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Sequence and Drug Susceptibility of Subtype C Reverse Transcriptase from Human Immunodeficiency Virus Type 1 Seroconverters in Zimbabwe

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Naturally occurring human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) variability has implications for the success of antiretroviral therapy. We determined the sequence of the polymerase-coding region of RT from virus isolates from 12 Zimbabwean individuals recently infected with HIV-1. The 12 RT sequences differed from the consensus B RT sequence at 10.5% of nucleotides and 5.8% of amino acids. Susceptibility testing of five isolates to zidovudine, didanosine, lamivudine, and nevirapine demonstrated susceptibilities similar to those of wild-type subtype B isolates. Phylogenetic analysis of 40 HIV-1 RT sequences, including the 12 Zimbabwean subtype C sequences, 11 subtype B sequences, and the 17 remaining published non-subtype B sequences showed sufficient intrasubtype RT sequence variation to differentiate subtype A, B, C, and D isolates. Five recently reported subtype C RT sequences from India grouped with the Zimbabwean subtype C sequences but had significantly less intraisolate sequence variation. Both intra- and intersubtype RT comparisons were notable for extraordinarily high ratios of synonymous to nonsynonymous differences. Although substitutions in the HIV-1 RT gene are limited by functional constraints, variation between RT sequences demonstrates phylogenetic relationships that parallel *env* and *gag* gene variation.

Genetic analysis of human immunodeficiency virus type 1 (HIV-1) isolates has revealed at least 10 distinct group M (main) subtypes (A to J), as well as several highly divergent (group O [outlier]) isolates (16, 25, 30, 31, 33, 48). Differences between group M subtypes are based on the approximately 30% intersubtype genetic divergence in the *env* region and 14% intersubtype distance in the *gag* region (16, 25, 30, 31, 33, 48). Although HIV-1 genetic variation is one of the major obstacles to the development of a successful vaccine, it is not known if such variation also influences the initial susceptibility of HIV-1 to antiretroviral drugs. For example, the evolutionarily related virus, HIV-2, is intrinsically resistant to non-nucleoside RT inhibitors (36), and there have been conflicting reports on the susceptibility of HIV-2 to nucleoside analog RT inhibitors (7, 39).

Subtype B is the most prevalent HIV-1 subtype in North America and Europe, and subtype B HIV-1 RT sequences have been extensively reported and studied (33). However, there are few published RT sequences of non-subtype B HIV-1 isolates. Subtype C is one of the most prevalent HIV-1 subtypes and is especially common in Africa and India (21, 47, 48). In 1996, one subtype C HIV-1 RT sequence from Ethiopia (41) and five subtype C HIV-1 RT sequences from India (47) were reported. We determined the RT sequence of the polymerase-coding region of 12 additional subtype C isolates from HIV-1-infected individuals in Zimbabwe and assessed the susceptibility of five of these isolates to nucleoside analog and nonnucleoside RT inhibitors.

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MATERIALS AND METHODS

Study population. The HIV-1-infected persons included 11 men, (ages, 21 to 45 years) and one woman (age, 24 years). The 11 men were recent seroconverters; each acquired HIV-1 infection via heterosexual transmission and seroconverted within the 6 months prior to contributing blood samples in March 1995 (28). The woman was a sexual partner of one of the men, but there were otherwise no epidemiologic links between the study subjects. The 11 men were factory workers in Harare and did not have a history of travel outside Zimbabwe (28).

Virus isolation. Peripheral blood mononuclear cells (PBMC) were cocultured with phytohemagglutinin-stimulated PBMC from HIV-seronegative blood donors. When the p24 antigen concentration in the culture exceeded 20 ng/ml, multiple aliquots of cell-free supernatant were harvested for drug susceptibility testing and the pellets of cultured cells were saved for DNA sequencing.

HIV-1 RT sequencing. Cell pellets of the cultured cells were digested with proteinase K, and the resulting lysate was subjected to nested PCR with primer pairs RT18-RT21 and RT19-RT20 (Table 1). Direct sequencing of PCR product was performed by using overlapping internal primers (Table 1), *Taq* polymerase, and dye-labelled dideoxy terminators (Applied Biosystems, Inc., Foster City, Calif.). The sequence between codons 26 to 245 of the RT gene was examined.

HIV-1 *env* heteroduplex mobility assay analysis. HIV-1 proviral DNA from cultured PBMC was amplified as previously described (1, 8, 9). First-round primers ED3 and ED14 and second-round primers ED5 and ED12 amplified an ≈1.3-kb fragment spanning the V1 to V5 region of gp120 (Table 1). Five microliters (approximately 100 to 250 ng of DNA) of second-round product from each sample was mixed with 5 μl of homologous PCR product from a panel of subtype references and 1.1 μl of 10× heteroduplex annealing buffer. The mixture was heated to 94°C for 2 min, cooled rapidly on ice, mixed with loading dye, and loaded onto a 5% acrylamide gel. Gel electrophoresis was performed with standardized conditions (1, 8, 9). To assign an uncharacterized isolate to a known subtype, the PCR fragment from the unknown isolate was reannealed with the corresponding fragment from multiple representatives of the previously identified subtypes. The heteroduplexes exhibiting the fastest mobilities between the unknown and the most closely related subtype indicated the likely envelope subtype of the isolate.

Drug susceptibility testing. The PBMC assay was identical to a previously described assay (44). A 50% tissue culture infectious dose of 30 to 100 infectious units of virus stock was used to infect one million PBMC in the presence or absence of increasing concentrations of the appropriate drug. After 4 days, the levels of p24 antigen produced were measured in the cell-free supernatant and the drug concentrations required to inhibit p24 antigen production by 50% (IC_{50}) and by 90% (IC_{90}) compared to the drug-free controls was determined by nonlinear regression. The drugs and the concentrations used were as follows:

TABLE 1. Oligonucleotide primers for PCR, sequencing, and heteroduplex mobility assay

