Dual Human Immunodeficiency Virus Type 1 Infection and Recombination in a Dually Exposed Transfusion Recipient

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We studied a case in which a 2-month-old premature infant was concurrently transfused with packed erythrocytes from two different human immunodeficiency virus type 1 (HIV-1)-seropositive donors in late 1984. The two donors also each singly infected a second infant. Inspection of sequences from portions of the HIV-1 genomes in each of the two donors showed a close relationship to the strain in their respective singly exposed recipients. Inspection of sequences from the dually exposed recipient provided evidence of an individual simultaneously infected with two distinct HIV-1 strains, as well as recombination of the two strains in vivo.

The generation of diversity during lentivirus replication is thought to play a major role in the pathogenesis of virus-induced disease. Individuals infected with human immunodeficiency virus type 1 (HIV-1) have been shown to harbor a large number of genetically related viral genomes, making up what has been called a quasispecies (13). Variants with enhanced host range and in vitro cytopathicity are apparently spawned in HIV-1-infected individuals over time and may contribute to onset of AIDS (3, 6, 26). Furthermore, this ongoing generation of variants within an individual over time may enable HIV both to overcome the immune response and to develop resistance to antiviral agents (1, 2, 19, 28). At the population level, rapid viral evolution is of concern with respect to long-term prospects for vaccine-induced immunity.

Both a mutation-prone reverse transcriptase and subsequent recombination between differing genomes are thought to contribute to the ability of lentiviruses to generate viral diversity. Mutations in reverse transcription have been demonstrated to occur in vivo in HIV-1-infected individuals (5, 17), and HIV-1 recombination has been well documented in cell culture, in which experimental manipulation can achieve dual infection by two defined parental strains differing at linked genetic loci (7, 25, 27).

A situation in which an infant in the United States was transfused in 1984 with 2 units of HIV-1-seropositive blood provided an analogous in vivo opportunity to look for dual infection and recombination between two defined parental genomes. We document both dual infection by and recombination between the two different HIV-1 subtype B strains that were detected in the two donors and that were transmitted to the transfusion recipient.

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MATERIALS AND METHODS

Patient specimens. Whole blood was donated for transfusion by donor 1 (D1) on 15 October 1984 and by donor 2 (D2) on 19 September 1984. The packed erythrocytes from each of these two donations were divided into aliquots for multiple transfusion to premature infants. Recipient 1 (R1) received an aliquot of erythrocytes from D1 on 19 October 1984 at age 3.5 weeks. Recipient 2 (R2) was given erythrocytes from D2 on 24 September 1984 at 2 months of age. The dual recipient (DR), an infant aged 2 months, was transfused with erythrocytes from both D1 and D2 on 19 October 1984. When R1, R2, and DR were identified in March 1986 as having been transfused with HIV-1-seropositive blood, their parents were contacted, the children were examined, and blood samples were obtained as part of the Transfusion Safety Study (10). R1 had slow weight gain; R2 developed meningitis (type unknown) in 1985 and had lymphadenopathy on study entry in 1986. DR had marked lymphadenopathy on entry.

For the study presented here, specimens of frozen plasma from the recipients (from 1986) and frozen serum from the donated units (from 1984) were obtained from the Transfusion Safety Study Repositories (10). Five specimens represented the two donors (D1 and D2), the singly exposed recipients of each (R1 and R2), and the dually exposed recipient (DR). All except the DR sample were sent under code. As controls, the Transfusion Safety Study coordinating center sent plasma or serum from another infected donor, his infected recipient, an infected recipient of another donor, and an uninfected recipient.

RNA extraction and cDNA synthesis. RNAs were extracted from frozen serum or plasma with the guanidine thiocyanate-RNA Matrix bead adsorption kit (RNaid Plus kit; Bio-101, La Jolla, Calif.) (20). cDNAs were synthesized at 42°C for 1 h with 5 U of avian myeloblastosis virus reverse transcriptase (InVitrogen, La Jolla, Calif.) and 1 μg of random hexamer primer (Boehringer Mannheim, Indianapolis, Ind.) in 20 μl of 100 mM Tris (pH 8.3)–40 mM KCl–10 mM MgCl$_2$–2 mM dithiothreitol containing 10 U of RNase inhibitor (Boehringer Mannheim). RNA-cDNA hybrids were denatured at 95°C for 3 min, and the sample was chilled on ice.

PCR amplification. cDNA preparations were serially diluted so that when used as targets for nested PCR, fewer than one in five reactions yielded PCR product. This technique of end point PCR was used so that PCR product would for the most part be derived from targets consisting of only single molecules of cDNA (23). cDNA molecules were amplified either for a region encompassing exon 1 of *tat* or for various portions of the *env* gene. PCR was performed in 10 mM Tris (pH 8.3)–50 mM KCl–200 μM each deoxynucleoside triphosphate–0.2 μM each primer–2.5 U of *Taq* polymerase (Perkin-Elmer) per 100-μl reaction mix. Nested *tat* amplification was performed with primers and conditions detailed in reference 17, except that the second-round inner primers were modified (to contain uracil instead of thymidine and to have additional nucleotides at the 5′ ends) for subsequent cloning with the CloneAmp system (GIBCO BRL, Gaithersburg, Md.). For samples from DR, R1, and R2, the V3-through-V5 region of *env* was amplified with nested primers (22) that had the map positions in the HIV HXB2 clone indicated in parentheses: TACAATGTACACATG GAATT (sense, positions 6957 to 6976), GCAGTCTAGCAGAAGAAGA (sense, positions 7009 to 7027), CTTCTCCAATTGTCCCTCATA (antisense, positions 7644 to 7665), and CGCCATAGTGCTTCCTGCTGCT (antisense, positions 7792 to 7814). The second-round inner primers were modified as above for subsequent cloning with the CloneAmp system. Thermal cycling conditions

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             -----------C3-----------||-----------------------V4---------------------||-----------------------------------------------C4----------

