

# Characterization of a Subtype D Human Immunodeficiency Virus Type 1 Isolate That Was Obtained from an Untreated Individual and That Is Highly Resistant to Nonnucleoside Reverse Transcriptase Inhibitors

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**Human immunodeficiency virus type 1 (HIV-1) isolates derived from HIV-infected, treatment-naive Ugandan infants were propagated and tested for sensitivity to antiretroviral (ARV) drugs. Although most subtype A and D isolates displayed inhibition profiles similar to those of subtype B strains, a subtype D isolate identified as D14-UG displayed high-level resistance to nevirapine in peripheral blood mononuclear cell cultures (>2,000-fold) and in MT4 cell cultures (~800-fold) but weaker resistance to delavirdine (~13-fold) and efavirenz (~8-fold) in MT4 cell cultures. To investigate the possible mechanism for this resistance to nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs), the RT coding region in *pol* was sequenced and compared to the consensus RT sequence of NNRTI-resistant and NNRTI-sensitive subtype A, B, and D HIV-1 isolates. D14-UG did not contain the classic amino acid substitutions conferring NNRTI resistance (e.g., Y181C, K103N, and G190A) but did have some putative sites associated with drug resistance, I135L, T139V, and V245T. Wild-type and mutated protease-RT genes from D14-UG and an NNRTI-sensitive subtype D isolate from Uganda (D13-UG) were cloned into pNL4-3 to produce recombinant viruses and to determine the effects of the mutations on susceptibility to ARV drugs, specifically, NNRTIs. The results showed that I135L and/or V245T mutations can confer high-level resistance to nevirapine and delavirdine as well as low level cross-resistance to efavirenz. Finally, *ex vivo* fitness analyses suggested that NNRTI-resistant sites 135L and 245T in wild-type isolate D14-UG may reduce RT fitness but do not have an impact on the fitness of the primary HIV-1 isolate.**

Current drugs effective at inhibiting human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) are classified as nucleoside RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs) (3, 19). NRTIs interfere with enzyme activity following conversion to the triphosphate forms and incorporation into the growing DNA strand, which causes premature chain termination (4, 8). In contrast, NNRTIs are noncompetitive inhibitors which bind a hydrophobic pocket adjacent to the polymerase active site, causing an allosteric change which effectively inhibits DNA polymerization (9). One of the impediments to the frequent use of NNRTIs in combination with at least two NRTIs is the rapid emergence of resistance.

Studies of drug resistance have mainly focused on HIV-1 subtype B, which is dominant in developed countries (26). A paucity of data on the emergence of antiretroviral (ARV) drug resistance in individuals or populations infected with other subtypes (e.g., A, C, D, and CRF01) likely is attributable to limited access to ARV drug treatment in developing countries (1, 2, 13, 15, 30–32, 39, 47, 53). However, the rapid implemen-

tation of new ARV drug treatment programs in these regions likely will precede thorough investigations of ARV drug resistance in non-clade B HIV-1 isolates. Interestingly, nevirapine, as opposed to the more expensive protease (PR) inhibitors (PIs), is included with two NRTIs in almost all first-line treatment regimens in developing countries (31). Some studies have suggested that nevirapine-based regimens have an antiviral potency similar to that of PI-based regimens while lacking the possible drawbacks inherent in PI-containing regimens, such as lipodystrophy and metabolic alterations (11, 41, 50, 52). In addition, the quick absorption of nevirapine after oral dosing, the ability to cross the placenta, detection in breast milk, a long half-life, and activity against cell-free virions make nevirapine an ideal drug for preventing mother-to-child transmission (23, 25).