Method and primer	Sequence	Orientation	Position[a]	Reaction
RT-PCR and sequencing				
RT18	GGAAACCAAAAATGATAGGGGGAATTGGAGG	Sense	2376–2406	External PCR
RT19	GGACATAAAGCTATAGGTACAG	Sense	2453–2474	Nested PCR
RT20	CTGCCAGTTCTAGCTCTGCTTC	Antisense	3461–3440	Nested PCR
RT21	CTGTATTTCTGCTATTAAGTCTTTTGATGGG	Antisense	3538–3508	External PCR
88	TAAAATTAAAGCCAGGAATGGATG	Sense	2577–2600	Sequencing
89	AATCTGACTTGCCCAATTCAATTT	Antisense	3335–3359	Sequencing
B	GGATGGAAAGGATCACC	Sense	3002–3018	Sequencing
B-reverse	GGTGATCCTTTCCATCC	Antisense	3002–3018	Sequencing
3W	ATGTTTTTTGTCTGGTGTGGT	Antisense	3191–3211	Sequencing
Heteroduplex mobility assay				
ED3	TTAGGCATCTCCTATGGCAGGAAGAAGCGG	Sense	5956–5985	External PCR
ED5	ATGGGATCAAAGCCTAAAGCCATGTG	Sense	6556–6581	Nested PCR
ED12	AGTGCTTCCTGCTGCTCCCAAGAACCCAAC	Antisense	7822–7792	Nested PCR
ED14	TCTTGCCTGGAGCTGTTTGATGCCCCAGAC	Antisense	7960–7931	External PCR

[a] Sequence positions correspond to bases of the HIV-1 HXB_2 genome (GenBank accession no. K03455).

zidovudine (AZT) 0.0005, 0.005, 0.05, 0.5, and 5 μM; didanosine (ddI) 0.6, 1.2, 2.5, 5, and 10 μM; lamivudine (2′,3′-dideoxy-3′-thiacytidine) (3TC) 0.016, 0.08, 0.4, 2, and 10 μM; and nevirapine, 0.016, 0.08, 0.4, 2, and 10 μM.

Nucleotide and amino acid sequence analyses. The RT sequences of 11 subtype B HIV-1 isolates isolated prior to 1990 (LAI, SF2, NL43, MN, JRCSF, OYI, CAM1, HAN, D31, RF, and YU2), the consensus subtype B sequence, the subtype D sequences (ELI, NDK, and Z2Z6), the subtype O sequences (ANT70 and MVP5180), two of the subtype A sequences (U455 and IBNG), and a presumed subtype A-D recombinant (MAL) were obtained from the Los Alamos HIV Sequence Database (33). Five recently reported Indian subtype C sequences (47) and four additional non-subtype B sequences were obtained from GenBank: CM240 (subtype A) (4), 90cf402 and 93th253 (subtype A) (12), and C2220 (subtype C) (41).

Nucleotide and amino acid sequences were aligned by using the Genetics Computer Group Wisconsin Package (13). Synonymous and nonsynonymous nucleotide distances were calculated by the method of Nei and Gojobori (34), using MEGA DNA analysis software (20). P_S is the number of observed synonymous substitutions divided by the number of possible synonymous substitutions; P_N is the number of observed nonsynonymous substitutions divided by the number of possible nonsynonymous substitutions. Phylogenetic distances were calculated from the sequence alignments by using the Kimura two-parameter model (13, 19). Dendrograms were created by neighbor-joining, maximum parsimony, and maximum likelihood methods (PHYLIP version 3.5 [10] and fastDNAml version 1.0.6 [35]). Bootstrap analysis was used to test the robustness of the neighbor-joining trees (10).

Nucleotide sequence accession numbers. Nucleotide and amino acid sequences of codons 26 to 245 of RT from 12 Zimbabwean HIV-1 subtype C isolates were submitted to GenBank (accession numbers U83603 to U83614).

RESULTS

HIV-1 *env* heteroduplex mobility assay analysis. Each of the 12 Zimbabwean HIV-1 isolates was categorized unequivocally as subtype C by the heteroduplex mobility assay. The subtype C reference strains used in the assay included MA959 (Malawi), ZM18 (Zambia), IN868 (India), and BR25 (Brazil) (9).

Amino acid alignment of Zimbabwean subtype C RT sequences. The amino acid alignment of codons 26 to 245 of the RT genes of primary isolates from 12 HIV-1-infected persons from Zimbabwe is shown in Fig. 1. The consensus subtype C sequence (in bold type) differed from the consensus subtype B sequence at 11 residues (V35**T**, E36**A**, T39**E**, S48**T**, K122**E**, D123**G**, K173**A**, D177**E**, T200**A**, Q207**A**, and V245**Q**). None of these 11 residues have been reported to confer drug resistance to current nucleoside analog or nonnucleoside RT inhibitors. Seven of these residues are naturally occurring variants reported in subtype B isolates (35T, 48T, 122E, 123G, 177E, 200A, and 207E) (33). Four residues (36A, 39E, 173A, and 245Q) have not been reported in subtype B isolates but were present in at least one of the five Indian subtype C sequences reported (47) and in related primate immunodeficiency viruses (33). Residues 36A and 48T occurred in most subtype C sequences but were rarely present in any of the other HIV-1 subtypes.

Inter- and intrasubtype genetic distances. The 12 subtype C Zimbabwean RT sequences differed from the consensus subtype B RT sequence at 10.5% (9.6 to 11.7%) of 660 nucleotides and 5.8% (4.1 to 6.8%) of 220 amino acids (Fig. 2). The intersubtype distances (Zimbabwean subtype C versus consensus subtype B) were significantly greater than the intrasubtype C sequence distances (10.5 versus 5.5% for nucleic acids [$P < 0.001$] and 5.8 versus 3.7% for amino acids [$P < 0.001$]) (Fig. 2).

The average intrasubtype nucleotide sequence divergence was significantly higher among the 12 Zimbabwean subtype C isolates (5.5%) than among the 5 recently reported Indian subtype C isolates (3.3%; $P < 0.001$) and 11 subtype B isolates (2.8%; $P < 0.001$).

Phylogenetic analysis of HIV-1 RT sequences. Phylogenetic analysis of RT nucleotide sequence data using neighbor-joining, parsimony, and maximum likelihood algorithms yielded essentially identical results. Figure 3 shows a neighbor-joining tree constructed by using the nucleotide sequence data of 11 subtype B HIV-1 RT sequences, the 12 subtype C Zimbabwean RT sequences, and the remaining published non-subtype B HIV-1 RT sequences. The high bootstrap values at the relevant nodes along the tree indicate that the subtype B, C, and D sequences each form a consistent clade. Four of the subtype A sequences also appear to form a clade (U455, CM240, 90cf402, and 93th253); in contrast, two of the putative subtype A sequences (IBNG and MAL) are on different branches of the tree.