R1_2     T....................................GG...G.....--....GG..CA.A.A.C.CTGT..GA..T.AC..C.....G.....A...........................................
R1_158   T....................................GG...G.....--....GG..CA.A.A.C.CTGT..GA..T..C.A....G.....A...........................................
R1_180   T....................................GG...GA....--....GG..CA....A.C.CTGT..GA..T..C.A....G.....A................................G..........
R1_124   T....................................GG...G.....--....GG..CA.A.A.C.CTGT..GA..TA.C.A....G.....A...........................................
R1_151   T....................................GG...G.....--....GG..CA.A.A.C.CTGT..GA..T..C.A....G.....A.....C.....................................
R1_157   T....................................GG...G.....--....GG..CA.A.A.C.CTGT..GA..T..C.A....G.....A...........................................
R1_162   T....................................GG...G.....--....GG..CA.A.A.C.CTGT..GA..T..C.A....G.....A...........................................
R1_164   T....................................GG...G.....--....GG..CA.A.A.C.CTGT..GA..T.AC..A....G.....A...........................................
R1_56    T....................................GG...G.....--....GG..CA.A.A.C.CTGT..GA..T.AC..A....G.....A...........................................

DR21     T....................................GG...G.....--....GG..CA.A.ACC.CTGT...A.TG.AC..A....G.....A...........................................
DR23     T....................................GG...G......--.A..GG..CA.G.ACC.CTGT..GA.GT.AC..A....G.....A...........................................
DR88     T....................................GG...G.....--....GG..CA.A.A...CTGT..GA..T.AC..A....G....AC............................................
DR68     T....................................GG...G.....--....GG..CA.A.A...CTGT..GA..T..C.A....G.....A...........................................
DR41     T....................................GG...G.....--.A..GG..CA.A.ACC.CTGT..GA.GT.AC..A....G.....A...........................................
DR8      T....................................GG...GC....--.A..GG..CA.A.ACC.CTGT...A.TG.AC..A....G.....A...........................................
DR95     T....................................GG...G.....--A...GG..CA.A.ACC.CTGT..GA..T..C.A....G.....A..............................C.............
DR62     T....................................GG...G.....--....GG..CA.A.A...CTGT..GA..T..C.A....G.....A..............................C.............
DR94     T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T.....A....G.....A...........................................
DR252    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T.AC..A....G.....A...........................................
DR232    T....................................GG...G.....--....GG..CA.A.ACC.CTGT..GA..T..C.A....G.....A...........................................
DR240    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T..C.A....G.....A...........................................
DR250    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T..C.A....G.....A.................................C..........
DR272    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T..C.A....G.....A.................................C..........
DR280    T....................................GG...G.C...--....GG..CA.A.A....CTGT...A..T.....A....G.....A...........................................
DR297    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T.AC..A....G.....A...........................................
DR228    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T.....A....G.....A...........................................
DR241    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T.....A....G.....A...........................................
DR259    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T.....A....G.....A...........................................
DR293    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA..T.....A....G.....A...........................................

D1_29    T....................................GG...G.....--....GG..CA.A.A.C.CTGT.AGA..T..C.A....G.....A...........................................
D1_19    T....................................GG...G.....--....GG..CA.A.A....CTGT..GA.....C.A....G.....A...........................................
                                                                                                                           *
DR106    ACAACACAACTGTTTAATAGTACTTTTAATATTACTAGGAATAGT--ACTGAAAGGTCAGATAACGGAACTATCACACTCCCCTGCAGAATAAAACAAATTATAAACATGTGGCAGGAAGTAGGAAAAGCAA
                     **  *    *     ** **   *   **** **    *  *    *    *
D2_64    .....................................G..G............................A.........................................C...........................
D2_40    .................A...................G..G............................A.........................................C...........................
D2_22    .....................................G..G............................A.........................................C...........................

R2_74    ...................................C...T....G...........T........A.........T...........................C...........................
R2_43    ...................................C...T....G..........TC........A.........................................C..........................C
R2_55    ...................................C...T....G..........TC........A.........................................C...........................
R2_29    ...................................C...T....G..........TC........A.........................................C...........................
R2_106   ................C..................C...T....G..........TC........A.........................................C...........................
R2_114   ...................................C...T....G..........TC........A.........................................C...........................
R2_107   ...................................C...T....G..........TC........A.........................................C...........................
R2_5     ...................................C...T....G..........T.T.......A.........................................C...........................
R2_82    ...................................C...T....G..........TC........A.........................................C...........................
R2_66    ...................................C...T....G..........TC........A.........................................C...........................
R2_56    ...................................C...T....G..........TC........A.........................................C...........................


             --------------------------------C4------------------------------------||-----------V5-----------||-------------C5---

R1_2     ..............G.....................................C....................G....G.................C...................
R1_158   ....................................................C....................GG......................C...................
R1_180   ..............G.....................................C....................GG......................C...................
R1_124   ...................................................................A..G...........................C...................
R1_151   ..........T.................C.......................C....................G....G.................C...................
R1_157   ....................................................C....................GG......................C...................
R1_162   ..............G.....................................C....................G....G.................C...................
R1_164   ...................................C................C.....................A..G.................C.......G..........
R1_56    ..........T.................C.......................C....................G....G.................C...................

DR21     .........................................................................T.........................................
DR23     ................................................................................................................
DR88     ..........................................................................T.......................................
DR68     ..........................................................................T.......................................
DR41     ................................................................................................................
DR8      ...............................A................................................................................
DR95     ...........................................................................................C....................
DR62     ................................................................................................................
DR94     .................................C..A...C..................................T.......C.............................
DR252    .........................................................................T.......................................
DR232    ................................................................................................................
DR240    ....C...........................................................................................................
DR250    ................................................................................................................
DR272    .........................................................................T.......................................
DR280    ................................................................................................................
DR297    .........................................................................T.......................................
DR228    ..................................C.....C...............................C.....................................
DR241    ..................................C.....C...............................C.....................................
DR259    ..................................C.....C...............................C.....................................
DR293    ..................................C.....C...............................C.....................................

D1_29    .....................................................................................A..C.....................
D1_19    ...............................................................................G.........C....................
                                           *                                  **  *  *  *   .        *
DR106    TGTATGCCCCTCCCATCAGAGGACAAAATTAAATGTTCATCAAATATTACAGGGCTGCTATTAACAAGAGATGGTGGTAATAGTGAGAACAACGAGACTGAGGTCTTCAGACCTGGAGGAGGAGA
D2_64    ........................G...G...................................GG..A.A.C.CG........C...A.........................
D2_40    ........................G...G...................................GG..A.A.CG.G........C...A.........................
D2_22    ........................G...G...................................GG..A.A.CG.G........C...A....AC...................

