

## Evidence for Coinfection by Multiple Strains of Human Immunodeficiency Virus Type 1 Subtype B in an Acute Seroconverter

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**Sequences encoding the envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) were amplified by PCR from plasma and peripheral blood mononuclear cells obtained at four time points from an acute seroconverter. Genetic analyses, including nucleotide sequencing and heteroduplex mobility studies, showed that the patient harbored three distinct populations of HIV-1 clade B envelope sequences, with nucleotide distances ranging from 9.2 to 17.2%. One population of sequences was clearly distinguishable from the others on the basis of phylogenetic analysis. In addition, sequences suggesting recombination between two of the three distinct viral populations were also found. This case of acute seroconversion provides clear and conclusive evidence that coinfection by multiple HIV-1 strains can indeed occur in vivo.**

The finding of distinct populations of human immunodeficiency virus (HIV) in a person would suggest coinfection or superinfection. A case of possible but unproven HIV type 1 (HIV-1) coinfection has been reported recently (19a), but superinfection of an HIV-1-infected person with another distinct viral strain has not been documented despite the existence of a large population of individuals with repeated exposures via sexual contacts or sharing of intravenous needles. However, several findings suggest that simultaneous infection of humans by at least two strains of HIV can occur. Dual infection by HIV-1 and HIV-2 has been well documented (6, 11, 18). Recombinant viruses between different subtypes of HIV-1 (1, 15, 15a) or HIV-2 (10, 10a) have also been described, implying that dual infection must have occurred at some point. Furthermore, successful superinfection has been achieved in an HIV-1<sub>SF2</sub>-infected chimpanzee by experimental inoculation of HIV-1<sub>Lai</sub> (9). Whether or not superinfection by a second strain of HIV-1 can occur in humans deserves more extensive studies, because it is important to know the effectiveness of the immunity induced by the initial infection in conferring protection against subsequent challenges.

During studies to characterize the virus in patients with primary infection (14, 23), we uncovered a case of coinfection by multiple subtype B HIV-1 in an acute seroconverter. Moreover, evidence of viral recombination between two of the variants was also found in this patient.

The patient, MR, is a 29-year-old Australian homosexual man who presented to a Sydney physician with fever, rash, fatigue, myalgia, and headache, along with a low CD4 cell count (224/mm<sup>3</sup>) and HIV-1 p24 antigenemia (359 pg/ml). The acute illness resolved spontaneously after 25 days and was followed by seroconversion (Fig. 1A), thus confirming the diagnosis of primary HIV-1 infection. In addition, a burst of viremia characteristic of acute infection (2, 3) was also docu-

mented by using a commercial assay (16) to measure HIV-1 RNA in sequential plasma samples (Fig. 1B).

Following the diagnosis of primary HIV-1 infection, a detailed sexual history was obtained. The patient has a regular sexual partner who is a known asymptomatic carrier of HIV-1. They had unprotected sexual intercourse on two recent occasions, once 1 month and another time 3 days before the onset of the acute illness. However, 17 days prior to the onset of illness, the patient also had unprotected sexual contact with an unknown number of persons in a sauna while under the influence of alcohol. Because of the findings described below, we have chosen to call this day day zero.

To examine the HIV-1 genotype in this acute seroconverter, initial studies focused on gp120 sequences V1 to V5 in uncultured peripheral blood mononuclear cells (PBMC) obtained on day 26. DNA was extracted and subjected to nested PCR by first using outer primers PE0 (5953 to 5979 of NL4-3; 5'-GGC TTA GGC ATC TCC TAT GGC AGG AAG-3') and P2 (7815 to 7786 of NL4-3; 5'-GAC GAA GCT TCC ATA GTG CTT CCT GCT GC-3') (23). Amplifications were done with a Perkin-Elmer 9600 Thermocycler at 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min with a final extension at 72°C for 5 min. A 5- $\mu$ l aliquot of the first PCR product was subjected to a second round of PCR with inner primers P1 (6528 to 6552 of NL4-3; 5'-GAT GGT ACC GGA TAT AAT CAG TTT ATG GG-3') and P4 (7661 to 7637 of NL4-3; 5'-ATT CAC TTC TAG AAT TGT CCC TCA T-3'). The resultant PCR products of approximately 1,100 bp containing sequences V1 to V5 of gp120 were then digested with restriction enzymes *Kpn*I and *Xba*I and cloned into M13mp19 as previously described (23).