Note that each of the Indian subtype C isolates evolved from within the cluster formed by the Zimbabwean subtype C isolates, which is consistent with the more-recent introduction of HIV-1 into India. As has previously been reported (25, 33, 47), subtype B and D isolates were closely related (their mean intersubtype nucleotide distance was 6.4% (range, 5.2 to 8.3%). The RT sequence of RF, a Haitian isolate, was the most deeply branching subtype B strain, an observation which has also been made previously based on an analysis of HIV-1 *env* sequences (24).

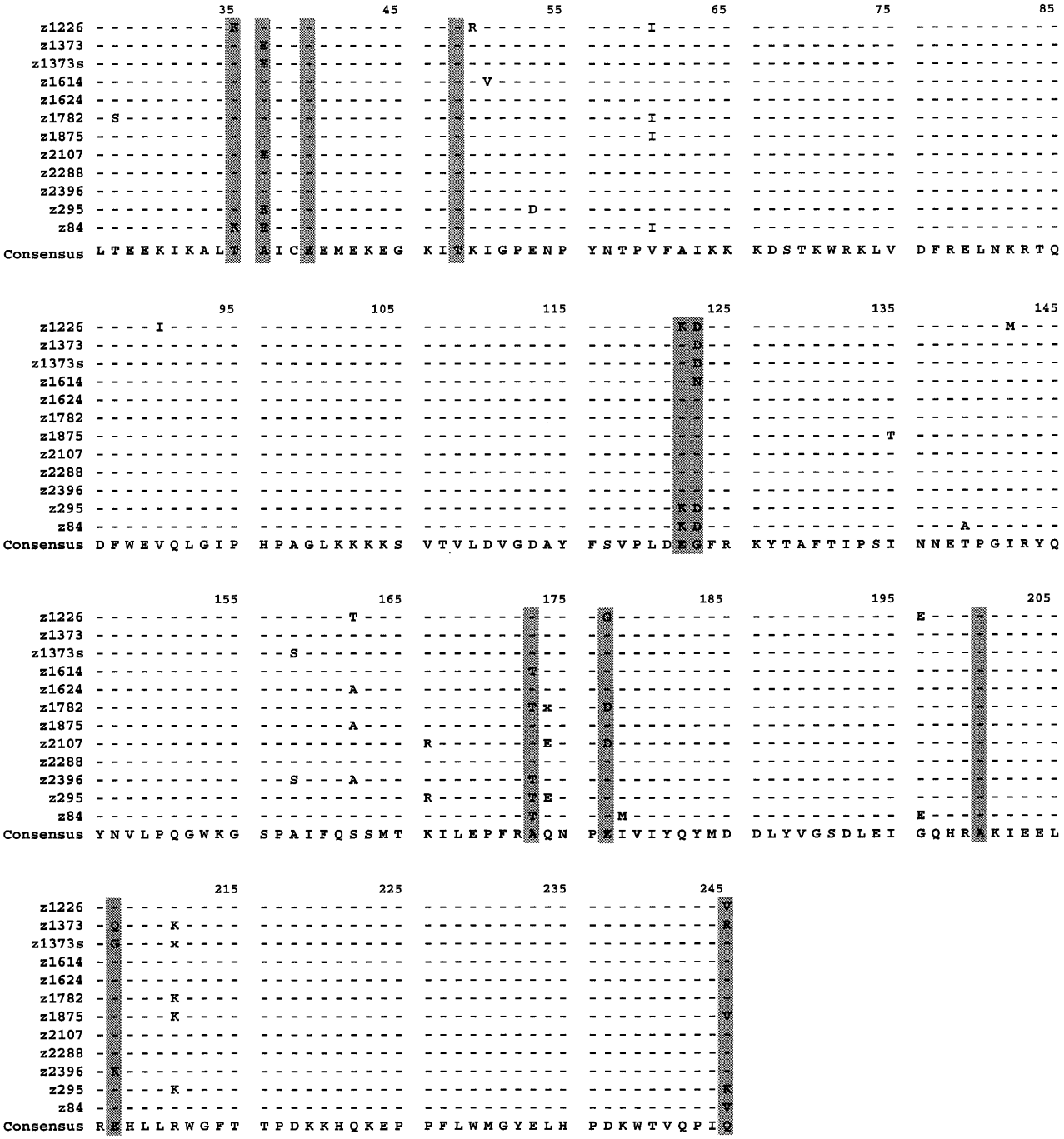

FIG. 1. Alignment of amino acid residues between codons 26 to 245 of 12 Zimbabwean HIV-1 subtype C RT sequences. A consensus sequence of the 12 sequences is shown. Conserved amino acid residues for the 12 sequences are indicated by dashes. The shaded residues are those in which the consensus sequence differs from the consensus subtype B sequence.

Synonymous and nonsynonymous nucleotide differences. There was a high ratio of synonymous (causing no amino acid change) to nonsynonymous (causing an amino change) nucleotide differences between the different HIV-1 subtypes (Table 2). Among subtypes A to D, mean intersubtype synonymous nucleotide differences (P_S) were present at 0.20 to 0.38 of potential synonymous sites, whereas mean intersubtype nonsynonymous nucleotide differences (P_N) were present at 0.01 to 0.04 of potential nonsynonymous sites (Table 2). Mean P_S and P_N values between subtype O and subtypes A to D ranged from 0.72 to 0.79 and from 0.09 to 0.11, respectively.

Synonymous nucleotide differences between subtypes B and C were present in several highly conserved amino acids and at residues involved in drug resistance. For example, the nucleotide triplet coding for 186D, one of the three catalytic aspartates, was GAC in each of the subtype C isolates compared with GAT for the subtype B sequences (Fig. 4). The nucleotides coding for 65K, 70K, 74L, 181C, 210L, and 219K, sites