R2_74    ........................G...G....C..............................GG..A.A.C.CG------..C...A.........................
R2_43    ........................G...G...................................GG..A.A.C.CG-----..C...A.........................
R2_55    ........................G...G...................................GG..A.A.C.CG-----..C...A.........................
R2_29    ........................G...G...................................GG..A.A.C.CG------..C...A.........................
R2_106   ........................G...G...................................GG..A.A.C.CG------..C...A.........................
R2_114   ........................G...G...........................G.......T.GG..A.A.C.CG-----..C...A...............A........
R2_107   ........................G...G.............................A...GG..A.A.C.CG------.TC...A.........................
R2_5     ........................G...G...................................GG..A.A.C.CG------..C...A.........................
R2_82    ........................G...G...................................GG.GA.A.C.CG------..C...A.........................
R2_66    ........................G.......................................GG..A.A.C.CG------..C...A.........................
R2_56    ........................G...G...................................GG..A.A.C.CG------..C...A.........................
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FIG. 1. Alignment of 46 sequences over 266 nucleotide positions from the V4/V5 region of *env* (HXB2 positions 7382 to 7644). Symbols: ·, identical nucleotide at a given position, with DR106 as the reference sequence; –, gap inserted to preserve alignment. All 21 genomes sequenced from DR resemble D1 and R1 genomes in the 3′ portion of the alignment (V5), but only 20 of the 21 resemble D1 and R1 in the 5′ portion of the alignment (V4). DR106 shows a V4 region related to D2 and R2 genomes, indicating that it is derived from a recombination event between the V4 region of a D2-derived genome and the V5 region of a D1-derived genome. Asterisks mark positions at which the nucleotide in DR106 is found in only one of the D1 + R1 or D2 + R2 sequence sets, indicating the likely source of that nucleotide for DR106.

for the V3-through-V5 region were 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 35 cycles, with a final extension at 72°C for 10 min. For samples from D1 and D2, the V3 and V4/V5 regions of *env* were amplified separately rather than as continuous molecules stretching from V3 through V5. V3 nested primers of Wolfs et al. (28) were modified as above for subsequent cloning with the CloneAmp system. V4/V5 nested primers of Zhang et al. (29) were used unmodified. Thermal cycling conditions were as described for V3 through V5.

Sequencing. *tat*, *env*-V3, and *env*-V3/V5 PCR products were annealed with the pAMP vector of the CloneAmp system and used to transform Subcloning Efficiency DH5alpha frozen competent cells (GIBCO BRL). Plasmid DNA minipreparations from individual bacterial colonies were prepared on Qiagen-tip 20 columns (Qiagen, Chatsworth, Calif.). Sequencing was performed with Sequenase V2.0 (U.S. Biochemical, Cleveland, Ohio). *env*-V4/V5 PCR products were sequenced without cloning as follows. Single-stranded PCR product was generated from 5 µl of nested PCR product in an additional 45-cycle asymmetric PCR with the second-round 3′ primer. Single-stranded DNA from this third PCR round was purified by ethanol precipitation and sequenced with the second-round 5′ primer in the dideoxy-chain termination procedure.

Heteroduplex formation and analysis. For the heteroduplex studies, a representative clone of V3-through-V5 PCR product from the three recipients (clones R1-2, R2-5, and DR297) was chosen at random, as well as the putative recombinant V3-through-V5 clone from DR (DR106). Heteroduplex formation and polyacrylamide gel electrophoresis were performed as described previously (9). Briefly, appropriate mixtures of the V3-through-V5 PCR products mentioned above were mixed, denatured at 94°C, and reannealed at 4°C. Homoduplexes

and heteroduplexes were separated by electrophoresis in 5% polyacrylamide gels and stained with ethidium bromide. The mobility of the heteroduplexes formed between divergent molecules is reduced relative to that of the fully complementary homoduplex molecules. The samples from R1 and R2 were also analyzed against known prototypes of subtypes A, B, C, D, E, and F for the purpose of subtype classification, as described by Delwart et al. (9).

Phylogenetic analysis. The D1, D2, R1, R2, and DR V4/V5 sequences resulted in an alignment of 266 nucleotide positions; the alignment was performed by hand to maximize the number of sites with nucleotide agreement between sequences and minimize the number of insertions and deletions. Two staggered gaps in the V4 region were inserted because of an apparent set of compensating frameshift mutations. Because the R2 sequences are 6 bases shorter than all other sequences, gaps were inserted into the R2 sequences so as to maximize the agreement between the R2 sequences and the epidemiologically linked D2 sequences. Alignment of the predicted protein sequences indicates the possibility of additional offsetting insertions and deletions in the V5 region (alternative alignment not shown); as discussed below, this alternative leads to nearly identical results.

RESULTS

On the basis of visual comparisons of the sequence data from the DR sample and the donor, recipient, and control samples in the coded panel, we were able to identify the ap-

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           ---C3---||------V4-----||--------------------C4----------------------||---V5----||--C5--
R1_2    S.......W.V.EG.NNTVG.D.....................................V.....................K.G.............
R1_158  S.......W.V.EG.NNTVG.............................................................KG.............
R1_180  S.......W.D.EG..NTVG.......................................V.....................KG.............
R1_124  S.......W.V.EG.NNTVG.S..........................................................NG.............
R1_151  S.......W.V.EG.NNTVG.........L..................................................K.G.............
R1_157  S.......W.V.EG.NNTVG............................................................KG.............
R1_162  S.......W.V.EG.NNTVG.......................................V.....................K.G.............
R1_164  S.......W.V.EG.NNTVG.D..........................................NG.......G.......
R1_56   S.......W.V.EG.NNTVG............................................................K.G.............