The rapid emergence of resistance is an obvious drawback of treatment with nevirapine-containing regimens or the use of this drug to prevent mother-to-child transmission (5, 23). Even a single dose of nevirapine can select for HIV-1 with high-level of resistance to nevirapine (28). In general, a few selected mutations (e.g., Y181C, K103N, and G190A) emerge in a conserved hydrophobic NNRTI binding pocket of RT and confer cross-resistance to all drugs in this class (e.g., delavirdine and efavirenz) (5, 38). One exception is the Y181C mutation, which is associated with over 1,000-fold resistance to nevirapine and

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delavirdine but only low-level resistance to efavirenz (6, 10). The genetic barrier to NNRTI resistance appears to be lower than the genetic barrier to resistance to other ARV drugs (5). In other words, the proportion of NNRTI-resistant clones in the HIV-1 population within an infected individual may be much higher in the absence of drug selective pressure. This notion is supported by the rapid emergence of NNRTI resistance during treatment (28) and the intrinsic NNRTI resistance of HIV-1 group O and HIV-2 isolates (21, 48).

Recent studies showed that ARV drug treatment of non-subtype B HIV-1 infections can select for novel drug resistance mutations or substitutions considered "rare" in drug-resistant HIV-1 subtype B isolates (13, 14). For example, a V106M substitution in the subtype C HIV-1 RT coding region was selected both during *in vitro* selection experiments with efavirenz and in subtype C-infected patients treated with efavirenz (13). In contrast, this mutation did not emerge in subtype B HIV-1 isolates under *in vitro* or *in vivo* efavirenz pressure.

To explore possible intrinsic resistance to ARV drugs in non-subtype B HIV-1 isolates, we determined the sensitivity of HIV-1 isolates derived from untreated Ugandans to inhibition by ARV drugs in all three drug classes. Most Ugandan isolates showed sensitivity to ARV drugs similar to that of wild-type subtype B HIV-1 isolates and even hypersensitivity to the NNRTIs. However, one subtype D isolate (D14-UG) derived in 1996 from an untreated Ugandan infant displayed high-level resistance to nevirapine and delavirdine but only low-level resistance to efavirenz. D14-UG did not contain any of the amino acid residues in RT typically associated with NNRTI resistance. Sequence alignments with RT sequences of NNRTI-sensitive subtype D isolates identified three putative amino acid residues associated with this high-level resistance: 135L, 139V, and 245T. Only substitutions at position 135 (I135T and I135L) have been associated with NNRTI resistance in subtype B isolates (14).

Wild-type and mutated PR-RT genes from D14-UG and other subtype A and D isolates were cloned into pNL4-3 to produce recombinant viruses. These viruses were tested for susceptibility to ARV drugs. Reversion mutations in D14-UG and "resistance" mutations in NNRTI-sensitive isolate D13-UG confirmed that both position 135 and position 245 were associated with resistance. *In vitro* fitness analyses were performed with both the primary isolates and their recombinant PR-RT counterparts. These experiments suggested that although L135 and T245 have a negative impact on RT fitness, this effect likely is compensated for in the primary isolates by other regions of the genome and/or replication steps.

#### MATERIALS AND METHODS

**Chemicals.** The NRTIs abacavir, didanosine, lamivudine, stavudine, zalcitabine, and zidovudine were obtained from the NIH AIDS Reagent Repository, the respective pharmaceutical company, or Sigma (didanosine, zidovudine, and zalcitabine). The NNRTIs delavirdine, efavirenz, and nevirapine and the PIs amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir were obtained from the same sources. Chemicals were dissolved in dimethyl sulfoxide, filtered with 0.22- $\mu$ m-pore-size filters, and diluted in media to the appropriate concentrations for use.