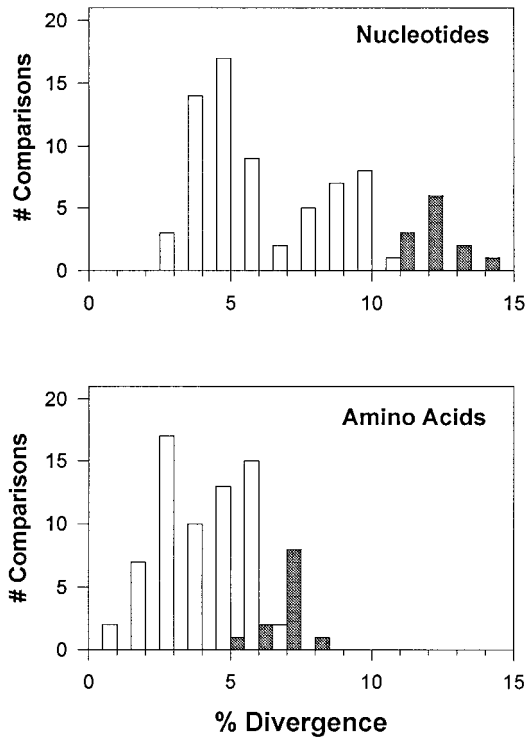

FIG. 2. Nucleotide and amino acid distances between the Zimbabwean subtype C and consensus subtype B HIV-1 RT sequences. Shaded bars represent the percent divergence between each of the 12 subtype C sequences and the consensus B sequence. White bars represent the percent divergence among the subtype C sequences.

involved in susceptibility to both nucleoside and nonnucleoside RT inhibitors, were also notable for intersubtype synonymous nucleotide differences (Fig. 4).

Susceptibility of subtype C sequences to RT inhibitors. Table 3 shows the susceptibilities of five of the Zimbabwean subtype C isolates to AZT, ddI, 3TC, and nevirapine. The mean AZT IC_{50} was 0.002 μM (range, 0.001 to 0.004 μM), the mean ddI IC_{50} was 0.4 μM (range, 0.2 to 0.7 μM), the mean 3TC IC_{50} was 0.01 μM (range, 0.002 to 0.03 μM), and the mean nevirapine IC_{50} was 0.01 μM (range, 0.003 to 0.1 μM). Each of the five strains had drug susceptibilities similar to those obtained using the same susceptibility assay on North American isolates from untreated individuals (wild-type subtype B isolates) (17, 44, 45).

DISCUSSION

Several factors contribute to the generation of HIV-1 genetic variation. First, HIV-1 RT lacks proofreading capability, and it is estimated that nearly one mutation occurs along the genome each replication cycle (6, 51). Second, HIV-1 replicates to high titers and undergoes many replication cycles in vivo (6). Third, HIV-1 is subject to multiple selective pressures (6, 32, 52). Finally, because the HIV-1 virion contains two tightly associated homologous RNA molecules, selection pressure operates on the genome itself, as well as on its products (5, 6).

Although the *pol* gene is the most conserved region of HIV-1 (29, 42, 43), variation occurs between different subtypes. However, the absence of known drug resistance mutations in the RT genes of subtype C isolates suggests that these mutations are not dominant, naturally occurring alleles. Indeed, the subtype C isolates were also found in vitro to be as susceptible as wild-type subtype B isolates to nucleoside and nonnucleoside RT inhibitors.

HIV-1 RT subtype variation and phylogenetic analysis. The subtype C RT nucleotide sequences from HIV-1-infected individuals in Zimbabwe differed from the consensus subtype B nucleotide sequence by 10.5%. By comparison, intersubtype differences in the *gag* and *env* genes are usually higher, approximately 14 and 30%, respectively (16, 25, 30, 31, 33). The lower rate of variability in RT than in *gag* and *env* is probably due to sequence requirements for enzymatic function (29, 43).

The close agreement between neighbor-joining, maximum parsimony, and maximum likelihood methods in our analysis supports the reliability of the phylogenetic tree of HIV-1 RT sequences shown in Fig. 3. The high bootstrap values at relevant nodes strongly suggest that the subtype classification scheme which is based on HIV-1 *env* and *gag* sequences also extends to the highly conserved RT gene. Bootstrap values higher than 70% in most cases represent a probability higher than 95% that the corresponding tree branch accurately represents the available data (15, 47).

Thirty-six of the 38 group M HIV-1 isolates clustered in the groups expected based on their *gag* and *env* phylogenies. Subtypes B, C, and D formed clades, and the previously noted close relationship between subtypes B and D was observed (25, 33, 46). Four of the subtype A isolates also formed a clade. This clade included three "subtype E" isolates, which are actually complex hybrids of subtypes A and E (4, 12, 25). Isolate MAL, which is considered an A-D recombinant based on its *gag* (subtype A) and *env* (subtype D) sequence (25, 33), did not consistently cluster with any of the other sequences. Isolate IBNG, which contains subtype A *env* sequence (33), also did not cluster with any of the other sequences. A recent analysis of IBNG suggests that it may also be a recombinant because its long terminal repeat clusters with subtype G isolates (12).

The lower intrasequence diversity among the recently published Indian subtype C isolates compared with that of the Zimbabwean subtype C isolates and the branching of the Indian sequences from within the Zimbabwean cluster are consistent with a founder effect caused by the more-recent introduction of HIV-1 into India. An analogous situation exists in Thailand, where the intrasubtype genetic distances for the prevalent subtypes (B and E) are much lower than the intrasubtype distances of subtypes B and E among African isolates (12, 31, 48).

Synonymous and nonsynonymous mutations. There was an extraordinary high ratio of synonymous/nonsynonymous nucleotide differences in the RT sequences both within and between different subtypes (Table 2). Indeed, the mean interpatient ratios of synonymous to nonsynonymous substitutions (P_S/P_N) ranged from 6.8 to 8.9 within subtypes A to D and from 8.3 to 10.9 between subtypes A to D (Table 2).

During reverse transcription, transitions are more common than transversions (27, 50, 51); this fact, together with the strict requirements for RT function, probably explain the high ratio of synonymous/nonsynonymous intersubtype RT differences. Higher rates of nonsynonymous mutations ($P_S/P_N \leq 1$) are observed in variable parts of the *env* region, and this phenomenon has been attributed to adaptive evolution in response to immune pressure (2, 40, 42, 52). Similarly, high rates of nonsynonymous mutations have been observed in the RT genes of patients receiving antiretroviral therapy with RT inhibitors (6, 18, 37).