DR21    S.......W.V.EG.NTTVEMD...........................................................
DR23    S.......W.V.KG.STTVGSD...........................................................
DR88    S.......W.V.EG.NKTVG.D.....R....................................................
DR68    S.......W.V.EG.NKTVG............................................................
DR41    S.......W.V.KG.NTTVGSD..........................................................
DR8     S.......W.A.EG.NTTVEMD........................M.................................
DR95    S.......W.V.EG.NTTVG.........L..................................................
DR62    S.......W.V.EG.NKTVG.........L..................................................
DR94    S.......W.V.EG.NKTVG...............................I............................
DR252   S.......W.V.EG.NKTVG.D..........................................................
DR232   S.......W.V.EG.NTTVG............................................................
DR240   S.......W.V.EG.NKTVE............................................................
DR250   S.......W.V.EG.NKTVG.........L..................................................
DR272   S.......W.V.EG.NKTVG.........L..................................................
DR280   S.......W.V.EG.NKTVE............................................................
DR297   S.......W.V.EG.NKTVG.D..........................................................
DR228   S.......W.V.EG.NKTVG............................................................
DR241   S.......W.V.EG.NKTVG............................................................
DR259   S.......W.V.EG.NKTVG............................................................
DR293   S.......W.V.EG.NKTVG............................................................

D1_29   S.......W.V.EG.NNTVR..........................................................K.............
D1_19   S.......W.V.EG.NKTVG.........................................................G.............
                                           *              *            ****    *
DR106   TTQLFNSTFNITRNSTERSDNGTITLPCRIKQIINMWQEVGKAMYAPPIRGQIKCSSNITGLLLTRDGGNSENNETEVFRPGGGDMR
                  * *    ****
D2_64   ...........GD.....N..........L...................VR...........GNNT....I.........
D2_40   .....N.....GD.....N..........L...................VR...........GNNE....I.........
D2_22   ...........GD.....N..........L...................VR...........GNNE....IL.........

R2_74   ..........T.W.G..S...R....L......L...............VR.P...........GNNT--..I.........
R2_43   ..........T.W.G..SP..R...........L...............VR............GNNT--..I.........
R2_55   ..........T.W.G..SP..R...........L.......R.........GNNT--..I.........
R2_29   ..........T.W.G..SP..R...........L...............VR............GNNK--..I.........
R2_106  ..........T.W.G..SP..R...........L...............VR............GNNT--..I.........
R2_114  ..........T.W.G..SP..R...........L...............VR.....A...VGNNT--..I.........
R2_107  ..........T.W.G..SP..R...........L...............VR............D.GNNT--I.I.........
R2_5    ..........T.W.G..SL..R...........L...............VR............GNNT--..I.........
R2_82   ..........T.W.G..SP..R...........L.......R.........GDNT--..I.........
R2_66   ..........T.W.G..SP..R...........L.......R.........GNNT--..I.........
R2_56   ..........T.W.G..SP..R...........L...............VR............GNNT--..I.........
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FIG. 2. Alignment of predicted protein sequences corresponding to the V4/V5 nucleotide sequences in Fig. 1. For details of format and symbols, see the legend to Fig. 1.
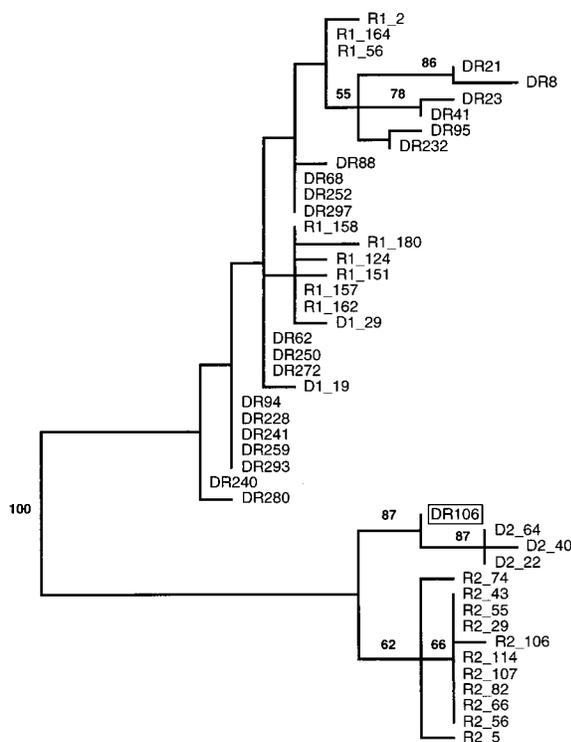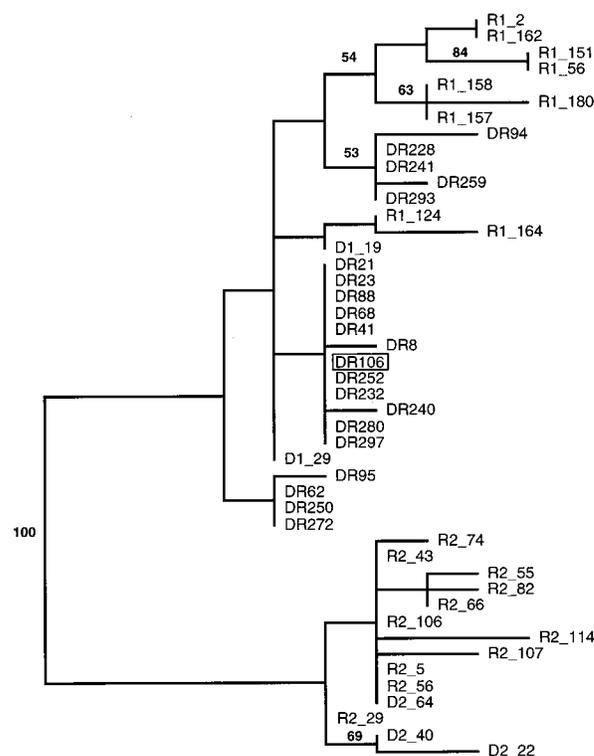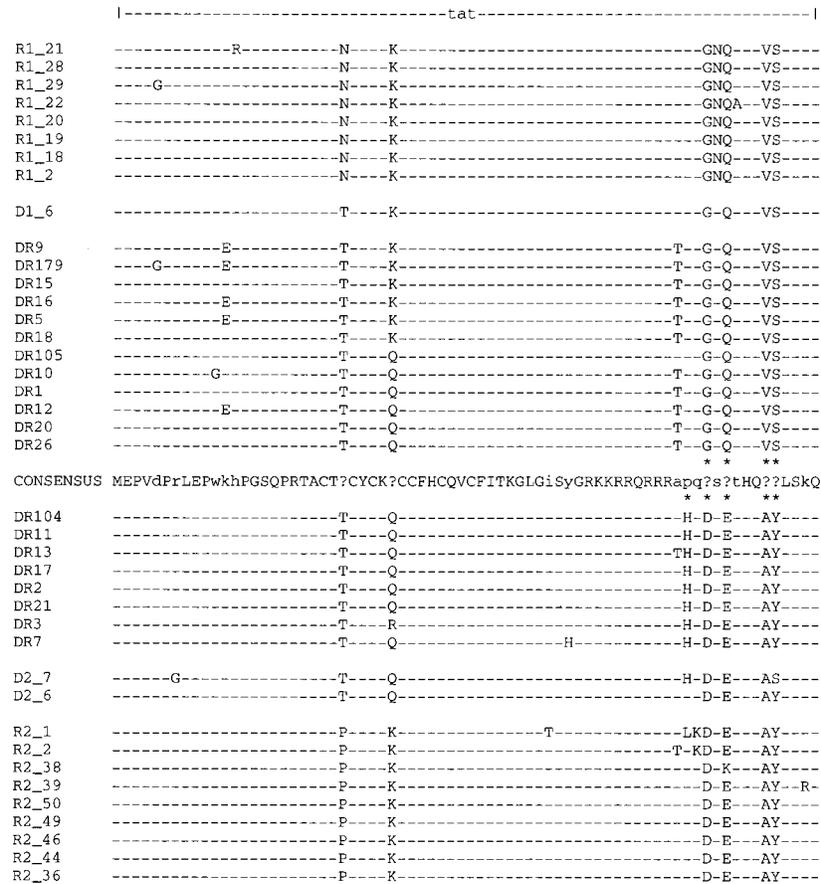
FIG. 3. Phylogenetic tree of the 46 sequences from the 5′ portion of the V4/V5 alignment. This is one representative of many equally parsimonious trees (51 steps), all showing the major grouping of D1 + R1 + DR (except DR106) versus D2 + R2 + DR106. Horizontal branch lengths correspond to the number of steps along the branches. The tree was rooted by the midpoint method. The putative recombinant sequence DR106 is boxed. Bootstrap values of >50% are indicated over the relevant branch, based on 100 bootstrap replicates, with 100 random sequence additions per replicate. The 100% bootstrap value for the branch separating D1 + R1 + most DR sequences from D2 + R2 + DR106 strongly supports the hypothesis that this portion of the DR106 genome is D2 derived.