**Cells and viruses.** Peripheral blood mononuclear cells (PBMCs) from HIV-seronegative blood donors were obtained by Ficoll-Hypaque density gradient centrifugation of heparin-treated venous blood. Prior to HIV-1 infection, cells

were stimulated with 2  $\mu$ g of phytohemagglutinin (PHA) (Gibco BRL) per ml for 3 to 4 days and maintained in RPMI 1640–2 mM L-glutamine medium (Cellgro) supplemented with 10% fetal bovine serum (Cellgro), 1 ng of interleukin-2 (IL-2) (Gibco BRL) per ml, 100 U of penicillin (Cellgro) per ml, and 100  $\mu$ g of streptomycin (Cellgro) per ml. Human T-cell lymphotropic virus type 1-infected cell line MT2 or MT4 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U of penicillin (Cellgro) per ml, and 100  $\mu$ g of streptomycin (Cellgro) per ml. PBMCs and MT2 and MT4 cells were incubated at 37°C in 5% CO<sub>2</sub>.

Five primary isolates (A14-UG, A15-UG, D13-UG, D14-UG, and D15-UG) were obtained from five treatment-naïve HIV-1-infected pediatric patients in Kampala, Uganda, in 1996. The patients were identified as HIV seropositive in plasma, and neither they nor their mothers had received any ARV treatment. The lack of ARV drug treatment and the availability of propagated virus from known patients were the rationale for selecting these HIV-1 isolates from a pediatric cohort rather than an adult cohort. However, screening for intrinsic ARV drug resistance in these viruses was not necessarily a systematic or planned study. HIV-1 was isolated and propagated by coculturing PBMC samples from the patients and from healthy donors. Prior to being cocultured, PBMC samples were prestimulated with PHA and cultured with IL-2 as described above. Every third day, virus production in the culture fluids was monitored by using an RT assay. Assays for 50% tissue culture infective doses were performed to determine virus titers in culture fluids at peak virus concentrations as described previously (44). Titers were expressed as infectious units per milliliter. For the strains listed above, the letter and number before the hyphen indicate the subtype of the virus and the strain number, and the letters after the dash indicate the country of origin; e.g., D14-UG is a clade D HIV-1 strain isolated in Uganda. The coreceptor usage of each primary HIV-1 isolate (A14-UG, A15-UG, D13-UG, and D14-UG) was determined by exposing CD4-positive U87 cells expressing CCR5 (U87.CD4.CCR5 cells) or CD4-positive U87 cells expressing CXCR4 (U87.CD4.CXCR4 cells) to virus and assaying for RT activity every third day. U87 cells expressing CD4 and a coreceptor were cultured in Dulbecco minimal essential medium containing 15% fetal calf serum, 100 U of penicillin (Cellgro) per ml, 100  $\mu$ g of streptomycin (Cellgro) per ml, 300  $\mu$ g of G418 per ml, and 1  $\mu$ g of puromycin per ml.

**Sequencing and phylogenetic analyses.** Cell pellets from virus cocultivation in PBMCs were lysed, and DNA was extracted with a QIAamp DNA blood kit (Qiagen). External nested PCR was used to amplify the HIV-1 Gag, Env, and PR-RT fragments from this DNA.