HIV-1 detection. The development and evaluation of the currently used nucleic acid-based diagnostic tests for HIV-1 have been based primarily on subtype B strains from North

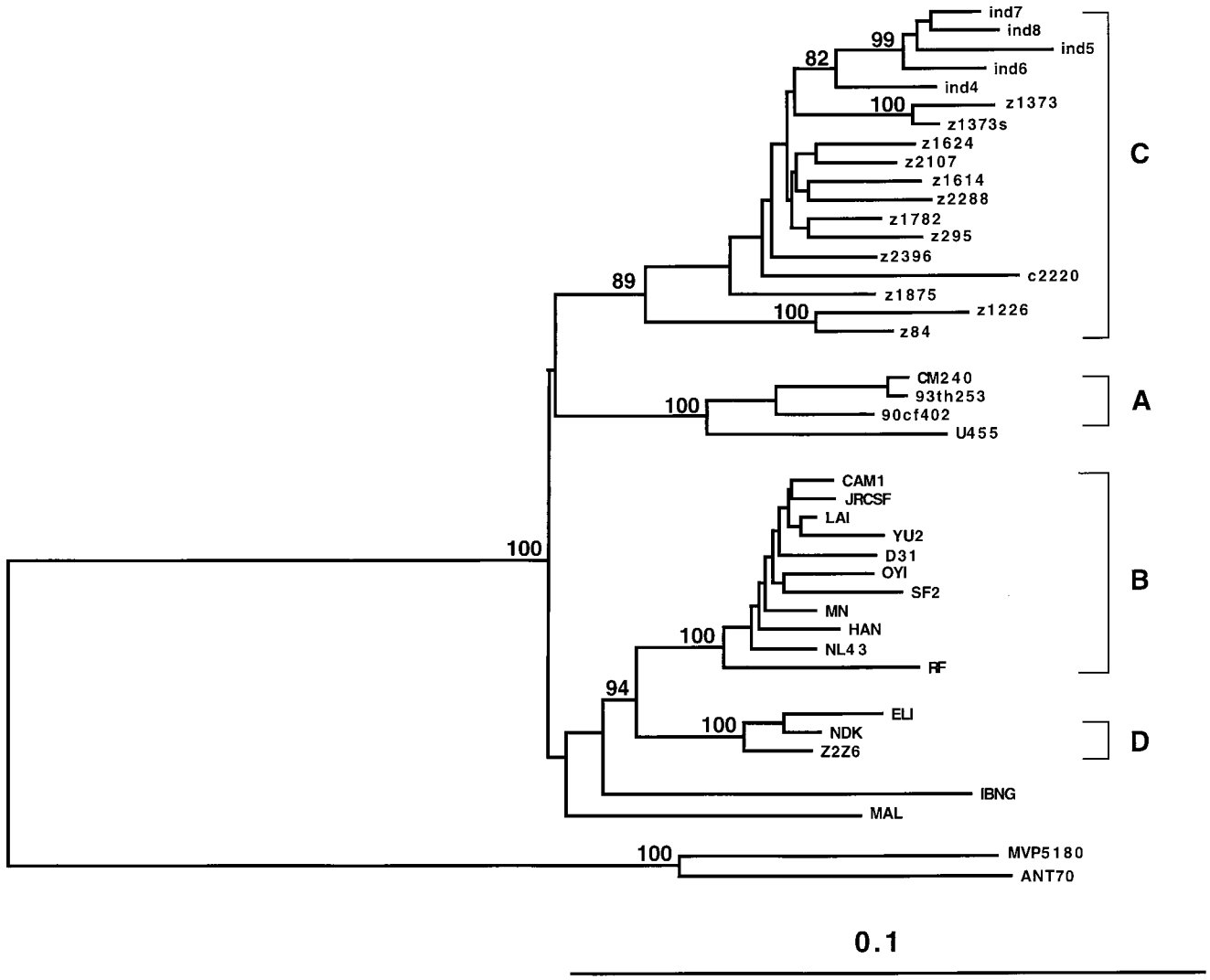

FIG. 3. Neighbor-joining tree demonstrating the genetic relationships between the RT genes (codons 26 to 245) of 40 HIV-1 isolates. These isolates included 18 subtype C isolates (12 Zimbabwean isolates, 5 Indian isolates [47], and C2220 [41]), 11 subtype B isolates isolated prior to 1990 (33), 6 subtype A isolates (CM240 [4], U455, IBNG, MAL, 90cf402 [12], and 93th253 [12]), 3 subtype D isolates (33), and 2 subtype O isolates (33). The subtype O isolates were treated as outgroups. CM240, 90cf402, and 93th253 are each A-E recombinant (subtype E *env*). MAL is a putative recombinant of *env* subtype D and *gag* subtype A. IBNG has a subtype A *env* gene but may also be a recombinant. Each number at a node is the percentage of bootstrap samples in which the cluster to the right is found. The tree and bootstrap values were determined by using PHYLIP version 3.5 (10).

TABLE 2. Intra- and intersubtype proportions of synonymous and nonsynonymous RT nucleotide differences[a]

Subtype	Synonymous differences (P_S) between subtypes					Nonsynonymous differences (P_N) between subtypes					P_S/P_N ratio[b] between subtypes				
	A	B	C	D	O	A	B	C	D	O	A	B	C	D	O
A	0.13	0.32	0.38	0.33	0.72	0.02	0.04	0.04	0.04	0.11	6.8	8.4	10.2	9.4	6.9
B		0.13	0.34	0.20	0.79		0.01	0.04	0.03	0.10		8.2	9.7	8.3	7.8
C			0.18	0.34	0.77			0.02	0.01	0.10			8.9	10.9	7.8
D				0.08	0.77				0.01	0.09				7.5	8.4
O					0.35					0.03					13.0

[a] Includes mean results obtained from 4 subtype A isolates, 11 subtype B isolates, 18 subtype C isolates, 3 subtype D isolates, and 2 subtype O isolates. IBNG and MAL were excluded because they did not cluster with subtype A, B, C, or D.

[b] The Jukes-Cantor transformations (adjustments for multiple substitutions) of the P_S/P_N ratios (D_S/D_N ratios) were slightly higher than the untransformed P_S/P_N ratios, because the transformation had a greater effect on the synonymous substitution rates than on the nonsynonymous substitution rates. However, this transformation could not be applied to the synonymous differences between subtype O and subtypes A to D because substitutions at these synonymous sites were fully saturated ($P_S > 0.7$).