FIG. 4. Phylogenetic tree of the 46 sequences from the 3′ portion of the V4/V5 alignment (45 steps). For details, see the legend to Fig. 3. The 100% bootstrap value for the branch separating D2 + R2 sequences from D1 + R1 + all DR sequences strongly supports the hypothesis that this portion of the DR106 genome is D1 derived.

propriate epidemiologic relationships among persons represented in the study. Figure 1 shows an alignment of genomes from D1, R1, D2, R2, and DR for a portion of the *env* gene spanning V4 through V5. Figure 2 shows the amino acid sequences predicted from Fig. 1. Visual inspection of these two figures shows that the V4 and V5 regions present in the donation serum of D1 from 1984 were distinct from those present in the donation serum of D2 from 1984. Furthermore, the samples obtained from R1 and R2 in 1986 matched those present in their respective donors in 1984. All 21 genomes sequenced from DR showed C4 and V5 regions corresponding to those present in D1 and R1. However, 1 of the 21 genomes in the DR (DR-106, the reference sequence in Fig. 1 and 2) had a V4 sequence corresponding closely to that present in D2 and in R2. These data support the hypothesis that HIV-1 from both donors did indeed infect DR and that genome DR106 was the product of recombination between genomes derived from D1 and D2.

Phylogenetic analysis supports this conclusion. The V4/V5 alignment shown in Fig. 1 was split into an early 5′ region (V4) and a late 3′ region (V5); the point of separation was determined by visual inspection. The early region consisted of the first 100 columns of the alignment, which, after removal of columns containing a gap in one or more sequences, contained 29 positions which were potentially informative for parsimony analysis. The late region consisted of the remaining 156 col-

umns of the alignment, which after removal of columns containing a gap in one or more sequences, contained 21 potentially informative positions. Maximum-parsimony analysis, using PAUP (24), of the early set indicates that the early portion of DR106 is most closely related to D2 and R2 sequences; conversely, analysis of the late set indicates that the late portion of DR106 is most closely related to D1 and R1. In both cases, multiple equally parsimonious trees were found; representative phylogenies are given in Fig. 3 and 4 (rooting of the trees is according to the midpoint method). Bootstrap analysis (11) indicates that these findings are robust: these groupings were perfectly reproduced in 100 bootstrap replications (100 random sequence additions per replicate). Analysis of the early portion of an alternative alignment which does not have the offsetting gaps also gives 100% bootstrap values for the branch separating D2 + R2 + DR106 sequences from the remaining sequences. Likewise, analysis of an alternative alignment of the late portion containing additional gaps to maximize agreement in the protein alignment (see Materials and Methods) does not affect the conclusion, grouping DR106 with the D1 + R1 + other DR sequences with a bootstrap value of 99%.

