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Genetic Characterization of Human Immunodeficiency Virus Type 1 in Blood and Genital Secretions: Evidence for Viral Compartmentalization and Selection during Sexual Transmission

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To explore the mechanism of sexual transmission of human immunodeficiency virus type 1 (HIV-1), we compared HIV-1 gp120 sequences in longitudinal samples from five acute seroconvertors with those from their corresponding sexual partners (transmitters). We used a quantitative homoduplex tracking assay to compare the overall genetic composition of HIV-1 quasispecies in each transmission pair and to track the transmitted viruses during the acute and asymptomatic stages of HIV-1 infection. In the chronically infected transmitters, HIV-1 variants in genital secretions differed from those in blood and variants in cells differed from those in cell-free plasma, indicating remarkable sequence heterogeneity in these subjects as well as compartmentalization of the virus in different bodily sites. Conversely, two of five seroconvertors had only a few related variants and three of five harbored only one viral population, indicating that in these subjects the transmitted viruses were typically homogeneous. Transmitted viruses were evident in the donor's seminal plasma (one of five cases) and even more so in their seminal cells (three of five cases), suggesting that both cell-associated and cell-free viruses can be transmitted. In every pair studied, the transmitted variant(s) represents only a minor population in the semen of the corresponding transmitter, thereby providing evidence that HIV-1 selection indeed occurs during sexual transmission.

Human immunodeficiency virus type 1 (HIV-1) is spread primarily by sexual, vertical, and parenteral transmission (27, 32, 37, 42, 53–55, 57). Several studies have shown that HIV-1 sequences in recently infected recipients are relatively homogeneous, even though the transmitters harbor heterogeneous genotypes (53–55, 57). Moreover, whereas HIV-1 isolates obtained from newly infected individuals are largely macrophage tropic and non-syncytium inducing (NSI) (41, 57), those from some of the corresponding donors are both syncytium inducing (SI) and NSI (48, 57). There is also evidence that the pressure to conserve sequences is stronger for gp120 than for gp41, *nef*, and p17 of *gag* during sexual transmission (55, 57). In aggregate, these data suggest that a selective mechanism may be involved in HIV-1 transmission (54, 55, 57).

Sexual transmission of HIV-1 in humans is a highly complex process. Among the variables involved are the precise transmission route and the interactions between multiple viral and host factors (4, 5, 22, 24, 25, 26, 30, 38, 39, 43, 48, 54, 55, 57). We previously proposed three models (low inoculum level, selective amplification, and selective penetration) to explain the discrepancy between peripheral blood virus populations in donors and those in newly infected recipients, as well as the observed sequence homogeneity in acute seroconvertors (57). Although some efforts have since been made to understand how HIV-1 variants are selected during parenteral and vertical transmission in humans (1, 11, 29, 33, 48), little has been done to elucidate this selection process in sexual transmission. One reason for this may be that all previous studies of sexual transmission have limited HIV-1 sequence analysis to peripheral blood mononuclear cells (PBMC) or plasma (54, 55, 57). However, in sexual transmission, certain virions or infected cells in genital secretions deposited on mucosal surfaces may have an advantage in initiating infection. Moreover, it is possible that HIV-1 strains in the genital secretions differ from those in plasma or PBMC. In this study, therefore, in order to better discern the selection mechanisms operating on HIV-1 during sexual transmission, we characterized and compared the virus strains present not only in the blood but also in the genital secretions. Another reason the selective mechanism in the sexual transmission of HIV-1 remains unclear is that previous investigations have tended to concentrate solely on samples taken at the time of seroconversion or immediately thereafter (53–55, 57). The process of HIV-1 variant selection, however, may begin much earlier and extend much later than that brief period. Consequently, in this study, samples collected from the transmission pairs before and after seroconversion were also examined.

The V1-V2 and V3 regions of the envelope gene have been reported to contain domains important for immune recognition (10, 21, 28), replication efficiency (45), and cellular tropism (8, 13, 20, 35, 44, 51, 52). Hence, assuming that the determinants influencing selective HIV-1 transmission are likely to be concentrated in the functional domains (57), we sought to compare these regions in five transmission pairs. Because of the high rate of variation in HIV-1 *env* sequences (14, 15, 18, 21, 23, 34, 49, 57) and the difficulty of quantitatively tracking transmitted viruses by current random sequencing procedures, we used a sensitive and quantitative technique, the quantitative homoduplex tracking assay (QHTA), to investi-

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gate the overall genotypic composition of HIV-1 quasispecies. Our findings suggest that a multiple-step process occurs during HIV-1 sexual transmission: first, compartmentalization occurs between blood and semen within the donor, and then selection occurs between the donor semen and the recipient mucosa and blood.

MATERIALS AND METHODS

Study subjects. This study involved four cases of homosexual transmission from male to male and one case of heterosexual transmission from male to female. Clinical and virological information on patients and samples is summarized in Table 1. All of the newly infected recipients had an acute, self-limited symptomatic illness with measurable viremia that was followed by seroconversion. In four of five recipients, longitudinal samples were collected before and after seroconversion, in one case as early as 59 days preseroconversion and in another as late as 183 days postseroconversion. In some of the donors, longitudinal samples were also obtained before or after the day of their sexual partners' seroconversion. Subject AD39, for example, had a 10-day seroconversion illness commencing on day −26, i.e., 26 days before the first antibody-positive sample. HIV-1 p24 antigen was first detected in his plasma on day −22 at a level of 47 pg/ml, which rose to 390 pg/ml on day −6 and fell to an undetectable level on day 0 (the day of seroconversion). He presumably was infected at day −36 by his homosexual partner, AD38, who had symptomatic HIV-1 infection (severe fatigue, a $CD4^+$ cell count of 56/mm^3, and a p24 antigen level of 42 pg/ml). Sera drawn on day −22 from both subjects and blood and genital secretions collected on day 0 from the recipient were therefore obtained 14 and 36 days, respectively, after the presumed time of sexual transmission. Serum samples from a heterosexual transmission case, that of subjects VF1 and VF2, were also obtained as early as 58 days before seroconversion (Table 1).