To amplify a 1,272-nucleotide (nt) fragment of the CA-MA region in Gag, the conserved external primers used were GS6 (5'-GTGCGAGAGCGTCAGTATT AAG; nt 794 to 815) and GA9 (5'-GGGCTATACATTCTACTATT; nt 1605 to 1625) or GS1 (5'-TAAACATATAGTATGGGCAAGC; nt 881 to 903) and GA4 (5'-TTGCCAAGAGTGACCTGAGGAA; nt 2250 to 2273), and the internal primers used were GS7 (5'-TAGTATGGGCAAGCAGGGAGCT; nt 890 to 911) and GA8 (5'-TCCCATTCTGACAGCTTCTCTATTG; nt 1404 to 1427) or GS2 (5'-GGGGGGACATCAAGCAGCCATGC; nt 1362 to 1384) and GA3 (5'-GGGGCTGTGGCTGTGGCTGTGCTGCTG; nt 2137 to 2162), respectively. To amplify a 2,040-nt fragment in the Env region, the external primers used were envB (5'-AGAAAGAGCAGAAGAGCTGGCAATGA; nt 6202 to 6228) and ED14 (5'-TCTTGCCTGGAGCTGTTTGTATGCCCCAGAC; nt 7932 to 7961) or E80 (5'-TCTTGCCTGGAGCTGTTTGTATGCCCCAGAC; nt 6858 to 6879) and envN (5'-CTGCCAATCAGGGAAGTAGCCTTGTGT; nt 60 to 86), and the internal primers used were KpnI-env (5'-TGTGGGTCACAGTCT ATTATGG; nt 6325 to 6346) and E125 (5'-CAATTTCTGGGTCCTCCCTCTG AGG; nt 7315 to 7338) or E110 (5'-CTGTTAAATGGCAGCTAGCAGAA; nt 7002 to 7025) and A/D gp41 envA (5'-GGTGATACCTGCCTAACTCTA TTTACTACAGAAAGCACAGC; nt 8322 to 8365), respectively. To amplify a 1,632-nt fragment in the RT region, the external primers used were univ-PS1 (5'-TTTTTTAGGGAAAATTTGGCCTTC; nt 2085 to 2108) and INTAA61 (5'-CCCAATCCCCCTTTTCTTTTAAAAATGTG; nt 4776 to 4806), and the internal primers used were univ-PS2 (5'-TCCCTCAAATCACTCTTTGGC AAC; nt 2251 to 2274) and HIVRTD51-6H3' (5'-GCCCCATCTACATAAAG CTTGAAAGTTTCTGC; nt 3858 to 3883).

The PCR products were purified with a QIAquick gel extraction kit (Qiagen) and then sequenced by Davis Sequencing, Inc. The primers used for sequencing were GS2, GA3, GS7, and GA8 for the Gag fragments; KpnI-env, E110, A/D gp41 envA, and ED14 for the Env fragments; and univ-PS2 and univ-RTA5 for the RT fragments (47). Confirmatory sequencing of the PR-RT region was performed with the ViroLogic assay (40). The Gag, Env, and RT sequences of the five HIV-1 isolates used in this study and the corresponding set of reference strains (Fig. 1) were aligned with Clustal X, version 1.83b. Phylogenetic trees based on nucleotide distance were constructed by the neighbor-joining method