One hundred M13 clones were then chosen for genetic analysis. The clones were first examined by the heteroduplex mobility assay (HMA) (4, 5; see details below). The PCR product derived from the first clone, MR1, was labelled and used as the probe to examine the PCR products derived from the other clones. Since seroconvertors generally harbor a relatively homogeneous population of viruses (21-23), it was unexpected that four different populations of envelope sequences would be found. Examples of each group are shown in the left panel of

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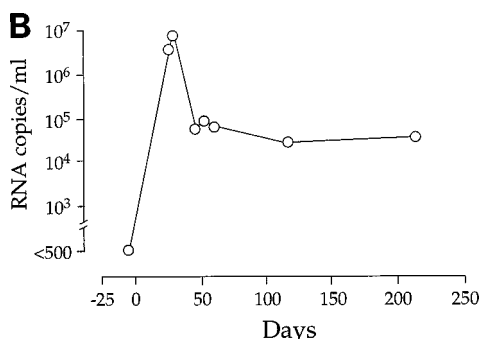
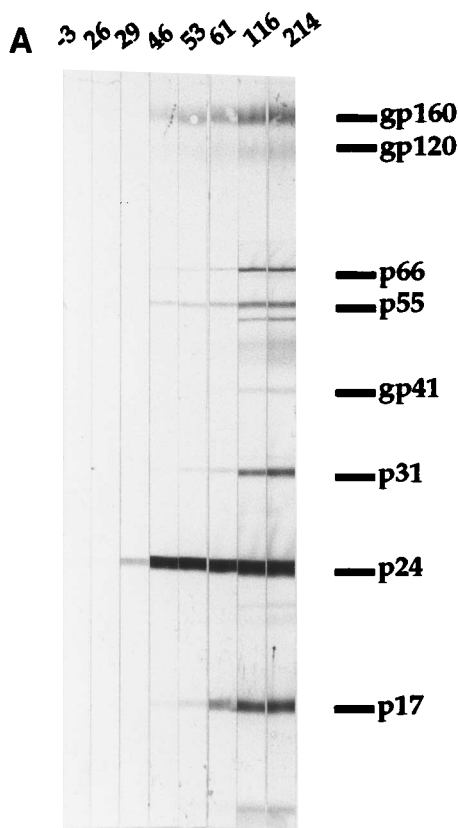


FIG. 1. (A) Western blot (immunoblot) analysis of sequential plasma samples from seroconverter MR. The numbers at the top are numbers of days after the presumed exposure to HIV-1. (B) Quantitation of HIV-1 RNA copy numbers in sequential plasma samples at and after seroconversion. The day -3 sample was available because the patient was monitored in a prospective study of discordant couples.

Fig. 2. Two to four clones from each group were then subjected to standard dideoxynucleotide sequencing. As shown in Fig. 3, four distinct sets of sequences were obtained. On the basis of sequence similarity, clones MR1, MR7, MR6, and MR8 fell into group I; MR2, MR21, and MR22 were in group II; and MR3, MR9, MR24, and MR25 were in group III. Clone MR4 was found to be a recombinant virus with a V1 sequence similar to that of MR1 and sequences V2 to V5 similar to those of MR2, while MR5 was a recombinant with V1 and V2 sequences similar to those of MR1 and C2 and V5 sequences similar to those of MR2. Following alignment of sequences with CLUSTAL V (12), genetic distances were determined by using the two-parameter model of Kimura (13), excluding all

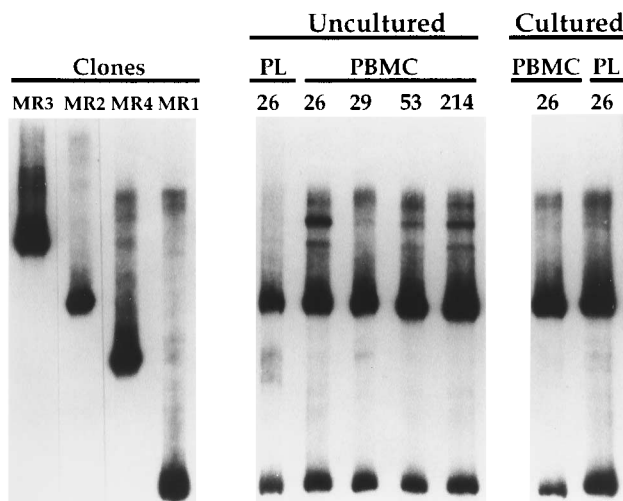


FIG. 2. Analysis of the HIV-1 envelope in patient MR by HMA. A  $^{32}$ P-labelled probe derived from the PCR product of clone MR1 was separately hybridized with corresponding PCR products from clones MR1, MR2, MR3, and MR4 and patient MR plasma (PL) and PBMC, as well as HIV-1 isolates obtained from day 26 plasma and PBMC by culture.

deletions or insertions. The average genetic distances between groups I and II, I and III, and II and III were 9.6, 16.5, and 18.4%, respectively. The average genetic distances within groups I, II, and III were 0.6, 0.8, and 0.5%, respectively.