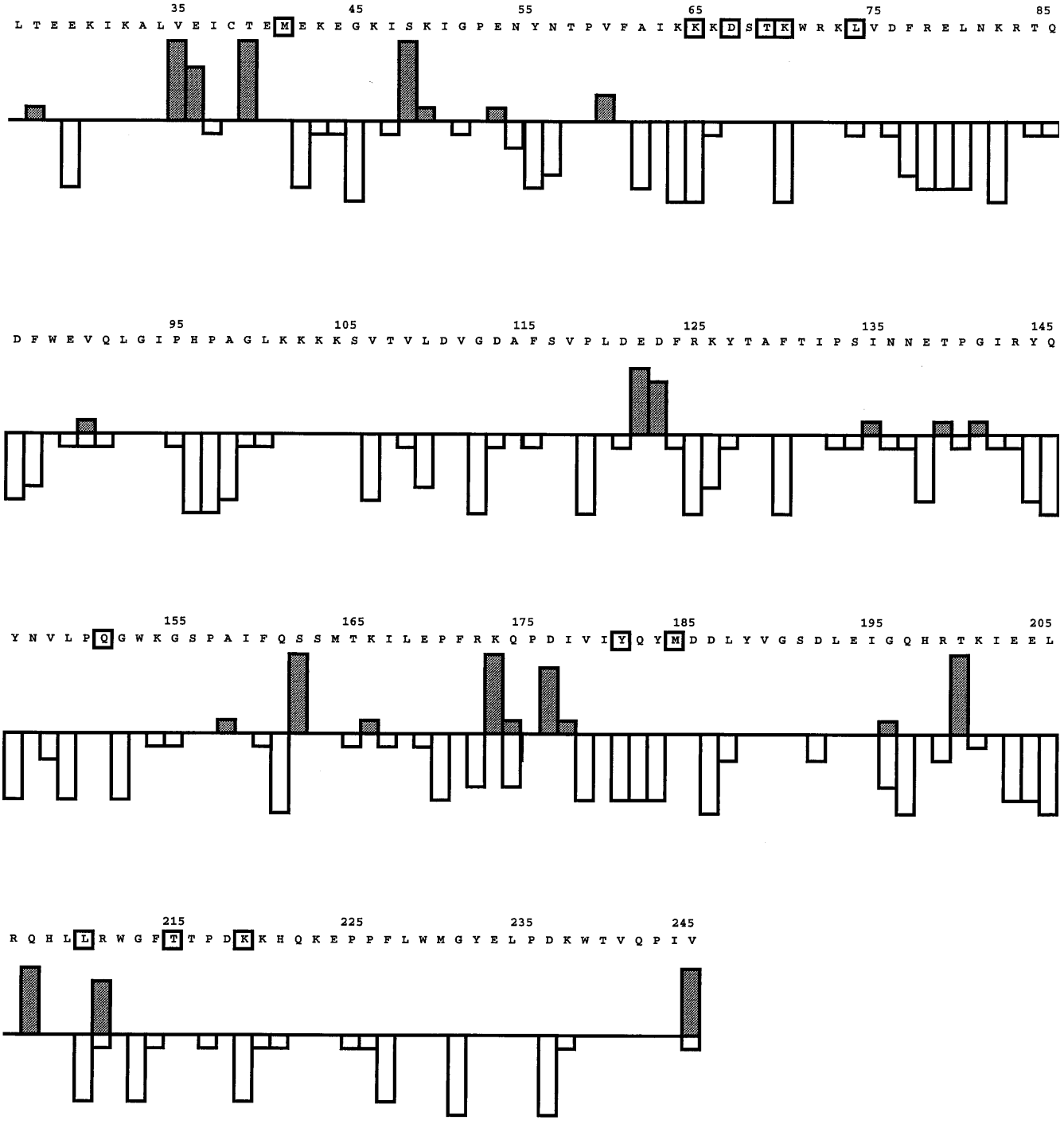

FIG. 4. Summary of synonymous versus nonsynonymous differences between each of the 12 Zimbabwean subtype C sequences and the consensus B sequence. The consensus B amino acid sequence is shown. The number of subtype C isolates with synonymous substitutions is shown by the length of the white bar below the horizontal line. The number of subtype C isolates with nonsynonymous substitutions is shown by the length of the shaded bar above the horizontal line. The 12 residues enclosed in squares are sites of drug resistance mutations. M41L, D67N, K70R, L210W, T215Y, and K219Q are associated with resistance to AZT; L74V is associated with resistance to ddI; K65R is associated with resistance to ddC; M184V is associated with resistance to 3TC; Q151M is associated with resistance to multiple nucleoside analog RT inhibitors; and Y181C is associated with resistance to nonnucleoside analog RT inhibitors.

America and Europe (16). The sensitivity of these tests in detecting divergent strains like group O and group M subtypes other than subtype B requires further evaluation. Most currently available data suggest that the most sensitive primers for detecting different HIV-1 subtypes lie in specific regions of *gag* and *pol* (14, 38, 49).

Our results, however, suggest that although the amino acid residues of HIV-1 RT are highly conserved, there is tremendous potential for synonymous mutation. Thus, it is likely that optimal strategies for HIV-1 RT detection may require PCR protocols using degenerate primers that take into account the redundancy of the genetic code.

TABLE 3. Drug susceptibilities of subtype C HIV-1 isolates[a]

Virus isolate	Drug susceptibilities (IC_{50}/IC_{90} [μM])			
	AZT	ddI	3TC	Nevirapine
z84	0.001/0.004	0.7/1.4	0.03/0.1	0.03/0.2
z1373	0.004/0.02	0.6/1.1	0.02/0.1	0.003/0.1
z1624	0.001/0.008	0.4/1.3	0.01/0.03	0.003/0.05
z1875	0.003/0.007	0.3/1.3	0.002/0.02	0.01/0.08
z2107	0.004/0.01	0.2/1.1	0.01/0.04	0.1/0.4

[a] Mean IC_{50} and IC_{90} for wild-type North American and subtype B HIV-1 isolates to different drugs were as follows: AZT, 0.001 to 0.002 and 0.01 μM, respectively; ddI, 0.4 and 1.2 μM, respectively; 3TC 0.01 and 0.05 μM, respectively; nevirapine, 0.03 and 0.1 μM, respectively (17, 44, 45).

HIV-1 treatment. Antiretroviral drug therapy, while currently beyond the means of most infected individuals in developing countries, may ultimately be used in the treatment and prevention of perinatal infection (26). In addition, non-subtype B viruses are already present in Europe and North America, and their prevalence in these areas is increasing (3, 11, 22, 23). Our study suggests that African subtype C HIV-1 isolates are susceptible to commonly used RT inhibitors. As the HIV-1 pandemic expands and the indications for drug therapy increase, further sequencing and susceptibility analysis of the molecular targets of therapy of global HIV-1 isolates will be needed.