DNA and RNA preparation, reverse transcription (RT), PCR, and proviral DNA and genomic RNA quantitation. DNA was extracted from PBMC, seminal cells, and vaginal cells by a standard technique (57, 58). Samples (from 300 to 1,000 μl) of plasma, seminal fluids, or vaginal fluids were diluted with 10 ml of precooled (4°C) phosphate-buffered saline and ultracentrifuged at 40,000 rpm for 4 h at 4°C in a swinging rotor (Sorvall SW40 TI, DuPont). RNA was then extracted from the pellets by the acid guanidinium thiocyanate method (9). RNA was eluted in 20 μl of sterile H_2O, incubated with RNase-free DNase in the presence of 15 mM $MgCl_2$ for 20 min at 37°C, and then incubated for 5 min at 80°C to stop the reaction. A 7-μl aliquot of DNase-treated RNA was used in an RT reaction with cDNA Synthesis System Plus (Amersham, Arlington Heights, Ill.) and 100 ng of antisense primer PE2 (shown in Table 2) to permit the synthesis of cDNA for the entire gp120-coding segment of the *env* gene. The RT reaction was terminated by heating the reaction mixtures for 5 min at 95°C. The primers and PCR conditions used in this study are summarized in Table 2. PCR reactions for the QHTA were carried out with AmpliTaq polymerase (Perkin-Elmer Cetus, Emeryville, Calif.), as described previously (57). cDNA and proviral DNA were subjected to PCR amplification to generate HIV-1 gp120 sequences (including V1-V5) spanned by primers PE1 and P2. A 5-μl aliquot of the first-round PCR product was subjected to a second round of PCR amplification with inner primers P1 and P10 to expand the V1-V2 sequences and, separately, with primers P5 and PV3 to expand the V3 sequences. Semiquantitation of HIV-1 copy numbers was carried out by quarterly serial dilutions of proviral DNA in PBMC, seminal cells, and vaginal cells and by dilutions of 1:5, 1:10, 1:20, and 1:40 of cDNA from RNA in plasma, seminal fluids, and vaginal fluids, and then the same nested PCR procedures used for the QHTA were performed. The last dilution used in each positive PCR reaction was determined to be the minimum HIV-1 copy number. The HIV-1 RNA copy numbers of some sera and plasma were also quantified by the branched DNA assay (6, 36), as shown in Table 1.

Long PCR, cloning, and sequencing. HIV-1 sequences coding for Rev, Tat, gp120, gp41, and Nef (3,289 bp) were amplified from uncultured patient PBMC and genital secretion cells in a total volume of 100 μl. The reaction mixtures contained 20 mM Tris-HCl (pH 8.55), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 150 μg of bovine serum albumin per ml, 300 μM deoxynucleoside triphosphates (dNTPs), 24 U of KlenTaq1 (Ab Peptides Inc., St. Louis, Mo.), 0.16 U of *pfu* DNA polymerase (Stratagene, La Jolla, Calif.), and 0.5 to 2 μg of DNA. Long-PCR products were digested by restriction enzymes *Eco*RI and *Xba*I, ligated to predigested pUC18, and then transformed into JM103 cells. Double-strand DNA extracted from recombinant clones was sequenced with a Sequenase 2.0 kit (U.S. Biochemical, Cleveland, Ohio) and used to generate the QHTA probe (see below).

QHTA. P1-P10 or P5-PV3 PCR products generated from a long-PCR clone or from PE1-P2 PCR products were visualized on a 1% agarose gel, and an appropriate-sized band was excised and purified with a GeneClean II kit (Bio 101, Inc., La Jolla, Calif.). Purified PCR products were subjected to an asymmetrical PCR with 40 pmol of primer P1 or PV3, 0.1 mM dNTP, 1 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 U of AmpliTaq (Perkin-Elmer Cetus), and 20 μCi of ^{32}P-dATP. A 1- to 2-μl aliquot of a ^{32}P-labeled asymmetrical PCR product (probe) was mixed with 10 to 20 μl of the corresponding P1-P10 or P5-PV3 PCR products from the mixtures of three to six separate nested PCR reactions. A 2-μl aliquot of 10× annealing buffer (1 M NaCl, 100 mM Tris-HCl [pH 7.8], 20 mM EDTA) was added, and the DNA molecules were denatured by heating at 95°C for 3 min and then reannealed at 22°C for 15 min and stored on ice. The resultant hybrid molecules were then subjected to electrophoresis in a 5% nondenaturing polyacrylamide gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA [pH 8.0]) at 200 V for 4 h in a vertical gel apparatus, model SE400 (Hoefer Scientific Instruments, San Francisco, Calif.). Homoduplexes and heteroduplexes were visualized by drying the polyacrylamide gels onto VWR238 blotting paper (VWR Co., Piscataway, N.J.) and exposing them on an X-ray film (Eastman Kodak Co., Rochester, N.Y.). To quantitate homoduplexes and heteroduplexes, the dried gel was scanned with a Betascope 603 blot analyzer (Betagen Corp., Mountain View, Calif.). Data were collected for 1 h and analyzed by the Manual Quantitation program, in which counts per minute of each band and the distances between each pair of bands were determined by the Auto Seek On program. Negative controls (a probe mixed with distilled water) and positive controls (a probe mixed with a sequence identical to its own sequence, i.e., a 100% homoduplex) were included in each experiment to determine quantitatively the amount of homoduplexes and heteroduplexes. Any homoduplex band in a tested sample that was stronger than the homoduplex band in the negative control was considered a real homoduplex since, theoretically, a negative control without the competition of template sequences would have more chances to form homoduplexes between complementary probe sequences.

Nucleotide sequence accession numbers. Nucleotide sequences generated from this study have been deposited in GenBank. Accession numbers are U50780 to U50815.

RESULTS

Quantitative detection of individual sequences by QHTA. The QHTA, a modification of the heteroduplex tracking assay (HTA) (14, 15), uses a ^{32}P-labeled single-strand probe generated from an asymmetrical PCR instead of the ^{32}P-labeled double-strand probe used in the HTA. An example of the use of the QHTA to detect homoduplexes and heteroduplexes from molecular clones and a comparison of the results with those of the HTA are shown in Fig. 1A. A ^{32}P-labeled single-strand (for the QHTA) or double-strand (for the HTA) probe was separately generated from a molecular clone (clone 1) and mixed with individual clones and mixtures of clones. As shown in Fig. 1, the QHTA produced one heteroduplex band from each clonal sequence, two bands from a mixture of two different molecular clones, and three bands from a mixture of three molecular clones. It has been demonstrated that heteroduplex mobility generally correlates with genetic distances obtained by sequencing (14, 15). In addition, mismatched nucleotide sequences that compose more than 1% of the total can be detected by the assay (data not shown). As depicted in Fig. 1A, a 3-bp insertion-deletion remarkably reduced heteroduplex mobility (14, 15). However, the HTA using a double-strand probe often showed two bands (because of the strand-specific composition of mismatched molecules) from a given divergent clone; four and six bands were produced from a mixture of two or three clones, thus rendering interpretation and quantitation difficult. An additional homoduplex band, caused by the reannealing of complementary probe sequences, was also observed on all of the HTA gels.

