtained 2 d (MA.0990) or 9 wk (MA.1190) after the birth of A3, but did appear in maternal sample MA.1089 obtained ~ 2 mo before conception of A3 (Fig. 1).

Many of the individual V1 sequences found in the eldest child A1 at 21 to 36 mo of age clustered together and, as a group, were more related to MA.1089.2 than to other maternal variants (Fig. 2 A, upper diagram). One variant, A1.1089.1, was distinct both from A1 sequences in the cluster and from other MA variants. This A1 variant may represent a virus that evolved independently in the child during the time after transmission. Alternatively, variant A1.1089.1 may reflect yet another maternal variant that was transmitted and persisted in the child.

Persistence of HIV-1 genotypes could be detected in Family B, as well. Although there was a striking similarity in V2 sequences among all members of family B, independent of the time that had elapsed between vertical transmission and subsequent samples, V1 sequences were clearly more variable (Fig. 2 B, upper and lower diagrams). V1 sequences in samples from

MB (MB.1091.1) and her infant B3 (B3.1091.2) were closely related to each other and to maternal variant MB.1290.3. The mother–infant samples designated 1091 were obtained 4 wk after the birth of B3, whereas maternal sample MB.1290 was obtained 10 mo earlier, during the first trimester of her pregnancy with B3 (Fig. 1). Even though MB's second infant, B2, was 1 yr of age when samples were first obtained, it was possible to distinguish two variants in B2 (B2.0291.1 and B2.0491.1) that clustered with maternal variants MB.0789.5 and MB.1290.3, or MB.0789.3, respectively (Fig. 2 B, upper diagram). These maternal variants were identified in samples obtained ~ 18 mo apart. One sample was obtained early in July 1989, during the first trimester of her pregnancy with B2 and the second, in December 1990, when B2 was 10 mo of age (Fig. 1). Similarly in MB's oldest child, one variant, B1.1290.1, was closely related to maternal variant MB.1290.1 (Fig. 2 B, upper diagram). These maternal–child samples were collected in December of 1990, when B1 was almost 4 yr of age (Fig. 1).

Discussion

The extent of nucleotide variation in env defined multiple HIV-1 genotypes in each of the mothers in our studies. A number of distinct maternal HIV-1 genotypes were detectable in the infants in each family. The most plausible explanation for the presence of multiple HIV-1 genotypes in infants is that multiple viruses can be transmitted from the mother. Our results, which indicate that pediatric HIV-1 infection does not always result from selective transmission of maternal HIV-1 variants, differ from other studies in which infants infected by vertical transmission were found to harbor homogeneous virus populations relative to maternal viruses (42, 43).

The families in our study were chosen because both mothers had transmitted HIV-1 to multiple infants. Vertical transmission to more than one infant is not unique among HIV-1 infected women with more than one child (1, 3, 6, 49, 50). The women in our study did not take zidovudine or other antiviral therapies, which might impact the frequency of vertical transmission. Two of the six children whom we studied were breast fed, but there was no evidence that they differed from the other HIV-1 infected children who were not. There were no apparent differences between other studies of vertical transmission and ours with regard to the ages of the infants, which ranged from birth to ~ 4 mo, their deliveries, or their clinical presentation (42, 43).

Because our studies were longitudinal, one explanation for our results could be that divergent HIV-1 genotypes evolved independently in infants and their mothers from a common progenitor sequence. Convergent evolution, in which the same amino acid was fixed independently at the same position in the V3 hypervariable domain in more than one HIV-1 variant, apparently developed in vivo over a period of years (51). However, there is no evidence that distinct HIV-1 genotypes, such as those identified in the mothers and infants in our study, can evolve independently within different individuals in a period of weeks. Moreover, different HIV-1 genotypes, which were identified in three of the infants in our studies, were detected before their births in earlier maternal samples.