We looked for evidence of dual infection and recombination in other portions of the genome, specifically the V3 region of *env* and exon 1 of *tat*. The V3 sequences in the five individuals (data not presented here; GenBank accession numbers U11124 to U11209) were quite similar to each other, and the differences in sequence between the two donors did not clearly point to linkage of the DR sequences with one donor rather than the other. Two clearly distinct groups of sequences were
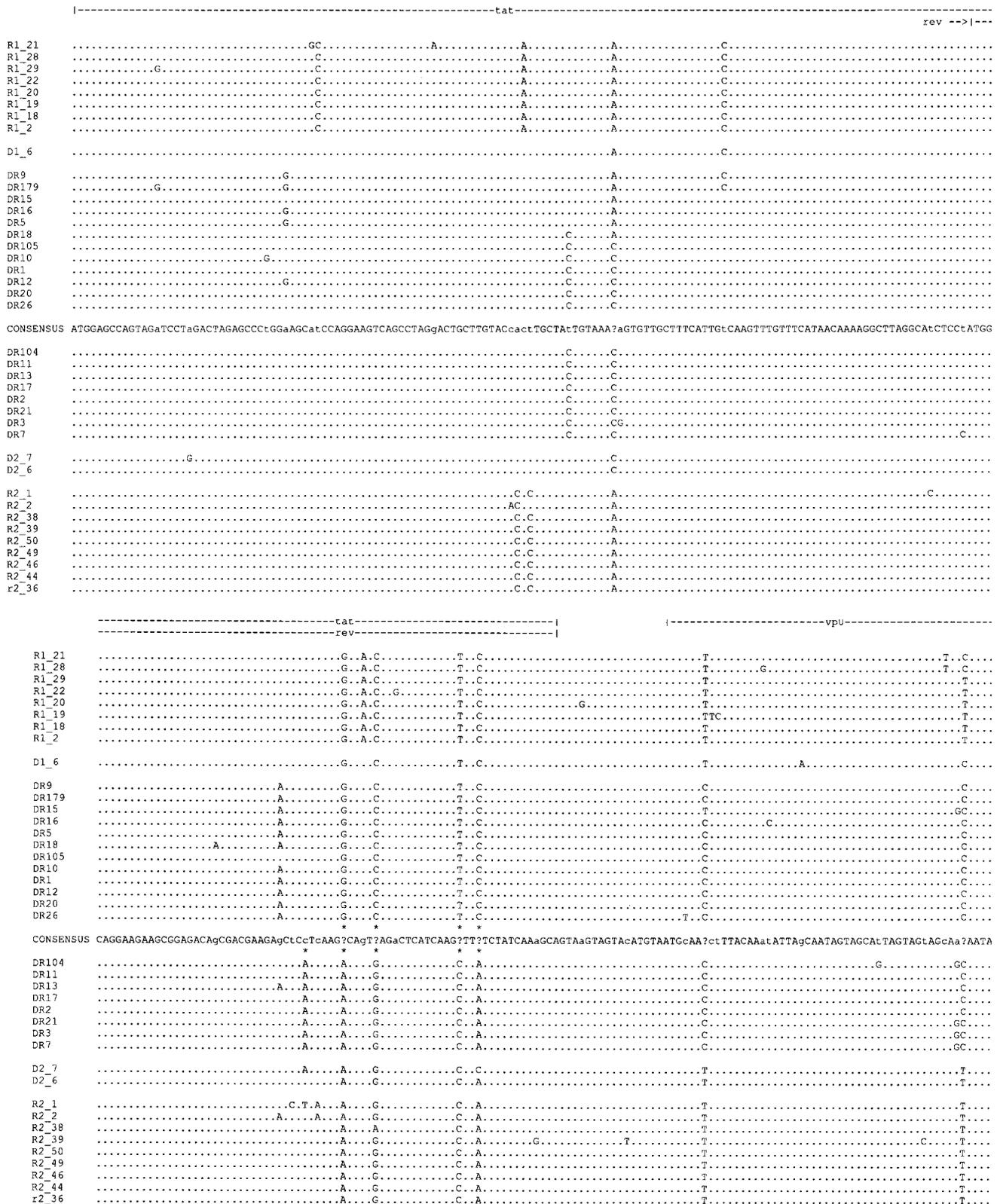
FIG. 5. Alignment of 40 sequences over 285 nucleotides, including the overlapping exons 1 of the *tat* and *rev* genes and a portion of the *vpu* gene (HXB2 positions 5830 to 6111). This figure has the same format as Fig. 1, except that the reference sequence is a consensus of all sequences and asterisks mark positions which consistently split the DR sequences into two groups, one sharing a base with all of the D1 + R1 sequences and almost none of the D2 + R2 sequences, and the other sharing a base with one or more of the D2 + R2 sequences and none of the D1 + R1 sequences.

```
                 |------------------------------tat----------------------------|
R1_21            -------------R----------N----K-------------------------------GNQ---VS----
R1_28            -----------------------N----K-------------------------------GNQ---VS----
R1_29            ----G------------------N----K-------------------------------GNQ---VS----
R1_22            -----------------------N----K-------------------------------GNQA--VS----
R1_20            -----------------------N----K-------------------------------GNQ---VS----
R1_19            -----------------------N----K------------------------------GNQ---VS----
R1_18            -----------------------N----K-------------------------------GNQ---VS----
R1_2             -----------------------N----K------------------------------GNQ---VS----

D1_6             -----------------------T----K-------------------------------G--Q---VS----

DR9              -----------E-----------T----K-----------------------------T--G-Q---VS----
DR179            ----G------E-----------T----K-----------------------------T--G-Q---VS----
DR15             -----------------------T----K-----------------------------T--G-Q---VS----
DR16             -----------E-----------T----K-----------------------------T--G-Q---VS----
DR5              -----------E-----------T----K-----------------------------T--G-Q---VS----
DR18             -----------------------T----K-----------------------------T--G-Q---VS----
DR105            -----------------------T----Q------------------------------G-Q---VS----
DR10             ----------G------------T----Q-----------------------------T--G-Q---VS----
DR1              -----------------------T----Q-----------------------------T--G-Q---VS----
DR12             -----------E-----------T----Q-----------------------------T--G-Q---VS----
DR20             -----------------------T----Q-----------------------------T--G-Q---VS----
DR26             -----------------------T----Q-----------------------------T--G-Q---VS----

CONSENSUS MEPVdPrLEPwkhPGSQPRTACT?CYCK?CCFHCQVCFITKGLGiSyGRKKRRQRRRapq?s?tHQ??LSkQ
                                        * *    **                             * * * **
DR104            -----------------------T----Q-----------------------------H-D-E---AY----
DR11             -----------------------T----Q-----------------------------H-D-E---AY----
DR13             -----------------------T----Q----------------------------TH-D-E---AY----
DR17             -----------------------T----Q-----------------------------H-D-E---AY----
DR2              -----------------------T----Q-----------------------------H-D-E---AY----
DR21             -----------------------T----Q-----------------------------H-D-E---AY----
DR3              -----------------------T----R-----------------------------H-D-E---AY----
DR7              -----------------------T----Q-----------------------H------H-D-E---AY----

D2_7             ------G----------------T----Q-----------------------------H-D-E---AS----
D2_6             -----------------------T----Q-----------------------------D-E---AY----

R2_1             ----------------------P----K--------------T------------LKD-E---AY----
R2_2             ----------------------P----K-------------------------T-KD-E---AY----
R2_38            ----------------------P----K--------------------------D-K---AY----
R2_39            ----------------------P----K-------------------------------D-E---AY--R-
R2_50            ----------------------P----K-------------------------------D-E---AY----
R2_49            ----------------------P----K-------------------------------D-E---AY----
R2_46            ----------------------P----K-------------------------------D-E---AY----
R2_44            ----------------------P----K-------------------------------D-E---AY----
R2_36            ----------------------P----K-------------------------------D-E---AY----
```