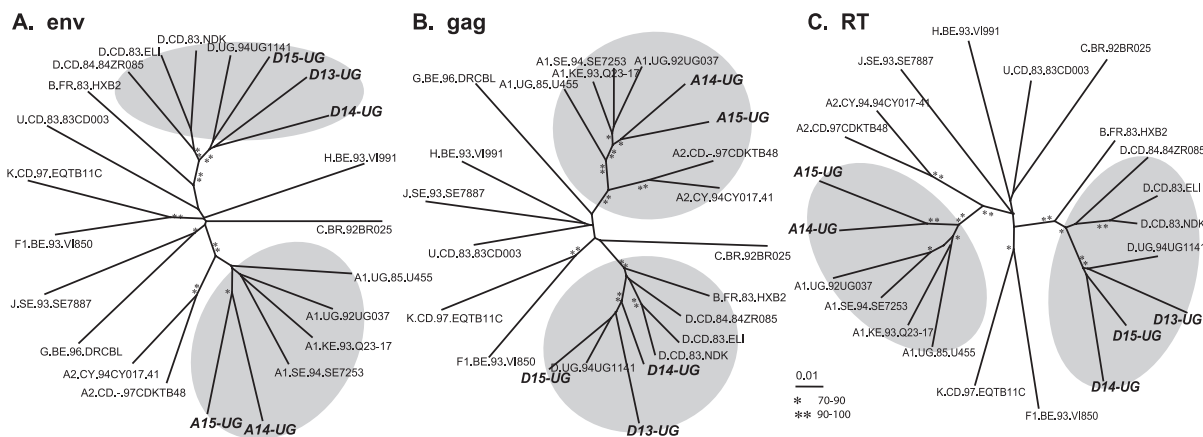


FIG. 1. Phylogenetic analyses of the *env*, *gag*, and RT gene sequences of five primary HIV-1 isolates from Uganda. A 1,900-bp region of the *env* gene (most of the gp120 coding region and a fragment of the gp41 coding region) (A), a 1,200-bp region of the *gag* gene (part of the MAP17 coding region and the coding regions for CAP24 to NCp7) (B), and a 910-bp region of the RT gene (C) were used to construct phylogenetic trees by the neighbor-joining method. *env*, *gag*, and RT subtypes are indicated and defined by clusters with reference HIV-1 sequences. Bootstrap resampling values of 70 to 90% and >90% are indicated by single asterisks and double asterisks, respectively. The branch lengths are drawn to scale. The scale bar represents 0.01 substitution per nucleotide.

as implemented in Clustal X, version 1.83b, with 1,000 bootstrap resamplings and were schematically represented with the TreeView program.

**Drug susceptibility and resistance assays for primary isolates.** Five primary HIV-1 isolates were used to infect MT4 cells or PHA- and IL-2-treated PBMCs from HIV-seronegative blood donors at a multiplicity of infection of 0.001 infectious unit/cell in a 96-well plate ( $5 \times 10^5$  cells/well). After 5 h, the cells were washed three times with phosphate-buffered saline and then incubated with various concentrations of ARV drugs (10-fold dilutions from 100  $\mu$ M to 0.001 nM) in a 96-well plate. Each isolate was evaluated in triplicate for susceptibility to NNRTIs (nevirapine, efavirenz, and delavirdine), NRTIs (zidovudine and lamivudine), and PIs (lopinavir, indinavir, and ritonavir). Drug inhibition of viral replication was determined by measuring relative RT activity in culture supernatants 5, 7, and 10 days after infection. The concentration required for 50% inhibition ( $IC_{50}$ ) was calculated from three different experiments and expressed as the mean and standard deviation.

**Determining the ARV drug susceptibility of the PR-RT region.** The drug susceptibility of the PR-RT region from the Ugandan HIV-1 isolates was measured with the PhenoSense HIV assay (40). Briefly, PR and RT coding sequences were amplified by RT-PCR and cloned into a recombinant HIV vector containing a luciferase reporter gene by using restriction enzymes *ApaI* and *PinAI*. The resistance test vectors were transfected into 293 cells; virus was harvested and used to infect fresh 293 cells in the presence or absence of drug. The concentration of drug required to inhibit viral replication by 50% in a single-cycle assay was determined with reference to that for a drug-sensitive reference strain (CNDO) containing PR and RT coding sequences from laboratory HIV strain NL4-3. Overall, the assay is reproducible within a 2.5-fold range (17). A panel of 15 ARV drugs were used in the PhenoSense HIV assay.

**Site-directed mutagenesis.** Mutations were introduced into the RT coding regions of D13-UG and D14-UG by using the megaprimer method (22). Briefly, a sense primer spanning the mutation is used in an amplification reaction with an antisense primer that anneals to sequences 3' of the mutated region and spans the *PinAI* site in the NL4-3 RT coding region. The product of the first amplification reaction is used as a megaprimer in a second PCR in combination with a sense primer that anneals to sequences 5' of the mutated region and spans the *ApaI* site in the *gag* coding region of NL4-3. The product generated in the second PCR and containing the mutated sequence is cloned into the reference vector by using the *ApaI* and *PinAI* sites. The sequence of the entire *ApaI*-*PinAI* segment of each clone is confirmed by DNA sequencing.