We then applied the QHTA to a series of mixtures with differing amounts of three molecular clones, as shown in Fig. 1B. From the sample in lane C on, clone 1 increased from 1 to 80.5% and clone 3 decreased from 99 to 0.5%. From the sample in lane F on, clone 2 increased from 1 to 19%. By this method, <1% of a heteroduplex and 0.5% of a homoduplex could be detected, and the percentage of each specific sequence contributed to the samples was clearly displayed on the gel. Thus, the QHTA demonstrated greater sensitivity to detect minor variants and superior ability to quantitatively detect specific sequences within a given sample.

Semiquantitation of HIV-1 copy number in uncultured patient samples. As mentioned above, the minimum HIV-1 copy number was determined by serial dilution. In this study, over

TABLE 1. Characteristics of sexual transmission pairs

Transmission type and subject	Sex[a]	Day of sampling[b]	Sample type	Amt of p24 in serum (pg/ml)	Antibody response to HIV-1	Amt of RNA in plasma (copies/ml)
Homosexual						
AD39 (recipient)	M	−22	Serum	47	Negative	ND[c]
		−13	Serum	129	Negative	ND
		−6	Serum	390	Negative	ND
		0	Plasma	0	Positive	ND
			PBMC			
		84	Plasma	0	Positive	ND
			PBMC			
AD38 (donor)	M	−22	Serum	42	Positive	ND
		−2	Plasma	45	Positive	ND
			PBMC			
			Seminal plasma			
			Seminal cells			
KR (recipient)	M	−59	Plasma	0	Negative	ND
		−7	Plasma	15	Negative	4.3×10^5
		0	Plasma	0	Positive	3.8×10^5
			PBMC			
		56	Plasma	0	ND	ND
			PBMC			
		146	Plasma	0	ND	ND
			PBMC			
KD (donor)	M	0	Plasma	0	Positive	2.8×10^5
			PBMC			
			Seminal plasma			
			Seminal cells			
AD13 (recipient)	M	−27	Plasma	0	Negative	ND
		0	Plasma	ND	Positive	7.1×10^4
		66	PBMC			
		183	Plasma	ND	ND	7.1×10^4
AD12 (donor)	M	0	Plasma	ND	ND	7.1×10^4
			PBMC			
			Seminal plasma			
			Seminal cells			
AD43 (recipient)	M	0	Plasma	19	Positive	ND
			PBMC			
AD42 (donor)	M	0	Plasma	0	Positive	ND
			PBMC			
			Seminal plasma			
			Seminal cells			
Heterosexual						
VF1 (recipient)	F	−58	Serum	ND	Negative	2×10^5
		0	Serum	ND	Positive	$<10^3$
		28	Plasma	0	Positive	ND
			PBMC			
			Vaginal plasma			
			Vaginal cells			
VF2 (donor)	M	28	Plasma	0	Positive	5.6×10^6
			PBMC			
			Seminal plasma			
			Seminal cells			
		83	Plasma	ND	ND	ND
			PBMC			
			Seminal plasma			
			Seminal cells			

[a] M, male; F, female.
[b] The day of the first antibody-positive sample from the recipient is called day 0; the sampling days before and after day 0 are marked − and +, respectively.
[c] ND, not determined.

40 HIV-1 copies from all samples (except the 20 to 40 copies from AD42 genital secretions) were added to each nested PCR and over 100 HIV-1 viruses from multiple PCR products were used for the QHTA. As shown in Table 1, using a branched DNA assay, we measured 40,000 to 600,000 HIV-1 RNA copies in 1 ml of serum or plasma. We estimate, therefore, that 4,000 to 100,000 viral RNA copies were present in the 0.1 to 0.3 ml of serum or plasma used in each RT-PCR. We can con-

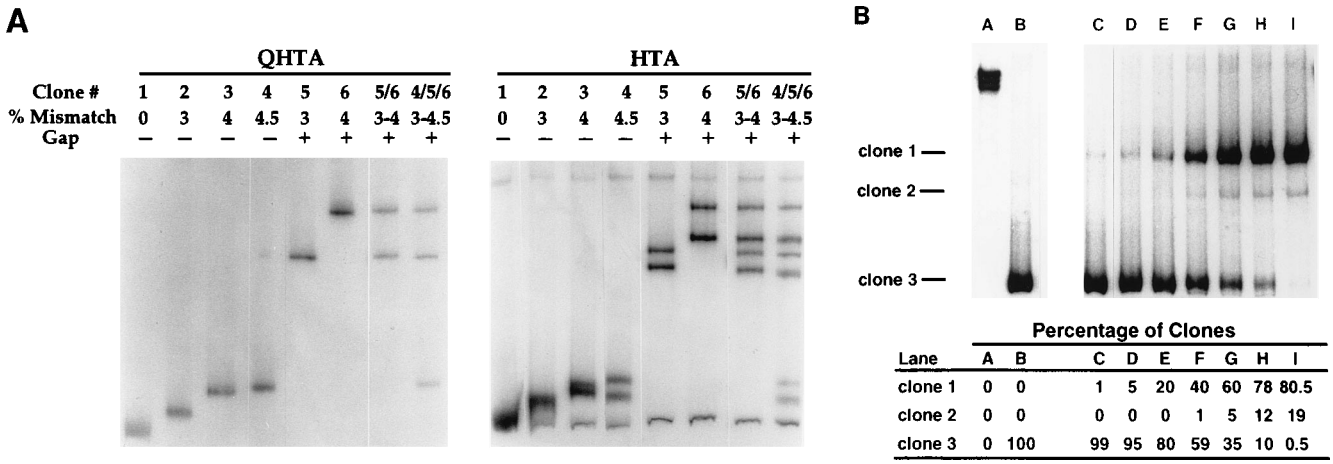
TABLE 2. PCR primers used for cloning and the QHTA