The most likely explanations for the difference between our results and those from other studies of vertical transmission are that different subgenomic regions of HIV-1 env were analyzed

Table VI. Frequency of Family Specific Amino Acid Signature Patterns in Sixteen Residues in V1-V2-C2

Family	No. of clones	V1																				V2											C2							
		T†	S	N	D	V	A	Y	-	N	V	L	T	I	G	M	V	I	E	K	G	V	M	Y	N	I	L	E	N	N	S	H	F	Y	E	D	K	Q	S	T
A	65	0	100	30	70	0	0	52	48	0	49	51	0	0	98	0	11	89	0	25	75	0	100	0	100	0	100	0	99	0	0	100	0	100	0	100	0	100	0	100
B	57	100§	0	100	0	74	26	0	0	99	0	0	78	12	0	99	1	0	98	2	0	100	0	100	0	100	0	98	0	58	40	0	100	0	100	0	100	0	100	0

Amino acid signature residues

* Numbers 1 to 16 in row correspond to the amino acid residues with signature patterns. † Letters in row are the single-letter amino acid code. § Numbers are percent.
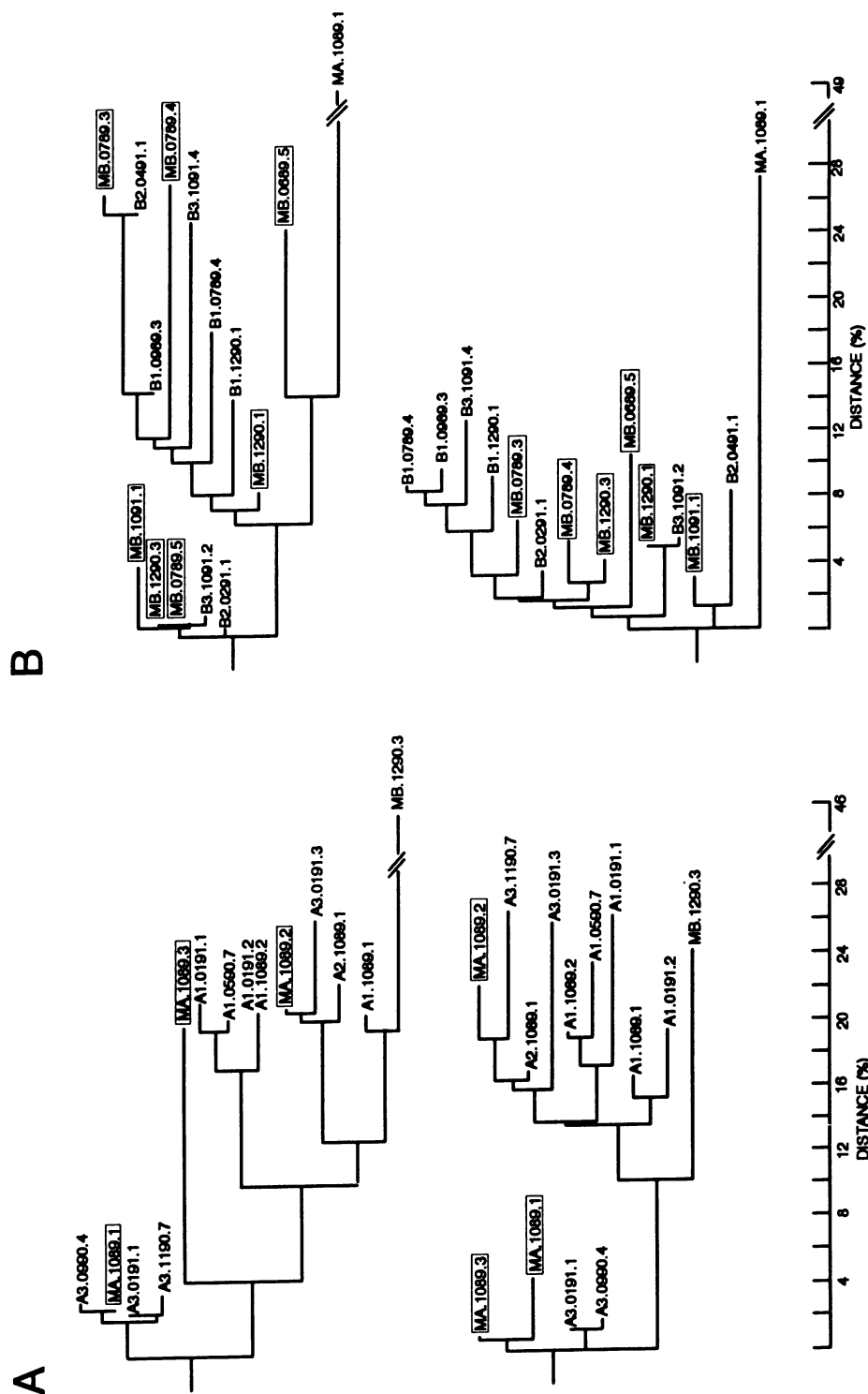
Figure 2. Phylogenetic trees of *env* nucleotide sequences in individual clones from maternal and pediatric samples in Family A (*A*) and in Family B (*B*). V1 sequences are represented in trees in the upper panels of *A* and *B*; V2 sequences are represented by trees in the lower panels. Maternal sequences in each family are boxed. Amino acid sequences of individual clones in the trees are designated by asterisks in Tables IV and V. Each tree includes a maternal sequence from the other family. Scale indicates distance in percent.