**HIV-1 fitness estimates from growth competition infections and heteroduplex tracking assays (HTAs).** All five primary HIV-1 isolates and NL4-3 were used for mono-infection and dual infection of PBMCs. Viruses were added alone or in pairs to PHA- and IL-2-treated PBMCs at a multiplicity of infection of 0.001 infectious unit/cell in a 24-well plate ( $2 \times 10^6$  cells/well). After 5 h of incubation at 37°C with 5%  $CO_2$ , cells were washed three times with phosphate-buffered saline and then resuspended in complete medium ( $10^6$  cells per ml). All mono-infection and dual-infection-competition experiments were performed in dupli-

cate with PBMCs from a single donor. Uninfected cultures were used as HIV-negative controls. Cell-free supernatants were assayed for RT activity at days 3, 5, 7, and 10 postinfection. Aliquots of cells were removed at days 5, 7, and 10 postinfection. Supernatants and cells were stored at  $-80^\circ C$  for subsequent analysis.

HTAs were used to monitor and quantify dual virus production in these competition experiments. For these HTAs, the HIV-1 *env* gene (C2-V3) was PCR amplified from each dual infection and mono-infection (43). The same *env* genomic region was also PCR amplified from a subtype A or subtype E HIV-1 *env* clone for use as a DNA probe. For this amplification, primer E80 was radiolabeled with T4 polynucleotide kinase and 2  $\mu$ Ci of [ $^{32}P$ ]ATP. Radiolabeled PCR-amplified probes were separated on 1% agarose gels and purified with a QIAquick gel extraction kit.

Reaction mixtures containing DNA annealing buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 2 mM EDTA), 10  $\mu$ l of unlabeled PCR-amplified DNA from the competition culture, and approximately 0.1 pmol of radioactive probe DNA were denatured at 95°C for 3 min and then annealed at 37°C for 5 min. After 30 min on ice, the DNA heteroduplexes were resolved on 6% nondenaturing polyacrylamide gels (acrylamide-bisacrylamide, 30:0.8) in Tris-borate-EDTA buffer for 4 h at 200 V. Gels were dried and exposed to X-ray film (Eastman Kodak Co., Rochester, N.Y.). Heteroduplexes representing the production of each isolate in a dual infection were quantified with a Bio-Rad PhosphorImager.

In HIV-1 competition experiments, the final ratio of the two viruses produced from a dual infection was determined by HTAs and relative to virus production in mono-infections. Relative fitness ( $w = f_0/i_0$ ) values then were derived from the production of individual HIV-1 isolates in a dual infection ( $f_0$ ) divided by the initial proportion in the inoculum ( $i_0$ ). The ratio of the relative fitness values for each HIV-1 variant in a competition experiment is a measure of the fitness difference ( $W_D$ ) between the two HIV-1 strains ( $W_D = w_M/w_L$ ) (16), where  $w_M$  and  $w_L$  correspond to the relative fitness values of the more fit and less fit viruses, respectively.

**Single-cycle assays to determine the replication capacity of recombinant HIV-1.** Viral replication capacity was measured by using a modification of the single-replication-cycle phenotype assay described previously (43). Briefly, recombinant virus stocks were used to infect 293T cells in the absence of drug. Viruses containing PR and RT sequences derived from wild-type viruses (D13-UG and D14-UG), site-directed mutants, or a drug-sensitive reference virus (NL4-3) were tested. The relative luciferase units (RLU) produced by cells infected with different viruses were routinely measured at 72 h postinfection. The amount of luciferase activity detected in the infected cells was used as a direct measure of infectivity or replication capacity, i.e., the ability of the virus to complete a single round of replication.

Three methods for normalizing the infection data to account for variations in transfection efficiencies were evaluated: (i) infection with a constant amount of virus input based on p24, (ii) postinfection normalization based on the amount of p24 in the virus stock, and (iii) postinfection normalization based on the

luciferase activity produced in transfected cells (the RTV transfected into 293T cells contains a luciferase expression cassette). All three methods for normalization of the infection data resulted in similar measures of replication capacity. Normalization was routinely performed by using transfection luciferase levels.