Experiment type and primer pair[a] (reference)	Nucleotide sequence[b]	Genomic region	Reaction condition[c]	Nucleotide position[d]	Fragment size (bp)
Long PCR for cloning					
Outer pair					
P11	5′-GGAAAGGTGAAGGGGCAGTAGTAATAC-3′	*pol*	94°C for 30 s, 68°C for 12 min; 10 cycles	4957–4983	4,752
P12-2	5′-TGCTAGAGATTTTCCACACTG-3′	3′-LTR[e]	94°C for 15 s, 68°C for 10 min; 20 cycles	9709–9688	
Inner pair					
P15 (57)	5′-GCCATAATAAGAATTCTGCAACAACTGCTGGG-3′	*vpr*	94°C for 30 s, 68°C for 8 min; 2 cycles	5733–5762	3,289
PE4	5′-ATTGGTCTAGAAGGTACCTGAGGTCTGACTG-3′	*nef*	94°C for 15 s, 68°C for 6 min; 33 cycles	9022–8992	
PE0 (58)	5′-GGCTTAGGCATCTCCTATGGCAGGAAG-3′	*rev*	94°C for 30 s, 68°C for 3 min; 5 cycles	5953–5979	1,994
PE2	5′-GCCTGGAGCTGTTTAATGCCCCA-3′	gp41	94°C for 15 s, 68°C for 2 min; 30 cycles	7947–7925	
PCR for QHTA					
Outer pair					
PE1	5′-AGAAAGAGCAGAAGACAGTGGCAATGA-3′	*tat*	94°C for 15 s, 55°C for 30 s, 72°C for 2 min; 30 cycles	6198–6224	1,617
P2 (57)	5′-GACGAAGCTTCCATAGTGCTTCCTGCTGC-3′	gp41		7815–7787	
Inner pair					
P1 (58)	5′-GATGGTACCGGATATAATCAGTTTATGGG-3′	C1 (gp120)	94°C for 15 s, 55°C for 30 s, 72°C for 1 min; 35 cycles	6528–6552	442
P10	5′-CCTAATTCCATGTGTACATTGTACTGT-3′	C2 (gp120)		6970–6944	
P5 (57)	5′-ACACATGGAATTCGGCCAGTAGT-3′	C2 (gp120)	94°C for 15 s, 55°C for 30 s, 72°C for 1 min; 35 cycles	6956–6978	412
PV3 (56)	5′-CAGTAGAAAAATTCCCCTCCACAATTAA-3′	C3 (gp120)		7368–7341	

[a] Primers were derived from the HIV consensus sequence (34). Those previously published are cited with the relevant reference number.
[b] Underlining indicates the sites for restriction enzymes.
[c] All conditions were followed by a final extension for 10 min at 72°C.
[d] Sequences are numbered according to the sequence of accession number NL4-3 (34).
[e] LTR, long terminal repeat.

clude, then, that this study employed a representative sampling of HIV-1 variants in vivo.

Overall genetic composition of HIV-1 quasispecies following homosexual transmission. HIV-1 gp160 sequences were amplified from the recipients' PBMC by long PCR and were cloned into a pUC18 vector. A clone was then used as the template DNA to generate a ^{32}P-labeled single-strand probe for the QHTA. The use of the QHTA with a clonal single-strand probe has the advantage of clearly showing on the gel the overall sequence population within a given sample. A nested PCR was employed to amplify HIV-1 gp120 sequences from uncultured patient samples for analysis by QHTA, which ensures coamplification of multiple HIV-1 sequences within a given sample. Half of the PCR products (by volume) were used for cloning into the TA vector (Invitrogen, San Diego, Calif.). Instead of random clonal sequencing, we first applied the QHTA to analyze the overall genetic compositions in PCR products from patient samples. Figure 2A depicts the HIV-1 V3 sequences in samples from a homosexual transmission pair. The ^{32}P-labeled single-strand probe generated from a molecular clone from the recipient's (AD39) uncultured PBMC was separately mixed with the corresponding bulk PCR products from the recipient's PBMC and plasma; with the PCR products from the donor's PBMC, plasma, seminal cells, and seminal plasma; and with the PCR products from representative clones from donor samples. One homoduplex was found in all of the



Percentage of Clones

Lane	A	B	C	D	E	F	G	H	I
clone 1	0	0	1	5	20	40	60	78	80.5
clone 2	0	0	0	0	0	1	5	12	19
clone 3	0	100	99	95	80	59	35	10	0.5

FIG. 1. Molecular clone detection by the QHTA. (A) Comparison of the QHTA and the regular HTA. Heteroduplexes with different mismatches in the absence (−) or presence (+) of a 3-base gap were analyzed on a 5% polyacrylamide gel. (B) Quantitative detection of individual sequences within a given sample. The actual percentages of clones within each sample before PCR-QHTA are shown in the lower table.