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REFERENCES

1. **Bachmann, M. H., E. L. Delwart, E. G. Shpaer, P. Lingenfelter, R. Singal, J. I. Mullins, and WHO Network for HIV Isolation and Characterization.** 1994. Rapid genetic characterization of HIV type 1 strains from four world health organization-sponsored vaccine evaluation sites using a heteroduplex mobility assay. AIDS Res. Hum. Retroviruses **10:**1345–1353.
2. **Balfe, P., P. Simmonds, C. A. Ludlum, J. O. Bishop, and A. J. Leigh Brown.** 1990. Concurrent evolution of human immunodeficiency virus type 1 in patients infected from the same source: rate of sequence change and low frequency of inactivating mutations. J. Virol. **64:**6221–6231.
3. **Brodine, S. K., J. R. Mascola, P. J. Weiss, S. I. Ito, K. R. Porter, A. W. Artenstein, F. C. Garland, F. E. McCutchan, and D. S. Burke.** 1995. Detection of diverse HIV-1 genetic subtypes in the United States. Lancet **346:** 1198–1199.
4. **Carr, J. K., M. O. Salminen, C. Koch, D. Gotte, A. W. Artenstein, P. A. Hegerich, D. St. Louis, D. S. Burke, and F. E. McCutchan.** 1996. Full-length sequence and mosaic structure of a human immunodeficiency virus type 1 isolate from Thailand. J. Virol. **70:**5935–5943.
5. **Coffin, J. M.** 1992. Genetic diversity and evolution of retroviruses. Curr. Top. Microbiol. Immunol. **176:**143–164.
6. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science **267:**483–489.
7. **Cox, S. W., K. Aperia, J. Albert, and B. Wahren.** 1994. Comparison of the sensitivities of HIV type 2 and HIV type 1 to antiviral drugs and drug combinations. AIDS Res. Hum. Retroviruses **10:**1725–1729.
8. **Delwart, E. L., E. G. Shpaer, J. Louwagie, F. E. McCutchan, M. Grez, H. Rubsamen-Waigmann, and J. I. Mullins.** 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 *env* genes. Science **262:**1257–1261.
9. **Delwart, E. L., B. Herring, G. H. Learn, A. G. Rodrigo, and J. I. Mullins.** 1996. Heteroduplex mobility analysis HIV-1 env subtyping kit. Protocol version 3. NIH AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, Md.
10. **Felsenstein, J.** 1993. PHYLIP (phylogeny inference package) version 3.5. University of Washington, Seattle.
11. **Fransen, K., A. Buve, J. N. Nkengasong, M. Laga, and G. van der Groen.** 1996. Longstanding presence in Belgians of multiple non-B HIV-1 subtypes. Lancet **347:**1043. (Letter.)
12. **Gao, F., D. L. Robertson, S. G. Morrison, H. Hui, S. Craig, P. M. Fultz, J. Decker, M. Girard, G. M. Shaw, B. H. Hahn, and P. M. Sharp.** 1996. The heterosexual human immunodeficiency virus type 1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. J. Virol. **70:**7013–7029.
13. **Genetics Computer Group, Inc.** 1994. Wisconsin package, version 8.1. Genetics Computer Group, Inc., Madison, Wis.
14. **Grankvist, O., A. Gustaffson, U. Bredberg-Raden, K. Pallangyo, F. Mhalu, P. Biberfeld, and G. Wadell.** 1991. Selection of primers of optimal sensitivity for the detection of HIV-1 from Africa and Europe by the polymerase chain reaction. AIDS **5:**575–578.
15. **Hillis, D. M., and J. J. Bull.** 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. **42:**182–192.
16. **Hu, D. J., T. J. Dondero, M. A. Rayfield, J. R. George, G. Schochetman, H. W. Jaffe, C.-C. Luo, M. L. Kalish, B. G. Weniger, C.-P. Pau, C. A. Schable, and J. W. Curran.** 1996. The emerging genetic diversity of HIV: the importance of global surveillance for diagnostics, research, and prevention. JAMA **275:**210–216.
17. **Iversen, A. K. N., R. W. Shafer, K. Wehrly, M. A. Winters, J. I. Mullins, B. Chesebro, and T. C. Merigan.** 1996. Multidrug-resistant human immunodeficiency virus type 1 strains resulting from combination antiretroviral therapy. J. Virol. **70:**1086–1090.
18. **Keulen, W., C. Boucher, and B. Berkhout.** 1996. Nucleotide substitution patterns can predict the requirements for drug-resistance of HIV-1 proteins. Antiviral Res. **31:**45–57.
19. **Kimura, M.** 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
20. **Kumar, S., K. Tamura, and M. Nei.** 1993. MEGA: molecular evolutionary genetic analysis, version 1.0. The Pennsylvania State University, University Park.
21. **Lalvani, A., and J. S. Shastri.** 1996. HIV epidemic in India: opportunity to learn from the past. Lancet **347:**1349–1350. (Editorial.)
22. **Lasky, M., J.-L. Perret, M. Peeters, F. Bibollet-Ruche, F. Liegeois, D. Patrel, S. Molinier, C. Gras, and E. Delaporte.** 1997. Presence of multiple non-B subtypes and divergent subtype B strains of HIV-1 in individuals infected after overseas deployment. AIDS **11:**43–51.
23. **Leitner, T., D. Escanilla, S. Marquina, J. Wahlberg, C. Brostrom, H. B. Hansson, M. Uhlen, and J. Albert.** 1995. Biological and molecular characterization of subtype D, G, and A/D recombinant HIV-1 transmissions in Sweden. Virology **209:**136–146.
24. **Li, W.-H., M. Tanimura, and P. M. Sharp.** 1988. Rates and dates of divergence between AIDS virus nucleotide sequences. Mol. Biol. Evol. **5:**313–330.
25. **Louwagie, J., F. E. McCutchan, M. Peeters, T. P. Brennan, E. Sanders-Buell, G. A. Eddy, G. van der Groen, K. Fransen, G.-D. Gershy-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.
26. **Mansergh, G., A. C. Haddix, R. W. Steketee, P. I. Nieburg, D. J. Hu, R. J. Simmonds, and M. Rogers.** 1996. Cost-effectiveness of short-course zidovudine to prevent perinatal HIV type 1 infection in a sub-Saharan African developing country setting. JAMA **276:**139–145.
27. **Mansky, L. M., and H. M. Temin.** 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J. Virol. **69:**5087–5094.
28. **Mbizvo, M. T., R. Machekano, W. McFarland, S. Ray, M. Bassett, A. Latif, and D. Katzenstein.** 1996. HIV seroincidence and correlates of seroconversion in a cohort of male factory workers in Harare, Zimbabwe. AIDS **10:** 895–901.
29. **McClure, M. A., M. S. Johnson, D.-E. Feng, and R. F. Doolittle.** 1988. Sequence comparisons of retroviral proteins: relative rates of change and general phylogeny. Proc. Natl. Acad. Sci. USA **85:**2469–2473.
30. **McCutchan, F. E., M. O. Salminen, J. K. Carr, and D. S. Burke.** 1996. HIV-1 genetic diversity. AIDS **10**(Suppl. 3)**:**S13–S20.
31. **Myers, G.** 1994. HIV: between past and future. AIDS Res. Hum. Retroviruses **10:**1317–1324.
32. **Myers, G., and B. Korber.** 1994. The future of human immunodeficiency virus, p. 211–232. *In* S. S. Morse (ed.), The evolutionary biology of viruses. Raven Press, Inc., New York, N.Y.
33. **Myers, G., B. Korber, S. Wain-Hobson, R. Smith, and G. N. Pavlakis.** 1995. Human retroviruses and AIDS: a compilation and analysis of nucleic and amino acid sequences. Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, N.Mex.
34. **Nei, M., and T. Gojobori.** 1986. Simple methods for estimating the numbers