The sequences shown in Fig. 3 were also subjected to phylogenetic analysis with the neighbor-joining method (19), the results of which were evaluated statistically with the bootstrap approach (7). To provide a broader perspective, a number of additional sequences were included in the analysis for comparison. These were viruses from subtypes A (SF170 and Z321), C (NOF), D (Z2Z6 and NDK), and E (TN243), as well as representatives of subtype B viruses (MN, SC, LAI, SF2, JRCSF, RF, and JRFL) (17). In addition, geographic controls comprising viruses from a seroconverter (AD39) and a chronically infected patient (AD38), samples from Sydney, Australia (our unpublished results), a virus from MR's regular sexual partner (D), and viruses from acute seroconvertors (A, L, F, V, and R) and chronically infected patients (C and M) from New York, N.Y. (23). An unrooted phylogenetic tree was constructed by using sequences C2 to V5 (Fig. 4). Envelope sequences from patient MR fell into three distinct phylogenetic groups, all of which are within HIV-1 subtype B. Groups I and II were distantly related (9.2 to 9.7% divergence) to all of the other subtype B sequences. Most importantly, group III was clearly distinct from groups I (16.0 to 17.2%) and II (18.0 to 18.7%). Furthermore, to increase our confidence in the analyses, two other methods, maximum likelihood (8) and maximum parsimony (20), were also used. In essence, the same tree was generated by these methods with either sequences C2 and V5 or V1 and V2 (data not shown).

In similar studies, several V1 to V5 sequences in PBMC from the patient's regular sexual partner (D) were determined. As shown in Fig. 3, these sequences differ substantially (12.3 to 19.4%) from those found in the acute seroconverter. In addition, on the basis of the phylogenetic analysis (Fig. 4), it is clear that sequences from the partner do not group with any set of sequences from the patient. Therefore, we conclude that the regular sexual partner was not the transmitter in this case; instead, transmission was presumably from unknown partners in the sauna.