FIG. 6. Alignment of predicted protein sequences corresponding to the *tat* coding portion of the nucleotide sequences in Fig. 5. For details of format and symbols, see the legend to Fig. 5.

also not present in DR. The observed similarities in *env* sequence between the two donors is not surprising, since these sequences were obtained from people in the same geographic area at an early time in the HIV pandemic.

The sequences detected in the two donors, encompassing the overlapping exons 1 of *tat* and *rev* (Fig. 5), also were very similar to each other, showing differences at only 10 of the 285 sites sequenced. Five of these differences, which are clustered (asterisks in Fig. 5 and 6), defined two subsets of nucleotide sequences in DR that corresponded to one or the other of the two donors. This pattern is reflected at the five sites in the *tat* amino acid reading frame (Fig. 6) but at only four of the five sites in the *rev* reading frame (results not shown). This cluster of differences enabled us to correctly identify each of the two donors and three recipients from among the blinded panel described in Materials and Methods. Phylogenetic analysis of the 285-nucleotide sequence alignment in Fig. 5 was consistent with the conclusion that *tat* sequences from each of the two donors were present in the dual recipient. A representative maximum-parsimony tree, rooted at the midpoint, is given in Fig. 7. Bootstrapping gives a value of 75%, in support of the branch separating D2 + R2 + some DR sequences from D1 + R1 + other DR sequences. (Bootstrap values in excess of 50% are an underestimate of the confidence that may be placed on the relevant branch [15].)

A discrepancy is seen between the ratios of DR sequences derived from D1 versus D2 in the *tat* region (12:8) and in the *env*-V4/V5 region (20:1) ($P = 0.008$ by the exact Fisher test). If

this is not simply due to an artifact of differential PCR priming in the two regions, it suggests that in a high proportion of the genomes, genetic exchange had taken place at a region between the *tat* and *env* genes in addition to recombination within the *env* gene evident in DR106.

The heteroduplex mobility assay, which has been used to assess the genetic relatedness of HIV-1 genomes, was used here as an additional method to test the hypothesis that DR106 is a recombinant genome. The representative genomes R1-2, R2-5, and DR297 were chosen, and the 700-bp PCR products spanning V3 through V5 of these genomes were analyzed pairwise with respect to each other. In this assay, increased mismatches between two genomes are predicted to result in heteroduplexes with decreased mobility compared with the faster-migrating homoduplexes (H in Fig. 8). Accordingly, each sample run by itself gives only faster-migrating homoduplex bands (lanes 7 through 10) but the R1-R2 mixture (lane 1) also shows two additional slowly moving heteroduplex bands, consistent with the known sequence differences between R1 and R2. The DR297-R1 heteroduplex (lane 2) shows a higher mobility than the DR297-R2 heteroduplex (lane 6), again consistent with the sequence homology of this representative DR297 genome with the R1 sequence rather than with the R2 sequence. The intermediate mobilities of the heteroduplexes of genomes paired with DR106 (lanes 3 to 5) are consistent with DR106 being a recombinant and having a closer relation to the R2 genome than to the R1 and DR297 genomes.

When tested against PCR-amplified viral DNA from the
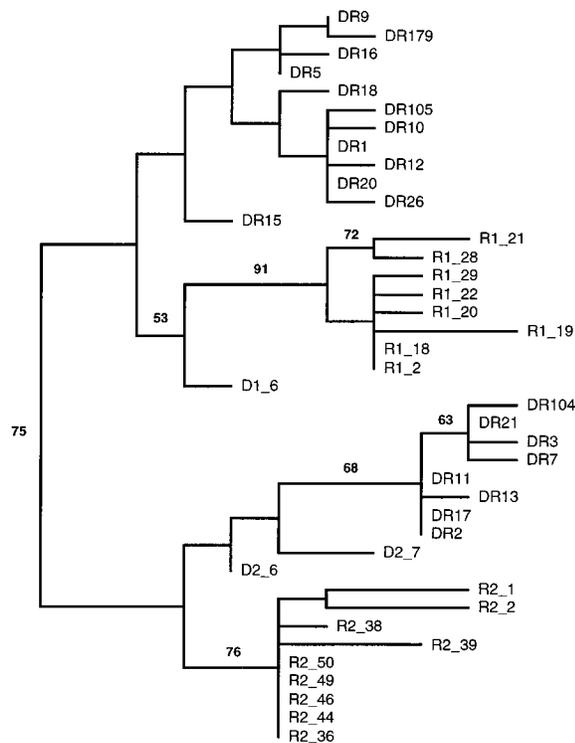
FIG. 7. Phylogenetic tree of the 40 sequences of 285 nucleotides encompassing exon 1 of *tat* from Fig. 5. This is one representative of four equally parsimonious trees (60 steps). For details, see the legend to Fig. 3. The 75% bootstrap value for the branch separating D1 + R1 + some DR sequences from D2 + R2 + the other DR sequences supports the hypothesis that there are two groups of DR *tat* sequences, one D1 derived and the other D2 derived.