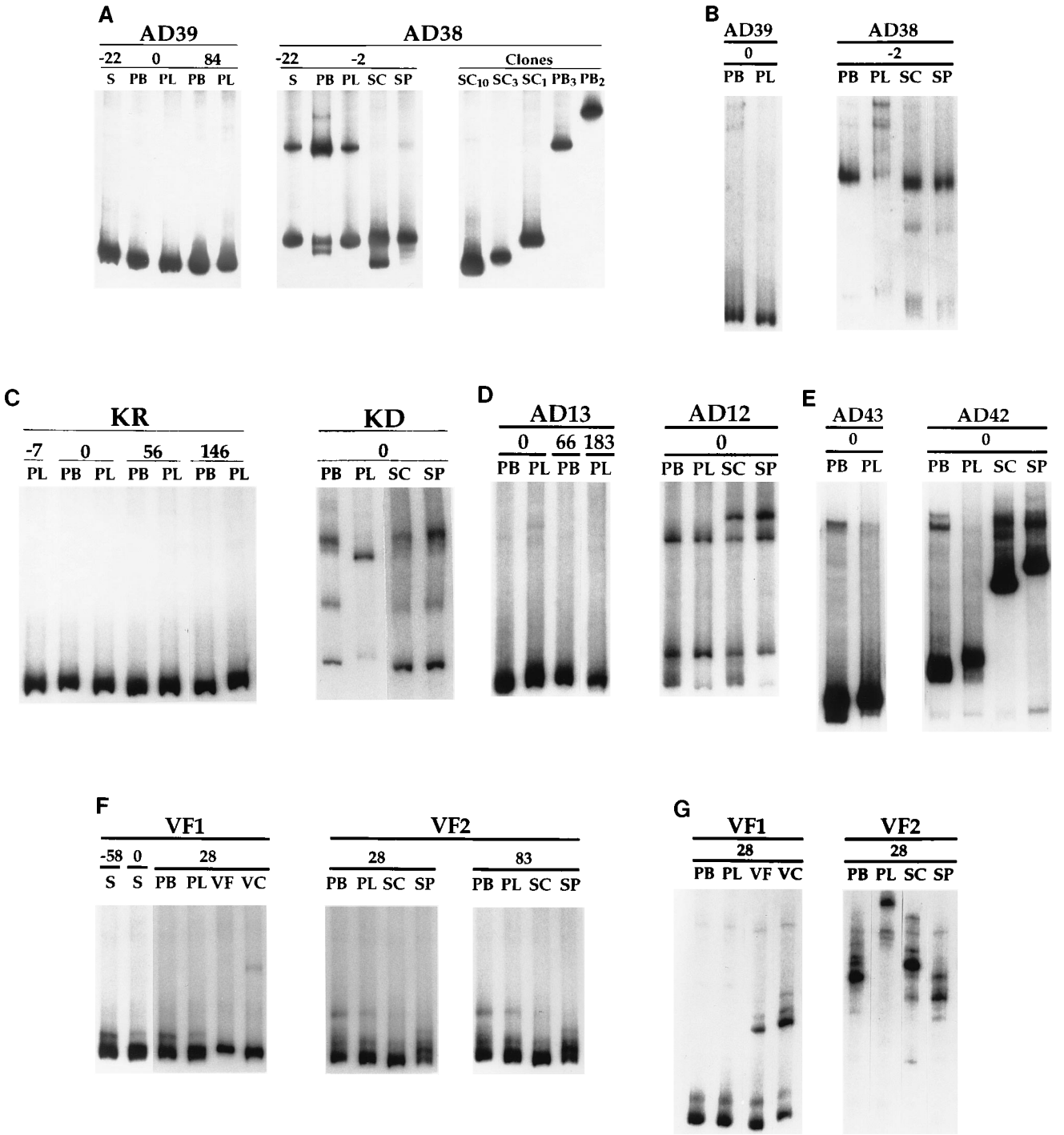


FIG. 2. Genetic variation of HIV-1 quasispecies following sexual transmission, as determined by the QHTA. A ^{32}P-labeled probe generated from a recipient PBMC clone was separately mixed with corresponding PCR products from patient samples (PB, PBMC; PL, plasma; SC, seminal cells; SP, seminal plasma; VC, vaginal cells; VF, vaginal fluid). Numbers above the samples denote the sampling day, with 0 indicating the day the sample first tested positive. (A) V3 sequences of AD38 (transmitter) and AD39 (recipient). (B) V1-V2 sequences of AD38 (transmitter) and AD39 (recipient). (C) V3 sequences of KD (transmitter) and KR (recipient). (D) V3 sequences of AD12 (transmitter) and AD13 (recipient). (E) V3 sequences of AD42 (transmitter) and AD43 (recipient). (F) V3 sequences of VF2 (transmitter) and VF1 (recipient). (G) V1-V2 sequences of VF2 (transmitter) and VF1 (recipient).

recipient's samples. In contrast, multiple bands with varying distances and densities were observed in all of the donor's samples (AD38). Transmitted viruses (homoduplex band) were most evident in donor seminal cells.

The clones corresponding to different bands were also sequenced (Fig. 3). Deduced amino acid sequences from all clones were aligned with the consensus sequence from the donor. Highly homogeneous sequences (0 to 0.3% nucleotide distances) were found in the recipient's PBMC and plasma, while heterogeneous sequences (0.7 to 5.4% nucleotide dis-

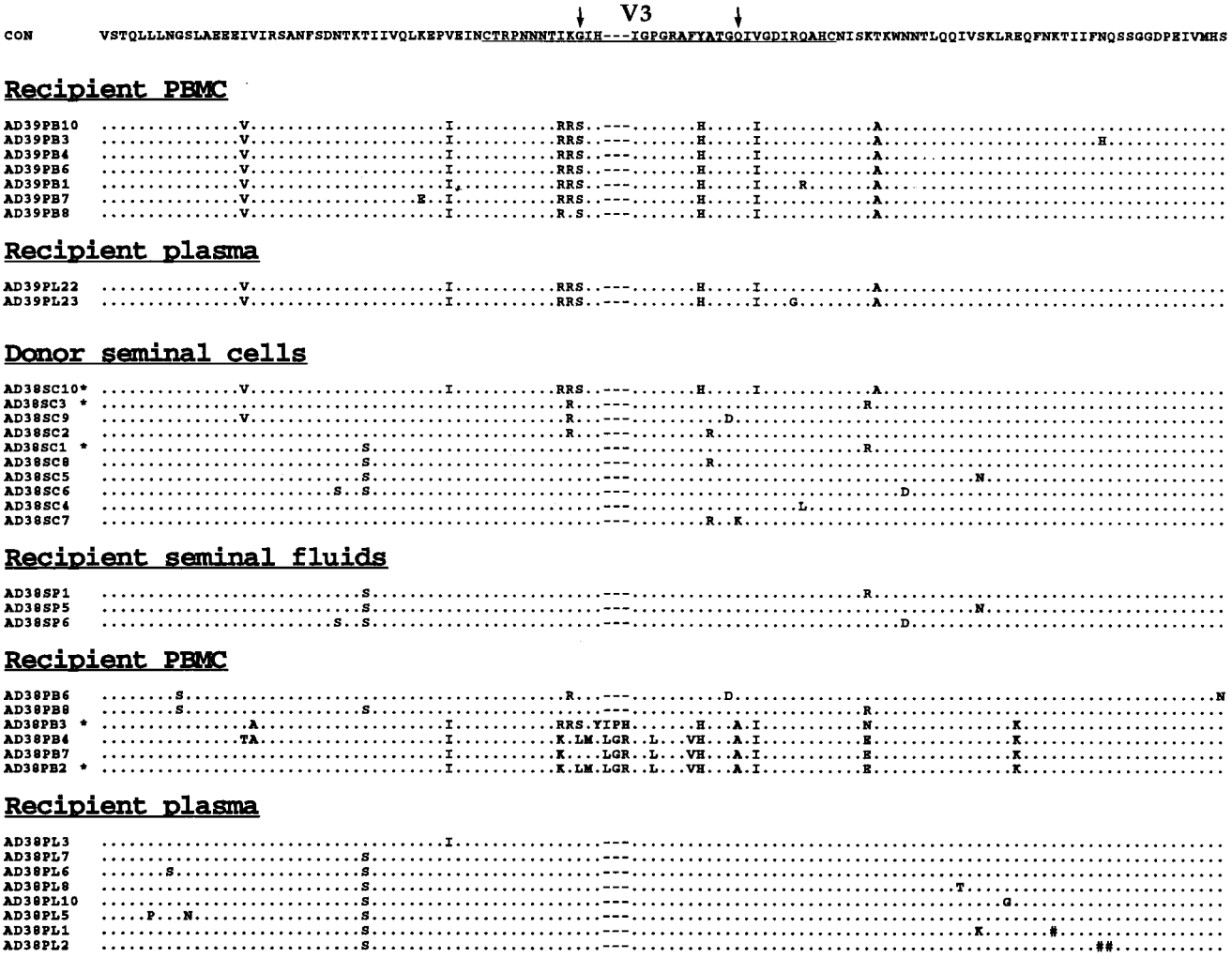


FIG. 3. Comparison of amino acid sequences of the V3 region from a recipient (AD39) and his corresponding sexual partner (AD38). The clones randomly detected from each sample were sequenced. All sequences are aligned with the transmitter's consensus sequence. AD39PB and AD39PL, PBMC and plasma, respectively, from seroconvertor AD39; AD38SC, SP, PB, and PL, seminal cells, seminal plasma, PBMC, and plasma, respectively, from transmitter AD38. Dots indicate sequence identity, dashes indicate deletion, and pound signs indicate stop codons. Clones marked with an asterisk are shown on the QHTA gel in Fig. 2A. The downward-pointing arrows indicate residue positions predicting SI and NSI phenotypes.