and that our studies were longitudinal. The V3 domain was a focus in other studies of maternal transmission of HIV-1 (42, 43). In contrast, both the V1 and V2 hypervariable domains of *env* were assessed in our studies. V1 and V2 vary independently in HIV-1 genomes in vivo and can display more genetic variability than the V3 domain (27, 52–55). Indeed, development of quasispecies of HIV-1 in vivo involved more genetic variability in a region 3' of the V3 loop, than in V3 itself (56). Because our studies analyzed virus sequences over time, rather than a single time point, the likelihood of identifying multiple HIV-1 variants within individuals was increased. Different conclusions could have been drawn if we had limited our studies to a single timepoint or to a single region of the HIV-1 genome; for example, the V2 domain, which was relatively homogeneous among all individuals in Family B.

Populations of HIV-1 variants were found to be more heterogenous in samples from two infants during the first few months after birth than in samples from their mothers at the same time. Increased sequence heterogeneity could reflect elevated levels of viremia in neonates early after infection by vertical transmission, similar to early viremia detected in adults after infection by other mechanisms (57–59). One infant in our study displayed homogeneous sequences in V1 and V2 at two separate time points, suggesting selective transmission of one maternal HIV-1 variant. Yet, restricted variation in one subgenomic region of HIV-1 does not necessarily indicate a homogenous population of virus because different regions within the same virus genome can vary independently (51, 53, 60, 61). An alternative explanation is that homogeneity of virus sequences in an infant at 7 or 14 wk of age could reflect selective outgrowth of one of several maternal variants that were transmitted.

HIV-1 genotypes not found in paired samples from a mother and one newborn close to the time of birth were detected subsequently, when all samples from the mother and her infant were analyzed. A representative profile of which maternal virus variants are actually transmitted may not be immediately evident, in part because of technical factors, such as the amount of target DNA amplified or the number of sequences analyzed in a sample set. In addition, biological factors undoubtedly contribute to the profile of variants detected because of fluctuations in frequencies of HIV-1 genotypes in peripheral blood cells (61–64). Particular HIV-1 variants can be sequestered in other tissues, such as the lymph nodes, before emerging in PBMCs (65, 66). Compartmentalization of the virus or activation of virus expression would explain the appearance of additional maternal HIV-1 genotypes in infants weeks after birth.

Timing of vertical transmission, that is, whether the infants were infected early in gestation or near birth, cannot be determined from our studies primarily because of the persistence of maternal HIV-1 genetic variants, in some cases, throughout pregnancy. Nonetheless, studies of genetic variation in HIV-1 do provide insights into mechanisms of transmission. Because multiple maternal viruses were identified in the infants, our studies indicate that vertical HIV-1 infection is not always limited to selective transmission of a single virion or a single virus genotype. Vertical transmission could occur by cell-free virus in maternal blood if the various maternal HIV-1 genotypes were expressed (43). Mother-to-infant transmission of HIV-1 may involve cell-associated virus, as well (11). In our studies, multiple HIV-1 variants detected in infants were closely related

to many of the virus variants in maternal peripheral blood cells. The extent of variation in HIV-1 genotypes that can be detected in maternal cells in small volumes of peripheral blood would be sufficient to account for transmission of cell-associated virus. Maternal lymphocytes have been identified in the blood of newborns, for example, in infants with severe combined immunodeficiency disease (67). The extent to which small numbers of maternal cells could be present in infants infected by vertical transmission of HIV-1 remains to be examined.

Genetically related viruses persisted for a number of years within members of each family. An unexpected result was the distinct pattern of amino acid signature residues that was conserved in V1 and V2 sequences among populations of viruses in each family. Persistence of the signature pattern independent of the overall extent of genetic variation in V1 and V2 contributed to an unambiguous fingerprint that linked epidemiologically related viruses for almost 4 yr after transmission. Results of our studies indicate that the V1 and V2 domains can be important for evaluation of the molecular epidemiology of HIV-1 transmission when the transmission link is in question.

Acknowledgments

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