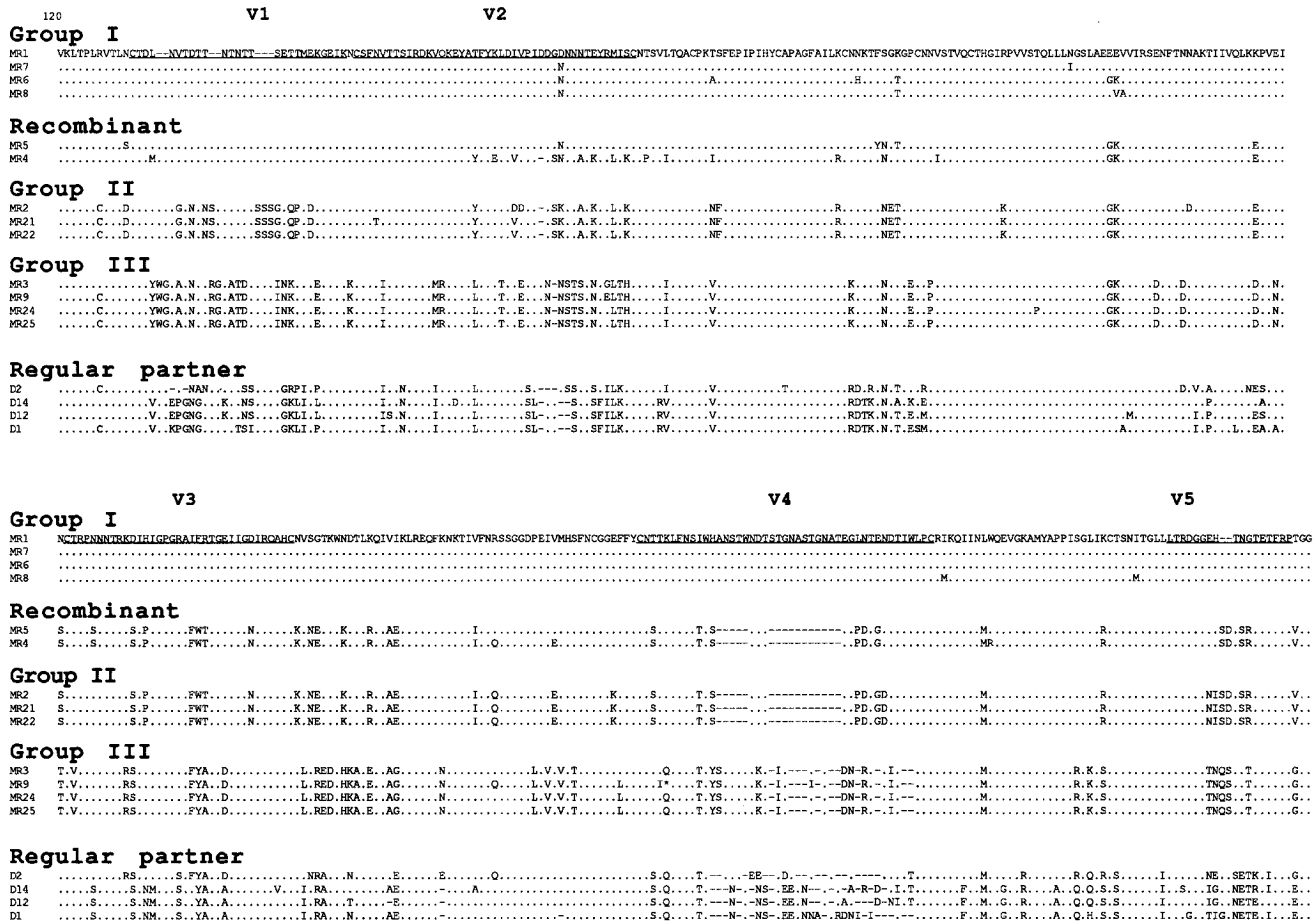


FIG. 3. Deduced amino acid sequence alignment of HIV-1 gp120 in day 26 PBMC from seroconverter MR and from his regular sexual partner (D). Dots denote identical sequences, and dashes denote deletions.

To confirm that multiple HIV-1 strains indeed exist in MR, we also examined the genotypes of HIV-1 in his plasma, as well as in sequential PBMC samples, by using a rapid genetic analysis method developed by Delwart et al. (4, 5), the HMA. The HMA can rapidly provide genetic information which closely resembles that obtained by DNA sequencing (4, 5). We have recently modified this assay by using a single-strand probe in place of the regular double-strand probe. Briefly, in the experiment whose results are shown in Fig. 2, the envelope PCR product from MR1 was subjected to asymmetrical PCR in the presence of [ $\alpha$ -<sup>32</sup>P]dATP. The probe was then mixed with the corresponding PCR products derived from PBMC obtained on day 26, 29, 53, or 214, as well as with reverse transcription PCR products derived from day 26 plasma or from HIV-1 isolates obtained by culture. The resultant hybrid molecules were then subjected to electrophoresis in a 5% neutral polyacrylamide gel. As shown in the right panel of Fig. 2, two dark bands corresponding to sequences similar to those of MR1 (group I) and MR2 (group II) were evident. In addition, faint but definite bands corresponding to sequences related to MR3 (group III) were seen in multiple samples. Furthermore, a faint band corresponding to the sequence of recombinant MR4 (group IV) was observed in several samples. These findings, showing the presence of the same set of HIV-1 strains in multiple samples separated in time and processed independently, suggest that the evidence of coinfection is real and not the result

of inadvertent contamination. Moreover, although not proven conclusively, the detection of MR4-like sequences by HMA in multiple samples (Fig. 2) suggests that this recombinant sequence is probably not due to random recombination events during the PCR.

In summary, patient MR appeared to harbor four groups of subtype B HIV-1 at the time of seroconversion. The phylogenetic relationship between viruses in groups I and II (Fig. 4) suggests that they represent divergent quaspecies derived from a common ancestral virus and that transmission of multiple variants from one individual had occurred, contrary to the usual situation reported for sexual transmission (21-23). However, it is also formally possible that groups I and II emerged from different ancestral strains from different donors. Notwithstanding the ambiguity involving groups I and II, the genetic distance (15.7 to 18.6%) between group III viruses and those from other groups (Fig. 4) leads us to conclude that MR was infected either by multiple persons or by one transmitter who harbored markedly divergent strains of HIV-1. In either case, these findings document the presence of coinfection by multiple distinct strains of HIV-1 in vivo. Moreover, the findings on this patient also illustrate two additional points. (i) Recombination between different HIV strains can occur in vivo, as has been suggested previously (10, 10a, 15). (ii) The mere presence of multiple strains of HIV-1 in a person may be due to coinfection and does not necessarily imply superinfection.