tances without calculating deletions or insertions) were present in the donor. The transmitted virus was found only in the donor's seminal cells (clone AD38SC10). On the basis of the good correlation between a positive charge at gp120 residues 306 and 323 (Fig. 3) and the SI property in culture (13, 16), the phenotypes of the recipient's HIV-1 variants were deduced to be NSI, while the donor's untransmitted HIV-1 included both potential SI variants (AD38SC7) and multiple populations of NSI variants. These results suggest that, in this case, HIV-1 infection was initiated by only one of the donor's several NSI variants. When V1-V2 sequences from this transmission pair were subjected to the QHTA (Fig. 2B), again, one homogeneous population of HIV-1 was observed in samples from the recipient. In contrast, multiple quasispecies were detected in the donor's samples and the transmitted virus was most evident in the donor's seminal cells. These findings suggest that, in this case, HIV-1 transmission may have been mediated by viruses produced in the donor's seminal cells.

We then used the identical strategy to compare V3 sequences from the three other homosexual pairs. The same pattern obtained with the V1-V2 sequences was observed for the second and third homosexual transmission pairs in that only one HIV-1 population was detected in recipient samples while multiple HIV-1 variants were present in all donor samples (Fig. 2C and D). In the fourth homosexual transmission case (Fig. 2E), however, more than one HIV-1 sequence population was observed in both the recipient's PBMC and plasma. Once again, the transmitted viruses corresponded to minor variants present in the donor samples obtained at seroconversion.

Overall genetic composition of HIV-1 quasispecies following heterosexual transmission. In a heterosexual transmission case (Table 1), we were able to amplify HIV-1 gp120 sequences directly from the vaginal cells, vaginal fluid, PBMC, plasma, and serum of the newly infected recipient (VF1) and from the PBMC, plasma, seminal cells, and seminal plasma of the corresponding transmitter (VF2). A ^{32}P-labeled single-strand probe generated from a molecular clone of the recipient's PBMC was separately mixed with the corresponding bulk PCR products from both recipient and donor samples. As shown in Fig. 2F, two related V3 sequence populations of HIV-1 found in recipient samples made up the majority of the sequences in

all donor samples. A distinct heteroduplex band was also observed in the recipient's vaginal cells. We then analyzed the V1-V2 sequences from this case (Fig. 2G). In contrast to the V3 sequences, the major HIV-1 V1-V2 sequences found in the recipient samples were minor variants in all donor samples. The difference between HIV-1 in recipient vaginal secretions and donor samples suggests that the selection of certain viral variants occurs at entry or that blood and mucosa compartmentalize the virus after HIV-1 dissemination.

Compartmentalization of HIV-1 in the donor. As shown in Fig. 2, HIV-1 sequence populations within each donor varied from one compartment to another. For example (Fig. 2A), in patient AD38's PBMC, the major V3 sequences detected were clone PB3-like variants that coexisted with minor populations of PB2-, SC1-, and SC3-like sequences, but in his plasma the major V3 sequences were SC1-like variants coexisting with PB3-like populations and much fewer PB2- and SC3-like sequences. Figure 2A makes it especially clear that variants in the blood differed from those in genital secretions and, within the latter, some viruses in seminal cells differed from those in seminal plasma. Marked HIV-1 compartmentalization was also observed in the other sexual transmitters, with cell-associated viruses in PBMC differing from cell-free viruses in plasma and cell-associated viruses in seminal cells differing from virions in seminal plasma (Fig. 2).

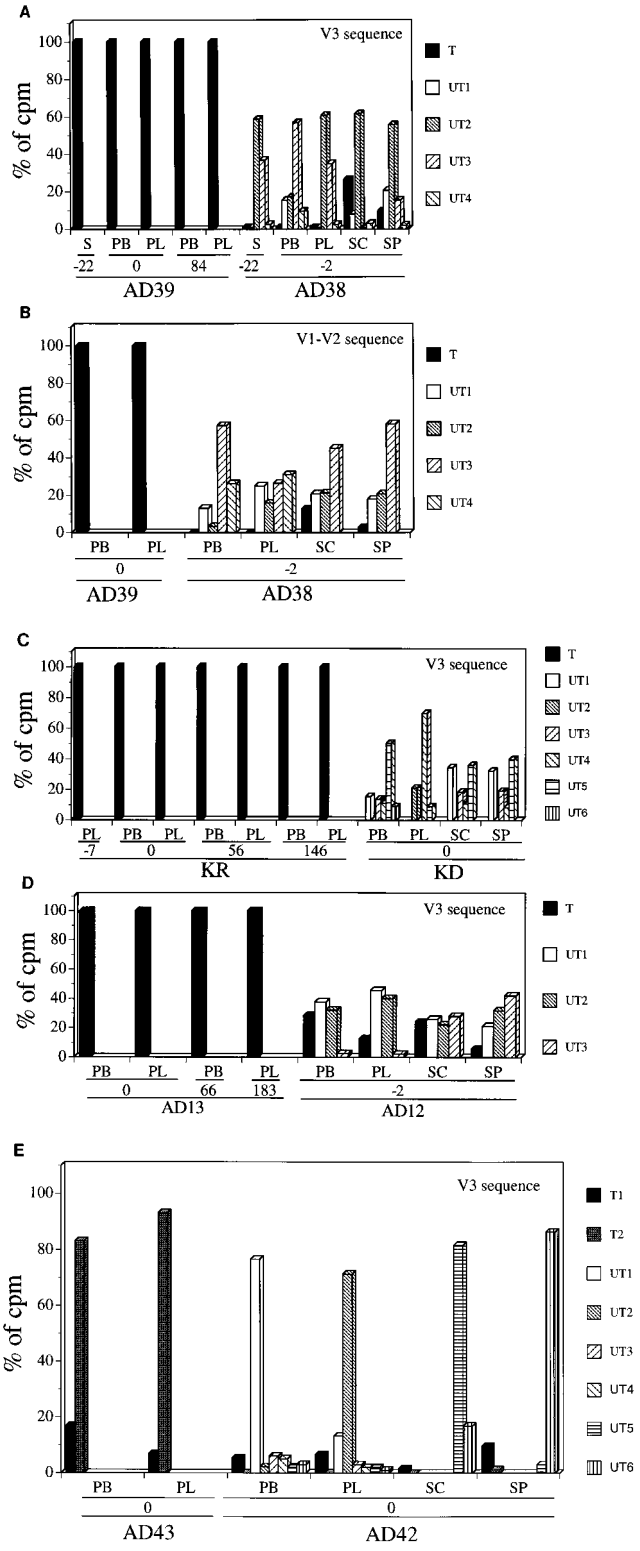
Longitudinal studies of HIV-1 genetic diversity following sexual transmission. It has been estimated that the interval between the initial HIV-1 infection and the first appearance of the HIV-1 antibody is 1 to 3 months (12, 19). In this study, we included longitudinal samples ranging from as early as 59 days preseroconversion from one recipient to as late as 183 days postseroconversion from another; in the case of donors, with reference to the date of the recipient's seroconversion, we included samples from 22 days preseroconversion from one transmitter to 83 days postseroconversion from another. Some samples were collected as early as 2 weeks after the presumed day of sexual transmission. In one homosexual transmission case (subjects AD38 and AD39) (Table 1 and Fig. 2A), multiple samples were obtained from both the donor and recipient at 14 days (day −22, before seroconversion) and from the recipient at 36 days (day 0, at seroconversion) and 120 days (day 84, after seroconversion) after the estimated time of transmission. That only one homoduplex was found in the recipient's sequential samples suggests that HIV-1 V3 sequences in this patient were homogeneous for more than 106 days during acute infection pre- and postseroconversion. HIV-1 genotypes in the donor's serum on day −22 were essentially the same as those in the donor's plasma on day −2. One homoduplex of a V3 sequence was also observed for 153 days (from 7 days preseroconversion to 146 days postseroconversion) and 183 days (from day 0 to day 183 postseroconversion) in newly infected recipients KR and AD13, respectively. In the heterosexual transmission case (Fig. 2F), HIV-1 V3 sequence populations in the recipient's (VF1) serum 58 days before seroconversion were essentially the same as those in her serum at seroconversion (day 0) and those in her plasma and PBMC 28 days after seroconversion. Thus, evidence from this study shows that homogeneous V3 sequences can persist for more than 6 months in the plasma and PBMC of the acute seroconvertor.