of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3:**418–426.
35. **Olson, G. J., H. Matsuda, R. Hagstrom, and R. Overbeek.** 1994. fastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput. Appl. Biosci. **10:**41–48.
36. **Pauwels, R., K. Andries, J. Desmyter, D. Schols, M. Kukla, H. Breslin, A. Raeymaeckers, J. Van Gelder, R. Woestenborghs, J. Heykants, K. Schellekans, M. Janssen, E. De Clercq, and P. Jannsen.** 1990. Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. Nature **343:**470–474.
37. **Quinones-Mateu, M. E., A. Holguin, J. Dopazo, I. Najera, and E. Domingo.** 1996. Point mutation frequencies in the *pol* gene of human immunodeficiency virus type 1 are two- to threefold lower than those of *env*. AIDS Res. Hum. Retroviruses **12:**1117–1128.
38. **Respass, R., A. Butcher, H. Wang, T. Chaowanachan, N. Young, N. Shaffer, T. Mastro, B. Biryahwaho, R. Downing, A. Tanuri, M. Schecter, R. Pascy, L. Zekeng, L. Kaptue, L. Gurtler, D. Ellenberg, C. Fridland, M. Rayfield, and S. Kwok.** 1996. Detection of HIV-1 group M and O virus by PCR, abstr. Th.A.4037. *In* Abstracts of the XI International Conference on AIDS, Vancouver, British Columbia, Canada.
39. **Richman, D. D.** 1987. Dideoxynucleosides are less inhibitory in vitro against human immunodeficiency virus type 2 (HIV-2) than against HIV-1. Antimicrob. Agents Chemother. **31:**1879–1881.
40. **Rodrigo, A. G., and J. I. Mullins.** 1996. Human immunodeficiency virus type 1 molecular evolution and the measure of selection. AIDS Res. Hum. Retroviruses **12:**1681–1685.
41. **Salminen, M. O., B. Johansson, A. Sonnerborg, S. Ayehunie, D. Gotte, P. Leinikki, D. S. Burke, and F. E. McCutchan.** 1996. Full-length sequence of an Ethiopian human immunodeficiency type 1 (HIV-1) isolate of genetic subtype C. AIDS Res. Hum. Retroviruses **12:**1329–1339.
42. **Seibert, S. A., C. Y. Howell, M. K. Hughes, and A. L. Hughes.** 1995. Natural selection on the *gag*, *pol*, and *env* genes of human immunodeficiency virus 1 (HIV-1). Mol. Biol. Evol. **12:**803–813.
43. **Seillier-Moiseiwitsch, F., B. H. Margolin, and R. Swanstrom.** 1994. Genetic variability of the human immunodeficiency virus: statistical and biological issues. Annu. Rev. Genet. **28:**559–596.
44. **Shafer, R. W., M. J. Kozal, D. A. Katzenstein, W. H. Lipil, I. F. Johnstone, and T. C. Merigan.** 1993. Zidovudine susceptibility testing of human immunodeficiency virus type 1 (HIV) clinical isolates. J. Virol. Methods **41:**297–310.
45. **Shafer, R. W., M. A. Winters, A. K. N. Iversen, and T. C. Merigan.** 1996. Genotypic and phenotypic changes during culture of a multinucleoside-resistant human immunodeficiency virus type 1 strain in the presence and absence of additional reverse transcriptase inhibitors. Antimicrob. Agents Chemother. **40:**2887–2890.
46. **Sharp, P. M., D. L. Robertson, F. Gao, and B. H. Hahn.** 1994. Origins and diversity of human immunodeficiency viruses. AIDS **8:**S27–S42.
47. **Soto-Ramirez, L. E., S. Tripathy, B. Renjifo, and M. Essex.** 1996. HIV-1 pol sequences from India fit distinct subtype pattern. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **13:**299–307.
48. **Subbarao, S., and G. Schochetman.** 1996. Genetic variability of HIV-1. AIDS **10**(Suppl. A)**:**S13–S23.
49. **Vandamme, A.-M., K. Fransen, L. Debaisieux, D. Marissens, S. Sprecher, D. Vaira, A. T. Vandenbroucke, C. Verhofstede, and the Belgian AIDS Reference Laboratories.** 1995. Standardization of primers and an algorithm for HIV-1 diagnostic PCR evaluated in patients harboring strains of diverse geographic origin. J. Virol. Methods **51:**305–316.
50. **Vartanian, J.-P., A. Meyerhans, B. Åsjö, and S. Wain-Hobson.** 1991. Selection, recombination, and G→A hypermutation of human immunodeficiency virus type 1 genomes. J. Virol. **65:**1779–1788.
51. **Williams, K. J., and L. A. Loeb.** 1992. Retroviral reverse transcriptases: error frequencies and mutagenesis. Curr. Top. Microbiol. Immunol. **176:**165–180.
52. **Wolinsky, S. M., B. T. M. Korber, A. U. Neumann, M. Daniels, K. J. Kunstman, A. J. Whetsell, M. R. Furtado, Y. Cao, D. D. Ho, J. T. Safrit, and R. A. Koup.** 1996. Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. Science **272:**537–542.