FIG. 8. DNA heteroduplex mobility analysis of the relationships of HIV-1 clones from representative sequences of DNA from R1 (R1-2), R2 (R2-5), DR (DR297), and the recombinant sequence DR106. PCR-amplified DNA fragments (700 bp) spanning V3 through V5 were denatured and reannealed by rapid cooling to maximize heteroduplex formation (see Materials and Methods). DNA was then subjected to gel electrophoresis through 5% polyacrylamide, stained with ethidium bromide, and photographed under UV illumination. The lane with the molecular weight marker is indicated by M. Lanes: 1, R1-R2 mixture; 2, R1-DR297; 3, R1-DR106; 4, DR297-DR106; 5, R2-DR106; 6, R2-DR297; 7, R1 alone; 8, R2 alone; 9, DR297 alone; 10, DR106 alone. Samples were loaded at L, S marks the location of single-stranded DNA bands, and H indicates the position of DNA homoduplexes.

various HIV-1 subtypes, the representative sequences of R1 and R2 were identified as subtype B by the more rapid relative migration of heteroduplexes with subtype B isolates than with other subtypes (Fig. 9).

DISCUSSION

We have used stored specimens from a study designed to document the outcome of transfusion of blood that could later be determined to be HIV-1 contaminated (10). In this instance, we analyzed an infant who was transfused in 1984 with 2 units of blood, both later identified as being HIV-1 seropositive, from different donors. We show that (i) double infection by HIV-1 occurred and (ii) virus recombination between the two different infecting HIV-1 subtype B strains took place in this dually infected individual. The analysis of virus present in stored aliquots from both of the donated blood units themselves, as well as from singly transfused recipients of blood from these two donors, was pivotal in allowing us to identify and distinguish parental and recombinant genomes in the dually transfused individual. This study thus complements prior studies reporting evidence of recombination between different HIV-1 (21) or HIV-2 (12) subtypes and between different variants of a single HIV-1 strain (8, 14, 16, 30), in which the likely parental strains were not identified and confirmed in transmitting individuals.

We believe that our results are not due to laboratory error, for several reasons. First, both *tat* and *env* sequences from the dual recipient were PCR amplified, cloned, and sequenced prior to the corresponding regions from the two donors and

single recipients. Second, the DR106 *env* sequence is different from any sequence seen in either of the two donors or singly exposed recipients. These two facts indicate that the presence of sequences from both donors in the dual recipient cannot be explained by PCR contamination. Third, the specimens, with the exception of that from the dual recipient, were analyzed from a coded panel with controls. Finally, several experiments show that the DR106 genome is not the result of in vitro recombination between two parental genomes during PCR amplification or subsequent cloning (18). End point PCR (23) was used to ensure that the great majority of PCRs used single cDNA molecules as targets for amplification. The particular end point PCR experiment from which the recombinant DR106 sequence was obtained yielded PCR product in only 5 of 40 tubes, making it unlikely that two or more cDNA molecules per tube gave rise to PCR product. In the unlikely event that two parental genomes were present in the DR106 reaction tube and that recombination did occur in vitro, the recombinant genome would be predicted to constitute only a small fraction of the final PCR product. This was not the case, since five of five clones of PCR product from the DR106 reaction tube gave the identical recombinant sequence and since heteroduplex analysis of DR106 PCR product (Fig. 8, lane 10) reveals only homoduplex molecules, indicating that the PCR product was homogeneous rather than a mixture. With respect to the approximately equal distribution of *tat* sequences derived from each donor in the DR specimen, the use of end point PCR product provides assurance that each sequence corresponded to a distinct genome present in the original sample and avoids the problem inherent in bulk PCR in which two or more sequences analyzed may be derived from the same input genome (23).
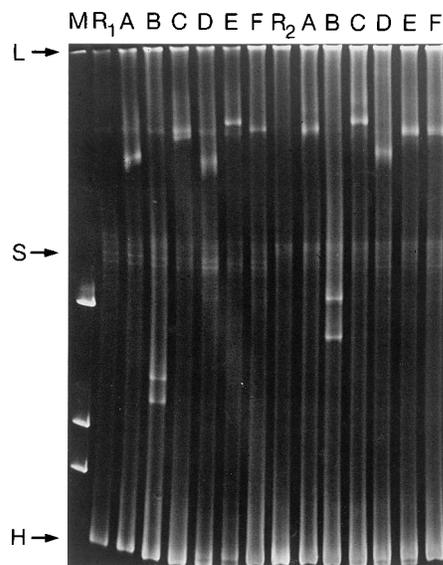
FIG. 9. Subtype classification obtained with the heteroduplex mobility assay. V3-through-V5 PCR-amplified DNAs (700 bp) from a representative sequence of previously identified envelope subtypes A through F were separately denatured and reannealed with analogous fragments derived from representative sequences of each of the two singly infected recipients (R1-2 and R2-5). The mobility of the resulting DNA heteroduplexes was determined by polyacrylamide gel electrophoresis (5% polyacrylamide). Samples were loaded at L, S marks the single-stranded DNA band, and H indicates the position of the DNA homoduplexes. The reference strains used are, from left to right, subtypes A (RW20, Rwanda), B (SF162, United States), C (MA959, Malawi), D (UG21, Uganda), E (TH22, Thailand), and F (BZ162, Brazil) (4).

Although the particular circumstance of this case, a nearly simultaneous dual exposure by blood transfusion, might seem unusual, persons engaging in high-risk behavior at frequent intervals may not be particularly uncommon. This study suggests that people who have not yet developed immunity to HIV-1 may well be susceptible to a second infection. A case of coinfection following homosexual activity (30) also supports this conclusion. It remains to be determined whether HIV-1 can establish a second infection in a previously infected seropositive individual.

The evidence for recombination presented here within *env* and between *tat* and *env* implies a large number of crossover events, given the relatively small regions and numbers of HIV-1 genomes analyzed. This suggests that recombination in vivo is a frequent event. This is consistent with the increasing reports of recombinants mentioned above. Taken together, these results indicate that recombination of viral genomes within the quasispecies present in an infected individual may constitute a significant factor in the generation of HIV-1 diversity (8).

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