QHTA detection of transmitted HIV-1 following sexual transmission. Because QHTA can measure the proportion of a particular sequence within a given sample, we used this technique to quantitatively analyze the transmitted viruses in samples from the transmission pairs. Each band on the radiograph was quantified and expressed as a percentage of the total signal in that lane. Results are shown in Fig. 4, in which each column represents the intensity of a band within a sample. In the first homosexual transmission case (Fig. 4A), 27 and 5% of transmitted V3 sequences were found in donor AD38's seminal cells and seminal plasma, respectively, and <0.5% was found in his serum, PBMC, and plasma. Comparable results for this pair of subjects occurred with their V1-V2 sequences: 13 and 3% of the transmitted viruses were measured in donor seminal cells and seminal plasma, respectively, and <0.5% was measured in donor blood (Fig. 4B). A similar pattern was observed in the third homosexual transmission case, in which V3 sequences of transmitted viruses were more evident in seminal cells (24%) than in seminal plasma (5%) (Fig. 4D). In the second homosexual transmission case, however, transmitted V3 sequences were present at a frequency of <0.5% in all donor samples (Fig. 4C). Although in the fourth homosexual transmission case (Fig. 4E) two V3 sequence populations were found in the seroconvertor (AD43), only one transmitted minor variant was detected in all donor samples (5 to 10%), and it was most evident in donor seminal plasma (10%). In the heterosexual transmission case, the two related V3 sequences of transmitted viruses were the major variants (up to 59%) in all donor (VF2) samples and were more evident in seminal cells than in seminal plasma (Fig. 4F). The percentage of transmitted variants in day 28 samples was essentially the same as that in the corresponding day 83 samples. In contrast to V3 sequences, multiple V1-V2 sequences of transmitted viruses from this case were the minor variants (down to 0.6%) in donor samples (Fig. 4G). Two of the major transmitted variants found in vaginal secretions were not detectable in the recipient's blood. Thus, using QHTA we found that either V3 or V1-V2 sequences of transmitted viruses were minor variants (from <0.5 to 27%) in samples from the corresponding transmitters. In the donors, transmitted viruses were more frequently detected in the semen than in the blood and more evident in seminal cells (3 of 5 subjects) than in seminal plasma (1 of 5 subjects).

DISCUSSION

The high variability of HIV-1 sequences presents a serious challenge to currently used PCR sequencing assays, in which the limited numbers of clonal sequences obtained are unlikely to guarantee a quantitative description of the proportion of specific sequences. On the other hand, HTA provides a simple screening tool to rapidly detect the sequence complexities of HIV-1 quasispecies (14, 15). We here demonstrate that a strategy combining QHTA (a modification of HTA) and selective sequencing can quantitatively describe the overall genetic populations and distances of HIV-1 quasispecies present during sexual transmission. This approach should be useful in quantitatively evaluating HIV-1 variants in vivo, in investigating HIV-1 genetic changes during the course of infection, and in identifying cases of infection by multiple strains of HIV-1 (58).

Previous sexual transmission studies (54, 55, 57) have restricted HIV-1 sequence analysis to serum or blood samples at or after seroconversion. However, in sexual transmission, it is the HIV-1 in the transmitter's genital secretions that is directly responsible for the infection of the new host. In this study, using QHTA and selective sequencing, we compared HIV-1 gp120 sequences in the serum, plasma, and PBMC (and in a heterosexual transmission case, in vaginal fluid and cells) of five seroconvertors with the serum, plasma, PBMC, seminal plasma, and seminal cells of the five corresponding transmitters. We found evidence for HIV-1 compartmentalization in chronically infected transmitters. Viral variants found in sam-