Replication capacity was calculated as follows:  $\{1 - [(infection\ RLU\ for\ the\ test\ virus \times CF_{TXN}) / (infection\ RLU\ for\ the\ control\ virus)]\} \times 100$ . In this equation,  $CF_{TXN}$  is the correction factor for the transfection efficiency and is calculated with the following equation:  $CF_{TXN} = \text{transfection RLU for the test virus} / \text{transfection RLU for the control virus}$ . Replication capacity is expressed as a percentage of the replication capacity of the reference virus. The in vitro replication capacity measurement has a reproducibility of  $\pm 0.2$  log unit for independent measurements.

## RESULTS

**Characterization of HIV-1 isolates.** A collection of HIV-infected pediatric PBMC was obtained from Kampala, Uganda, in 1996. Sequence analyses of the PR-RT fragment from 20 untreated patients suggested the absence of amino acids associated with ARV drug resistance. HIV-1 was propagated by cocultivation from five of the 20 patient PBMC samples. HIV-1 isolates A14-UG, A15-UG, D13-UG, D14-UG, and D15-UG were subjected to thorough genotypic and phenotypic assays described below.

The HIV-1 C2-C4 region of Env, portions of the PR-RT coding sequence, and the MA-CA region of Gag were PCR amplified, sequenced, and aligned with several HIV-1 reference sequences. Neighbor-joining phylogenetic trees of sequences from these three regions are presented in Fig. 1 and clearly show clustering of A14-UG and A15-UG with subtype A and clustering of D13-UG, D14-UG, and D15-UG with subtype D. There was no evidence of intersubtype recombination between or within any gene region when bootscanning analysis (window size, 200 bp) and the recombination identification program were used. Coreceptor utilization (CXCR4 tropic [X4] or CCR5 tropic [R5]) and biological phenotype (syncytium inducing [SI] or non-SI [NSI]) were predicted from the amino acid sequence in the V3 region of Env, i.e., positively charged amino acids at positions 306 and 322 in the loop as well as an overall positive charge. A14-UG, A15-UG, D13-UG, and D14-UG were predicted to be X4/SI, whereas only D15-UG appeared to be R5/NSI. Coreceptor utilization was confirmed for these isolates by using U87.CD4.CXCR4 and U87.CD4.CCR5 cells. Usage of coreceptors was as predicted by the sequences, except for A14-UG, which appeared to be dualtropic; i.e., it infected both U87.CD4.CCR5 and U87.CD4.CXCR4 cells.

**Susceptibilities of the Ugandan primary HIV-1 isolates to ARV drugs.** The susceptibilities of five primary HIV-1 isolates to NNRTIs (nevirapine, efavirenz, and delavirdine), NRTIs (zidovudine and lamivudine), and PIs (lopinavir, indinavir, and ritonavir) are shown in Tables 1 and 2. The  $IC_{50}$  of nevirapine for D14-UG was 32.5  $\mu$ M in PBMCs, i.e., at least 2,000- to 10,000-fold higher than the  $IC_{50}$  of nevirapine for other subtype A or D isolates. Complete inhibition of D14-UG was not observed with even 100  $\mu$ M nevirapine (Fig. 2A). In MT4 cell cultures, this level of nevirapine resistance in D14-UG was somewhat muted (an  $IC_{50}$  of 2.3  $\mu$ M;  $\sim$ 1,000-fold resistance) compared to that in the other subtype A or D isolates (Table 2 and Fig. 2B). As described below, this level of nevirapine resistance was derived from an ARV drug treatment-naive Ugandan and is as high as that reported for any HIV-1 strains

TABLE 1. Susceptibilities of subtype A and D HIV-1 isolates to selected ARV drugs in PBMC cultures