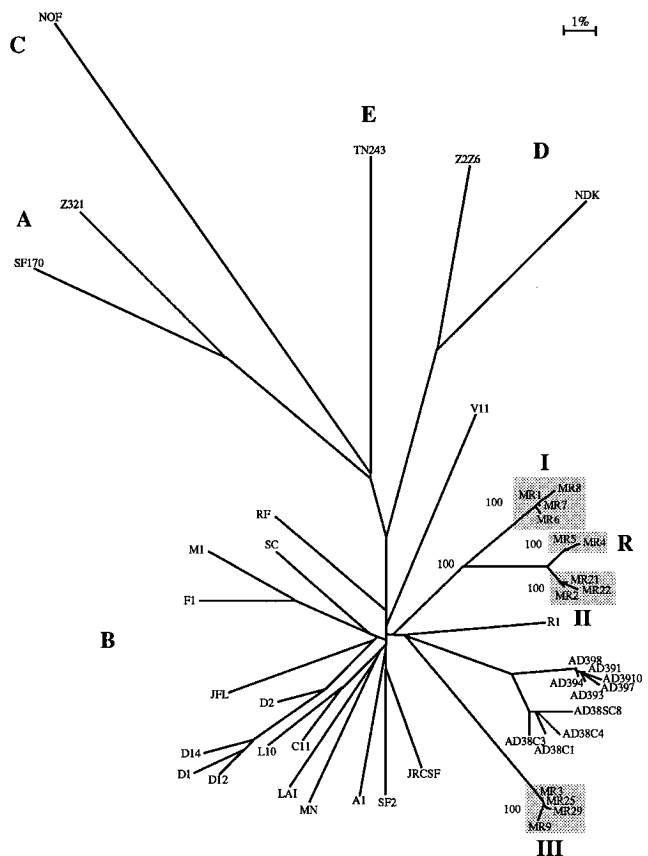


FIG. 4. Phylogenetic relationship of HIV-1 envelope (regions C2 and V5) nucleotide sequences from seroconvertor MR and other HIV-1 strains as constructed by the neighbor-joining method. A number at a node is the percentage of 1,000 bootstrap samples in which the distal cluster is found; only values greater than 95% are shown. Shaded boxes I, II, III, and R represent group I, II, and III and recombinant sequences, respectively.

**Nucleotide sequence accession numbers.** The GenBank accession numbers of the nucleotide sequences in Fig. 3 are as follows: D1, U16372; D2, U16373; D12, U16374; D14, U16375; MR1, U16376; MR2, U16377; MR3, U16378; MR4, U16379; MR5, U16380; MR6, U16381; MR7, U16382; MR8, U16383; MR9, U16384; MR21, U16385; MR22, U16386; MR24, U16387; MR25, U16388.

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REFERENCES

1. Alizon, M., S. Wain-Hobson, L. Montagnier, and P. Sonigo. 1986. Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. *Cell* **46**:63-74.
2. Clark, S. J., M. S. Saag, W. D. Decker, S. Campbell-Hill, J. L. Roberson, P. J. Veldkamp, J. C. Kappes, B. H. Hahn, and G. M. Shaw. 1991. High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *N. Engl. J. Med.* **324**:649-654.
3. Daar, E. S., T. Moudgil, R. D. Meyer, and D. D. Ho. 1991. Transient high

- levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **324**:961-964.
4. Delwart, E. L., H. W. Sheppard, B. D. Walker, J. Goudsmit, and J. I. Mullins. 1994. Human immunodeficiency virus type 1 evolution in vivo tracked by DNA heteroduplex mobility assays. *J. Virol.* **68**:6672-6683.
5. Delwart, E. L., E. G. Shpaer, J. Louwagie, F. E. McCutchan, M. Maran, K. Rubsamen-Waigmann, and J. I. Mullins. 1993. Evolutionary relationships between HIV env genes determined by a quantitative heteroduplex mobility assay. *Science* **262**:1257-1261.
6. Evans, L. A., K. Odehouri, G. Thomson-Honnieber, A. Barboza, J. Moreau, D. Seto, H. Legg, C. Cheng-Mayer, and J. A. Levy. 1988. Simultaneous isolation of HIV-1 and HIV-2 from an AIDS patient. *Lancet* **ii**:1389-1391.
7. Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783-791.
8. Felsenstein, J. 1992. Phylogenies from restriction sites: a maximum-likelihood approach. *Evolution* **46**:159-173.
9. Fultz, P. N., A. Srinivasan, C. R. Greene, D. Butler, R. B. Swenson, and H. M. McClure. 1987. Superinfection of a chimpanzee with a second strain of human immunodeficiency virus. *J. Virol.* **61**:4026-4029.
10. Gao, F., L. Yue, D. L. Robertson, S. C. Hill, H. Hui, R. J. Biggar, A. E. Neequaye, T. M. Whelan, D. D. Ho, G. M. Shaw, P. M. Sharp, and B. H. Hahn. 1994. Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. *J. Virol.* **68**:7433-7447.
- 10a. Gao, F., L. Yue, A. T. White, P. G. Pappas, J. Barchue, A. P. Hanson, B. M. Greene, P. M. Sharp, G. M. Shaw, and B. H. Hahn. 1992. Human infection by genetically diverse SIVSM-related HIV-2 in West Africa. *Nature (London)* **358**:495-499.
11. Grez, M., U. Dietrich, P. Balfe, H. V. Briesen, J. K. Maniar, G. Mahambre, E. L. Delwart, J. I. Mullins, and H. Rubsamen-Waigmann. 1994. Genetic analysis of human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2) mixed infection in India reveals a recent spread of HIV-1 and HIV-2 from a single ancestor for each of these viruses. *J. Virol.* **68**:2161-2168.
12. Higgins, D. G., and P. M. Sharp. 1989. Fast and sensitive multiple sequence alignments on a microcomputer. *Comp. Appl. Biosci.* **5**:151-153.
13. Kimura, M. 1980. A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**:111-120.
14. Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68**:4650-4655.
15. Li, W. H., M. Tamamura, and P. M. Sharp. 1988. Rates and dates of divergence between AIDS virus nucleotide sequences. *Mol. Biol. Evol.* **5**:313-330.
- 15a. Louwagie, J., F. E. McCutchan, M. Peeters, T. P. Brennan, E. Sanders-Buell, G. A. Eddy, G. van der Groen, K. Fransens, G.-M. Gershly-Damet, R. Deleys, and D. S. Burke. 1993. Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. *AIDS* **7**:769-780.
16. Mulder, J., N. McKinney, C. Christopherson, J. Sninsky, L. Greenfield, and S. Kwok. 1994. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. *J. Clin. Microbiol.* **32**:292-300.
17. Myers, G., B. Korber, S. Wain-Hobson, R. F. Smith, and G. N. Pavlakis. 1993. Human retroviruses and AIDS: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
18. Rayfield, M., K. De Cock, W. Heyward, L. Goldstein, J. Krebs, S. Kwok, S. Lee, J. McCormick, J. M. Moreau, K. Odehouri, G. Schochetman, J. Sninsky, and C.-Y. Ou. 1988. Mixed human immunodeficiency virus (HIV) infection in an individual: demonstration of both type 1 and type 2 proviral sequences by using polymerase chain reaction. *J. Infect. Dis.* **158**:1170-1176.
19. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406-425.
- 19a. Sala, M., G. Zambruno, J.-P. Vartanian, A. Marconi, U. Bertazzoni, and S. Wain-Hobson. 1994. Spatial discontinuities in human immunodeficiency virus type 1 quasispecies derived from epidermal Langerhans cells of a patient with AIDS and evidence of double infection. *J. Virol.* **68**:5280-5283.
20. Swofford, D. L. 1984. Phylogenetic analysis using parsimony (PAUP), version 3.1. Illinois Natural History Survey, Champaign.
21. Wolfs, T. F. W., G. Zwart, M. Bakker, and J. Goudsmit. 1992. HIV-1 genomic RNA diversification following sexual and parenteral transmission. *Virology* **189**:103-110.
22. Zhang, L. Q., P. MacKenzie, A. Cleland, E. C. Holmes, A. J. Leigh Brown, and P. Simmonds. 1993. Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection. *J. Virol.* **67**:3345-3356.
23. Zhu, T., H. Mo, N. Wang, D. S. Nam, Y. Cao, R. A. Koup, and D. D. Ho. 1993. Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. *Science* **261**:1179-1181.