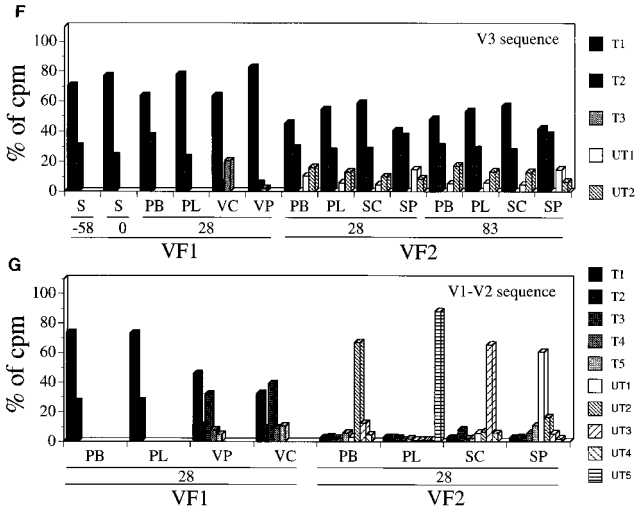


FIG. 4. Quantification of transmitted viruses following sexual transmission. Each column represents the percentage (cpm) of one band within a sample on the gel. (A) V3 sequences of AD38 (transmitter) and AD39 (recipient). (B) V1-V2 sequences of AD38 (transmitter) and AD39 (recipient). (C) V3 sequences of KD (transmitter) and KR (recipient). (D) V3 sequences of AD12 (transmitter) and AD13 (recipient). (E) V3 sequences of AD42 (transmitter) and AD43 (recipient). (F) V3 sequences of VF2 (transmitter) and VF1 (recipient). (G) V1-V2 sequences of VF2 (transmitter) and VF1 (recipient). T, transmitted virus; UT, untransmitted virus.

ples from seroconvertors were relatively homogeneous, but those found in samples (including semen) from the corresponding donors were heterogeneous. Moreover, the recipient viruses were minor variants (from <0.5 to 27%) of those in donor seminal secretions and blood. Also, in the heterosexual transmission case, we observed that the HIV-1 quasispecies present in the transmitter's semen differed from those in the recipient's blood, which in turn differed from those in her vaginal secretions.

Because HIV-1 has a short half-life and undergoes rapid turnover in vivo (17, 50), the possibility remains that the HIV-1 genetic discrepancy observed in different specimens around the time of seroconversion could be caused by HIV-1 evolution in vivo from the period of transmission to sampling. The relative homogeneity of recipient HIV-1 at seroconversion could then be due to the outgrowth of only one viral variant during the period from transmission to seroconversion. To address this issue, we analyzed HIV-1 longitudinally from four of the five sexual transmission pairs. In one pair, samples were even obtained from both the recipient (AD39) and the donor (AD38) as early as 14 days after the presumed time of sexual transmission (Table 1). Moreover, our finding that HIV-1 V3 sequences in the blood of newly infected recipients were largely homogeneous during the early period of more than 6 months suggests that no selective outgrowth of HIV-1 occurred before seroconversion in either the PBMC or plasma of these subjects. Furthermore, HIV-1 V3 sequences in each of the transmitters' compartments were relatively stable for up to 2 months. Thus, it is unlikely that HIV-1 genotypes from the study samples would differ substantially from viruses present at the time of transmission because of HIV-1 evolution in vivo in the donors and recipients. Indeed, it is likely that our findings provide a reasonable reflection of the genetic compositions of HIV-1 at the time of sexual transmission and during the period of acute infection.

The results of this study enable us to propose a multiple-step process of HIV-1 sexual transmission. First, HIV-1 compartmentalization occurs in the donor as the virus disseminates from blood to genital secretions, evident in all five of the study's sexual transmission cases. Other researchers have also shown HIV-1 genetic differences in the PBMC and seminal cells of two AIDS patients but not in one asymptomatic HIV-1-infected individual (31). That the transmitted virus was

found in the donor's (AD38) semen but not his blood (Fig. 2A) suggests that this compartmentalization is significant in HIV-1 sexual transmission. Second, HIV-1 penetration occurs as the virus passes from donor to host. Some variants in the transmitter's semen may have an advantage in infecting cells at entry, which can then disseminate the virus in the new host. Third, HIV-1 amplification occurs in the host as the virus spreads systemically from the mucosa. Some strains, because of their biological properties, may replicate so efficiently that they become the dominant variants in blood. The consistent finding that the transmitted virus represents only a minor variant in the semen of the transmitter suggests that viral selection operates on the second or third step or both.

In an intramuscular injection case, Cornelissen et al. (11) found evidence in blood samples for a selective amplification of SI viruses before seroconversion and a replacement by NSI viruses at seroconversion, suggesting that HIV-1 selection can occur in blood after transmission. However, there was no evidence for selection of this kind in another case examined by Cornelissen et al. (11) or in our study, indicating that such selection does not always occur and has not yet been observed in cases of sexual transmission.

The relative homogeneity of the phenotypes and genotypes of recipient HIV-1 is therefore most likely to be due to selection by the virus during sexual transmission. While most recipients possess relatively homogeneous HIV-1 (53–55, 57), others, because of various influences at different selection stages, may have several variants, most of which will be highly related (27). Some recipients may occasionally be coinfected with distinct HIV-1 subtype B viruses (58) or with viruses of different subtypes (2). However, even in a patient coinfected with multiple distinct HIV-1 subtype B viruses, selection during sexual transmission was suggested by the low level of sequence diversity observed within each group (58). Interestingly, less evidence for selection was found in experimental simian immunodeficiency virus sexual transmission in macaques (7, 47) than in natural HIV-1 sexual transmission in humans (53–55, 57). Further studies are needed to resolve this apparent discrepancy.

As predicted from the V3 sequences (Fig. 3), one transmitter (AD38) had an SI variant and multiple NSI variants, but only one of his multiple NSI variants was detected in the recipient (AD39). Other studies have also provided evidence that the transmitted virus was one of the multiple NSI variants found in the corresponding transmitter (48, 57). Taken together, these findings suggest that in sexual transmission, some, but not all, NSI viruses are selected to establish infection.

Observations that CD4-negative epithelial cells are efficiently infected by direct contact with HIV-infected cells in culture (46), that conjugates of dendritic cells and T cells facilitate productive HIV-1 infection (40), and that HIV-1 is present in vivo in epithelial cells (31) and seminal cells (3, 43) suggest a potential cell-associated mechanism for mucosal transmission. It has been hypothesized that productively infected epithelial cells could subsequently produce viruses that would infect macrophages and $CD4^+$ lymphocytes in the connective tissue below the epithelium (46). The extent to which HIV-1 is transmitted by cell-free virions or HIV-1-infected cells may be influenced by the relative amounts of each. That the transmitted viruses were more evident in either the seminal cells (three of five cases) or the seminal plasma (one of five cases) of the transmitters suggests that both cell-associated and cell-free viruses can be transmitted, but the former may be slightly more often involved in HIV-1 sexual transmission. However, interestingly, experimental mucosal inoculations of macaques with infected cells have not resulted in the infection of the animals (29a).

In conclusion, this study demonstrates that HIV-1 selection can occur during sexual transmission. There is evidence for compartmentalization from blood to semen in the transmitter, as well as for the selection of HIV-1 in the new host, either at the level of penetration or amplification or both. These findings may have important implications for, among other pursuits, developing an AIDS vaccine, because a successful vaccine strategy must first carefully consider the selective mechanism of HIV-1 transmission and the properties of transmitted viruses.

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