Virus	Sub-type	Mean $\pm$ SD $IC_{50}$ (nM) of the following drug in PBMC cultures <sup>a</sup> :			
		AZT	3TC	NVP	IDV
A14-UG	A	0.18 $\pm$ 0.07	0.5 $\pm$ 0.007	3.6 $\pm$ 2.9	3.7 $\pm$ 0.78
A15-UG	A	0.21 $\pm$ 0.13	0.95 $\pm$ 0.64	3.1 $\pm$ 1.2	2.5 $\pm$ 0.7
D13-UG	D	0.36 $\pm$ 0.18	0.23 $\pm$ 0.11	5.8 $\pm$ 0.28	3.3 $\pm$ 0.21
D14-UG	D	2.1 $\pm$ 0.35	4.2 $\pm$ 0.14	32,500 $\pm$ 212	3.5 $\pm$ 0.07
D15-UG	D	0.14 $\pm$ 0.14	5.5 $\pm$ 0.64	15 $\pm$ 2.8	2.9 $\pm$ 2.7

<sup>a</sup> Data were calculated as described in Materials and Methods. Each value is the mean of three independent experiments. Abbreviations: AZT, zidovudine; 3TC, lamivudine; NVP, nevirapine; and IDV, indinavir.

containing known NNRTI resistance mutations (e.g., K103N or Y181C). D14-UG was also cross-resistant to delavirdine (13-fold) and efavirenz (8-fold) relative to another subtype D isolate, D13-UG, in MT4 cells (Table 2 and Fig. 2C and D). In contrast, all subtype A and D isolates, including D14-UG, displayed similar susceptibilities to both PIs and NRTIs in both PBMC and MT4 cell cultures (Tables 1 and 2). The only exception was a slightly decreased susceptibility to lamivudine in D14-UG and D15-UG relative to A14-UG, A15-UG, and D13-UG (5- to 10-fold). The sequence analyses described below provide no explanation for this decreased susceptibility or possible intrinsic resistance. Unlike the NNRTI resistance displayed by D14-UG, the low level of lamivudine resistance observed with PBMC and MT4 cell cultures was not reproduced in the PhenoSense assay (i.e., testing the drug sensitivity of the D14-UG PR-RT coding region cloned into the NL4-3 virus).

**Determining the ARV drug susceptibility of the PR-RT region of subtype A and D isolates.** The PR-RT region of the five HIV-1 isolates was cloned into an NL4-3 backbone and used in the PhenoSense assay to determine susceptibility to ARV drug inhibition. Although all approved NRTIs, NNRTIs, and PIs were used in the PhenoSense assay, we report only hypersusceptibility ( $<0.5$ -fold) and resistance ( $>3$ -fold) to specific ARV drugs relative to NL4-3 sensitivity (Table 3). Recombinant viruses with the PR-RT region of D14-UG were 19.8-fold resistant to nevirapine, 18.3-fold resistant to delavirdine, and 3.7-fold resistant to efavirenz compared to the reference virus, NL4-3 (Table 3). As with the primary HIV-1 isolates, all subtype A and D isolates remained as sensitive to ARV drugs as NL4-3 and even became hypersusceptible to specific PIs (i.e., amprenavir and loprinavir) and almost all NNRTIs. Taking into account the NNRTI hypersusceptibility of the subtype D isolates, D14-UG was approximately 100-, 50-, and 12-fold resistant to nevirapine, delavirdine, and efavirenz, respectively. It should be noted that subtype A and D PR-RT sequences were cloned into a subtype B laboratory strain, a strategy which may account for the minor discrepancies in the NNRTI sensitivities of the primary and PR-RT-cloned HIV-1 isolates.

**Comparison of the PR-RT amino acid sequences to identify putative sites conferring NNRTI resistance in D14-UG.** The PR-RT amino acid sequences of five primary isolates were compared with the consensus PR and RT sequence of subtype A, B, and D isolates along with subtype B NNRTI-resistant isolates. Table 3 lists the PR-RT amino acid substitutions that differed between the consensus subtype B isolate and each of the five primary HIV-1 isolates. Bold type in Table 3 indicates

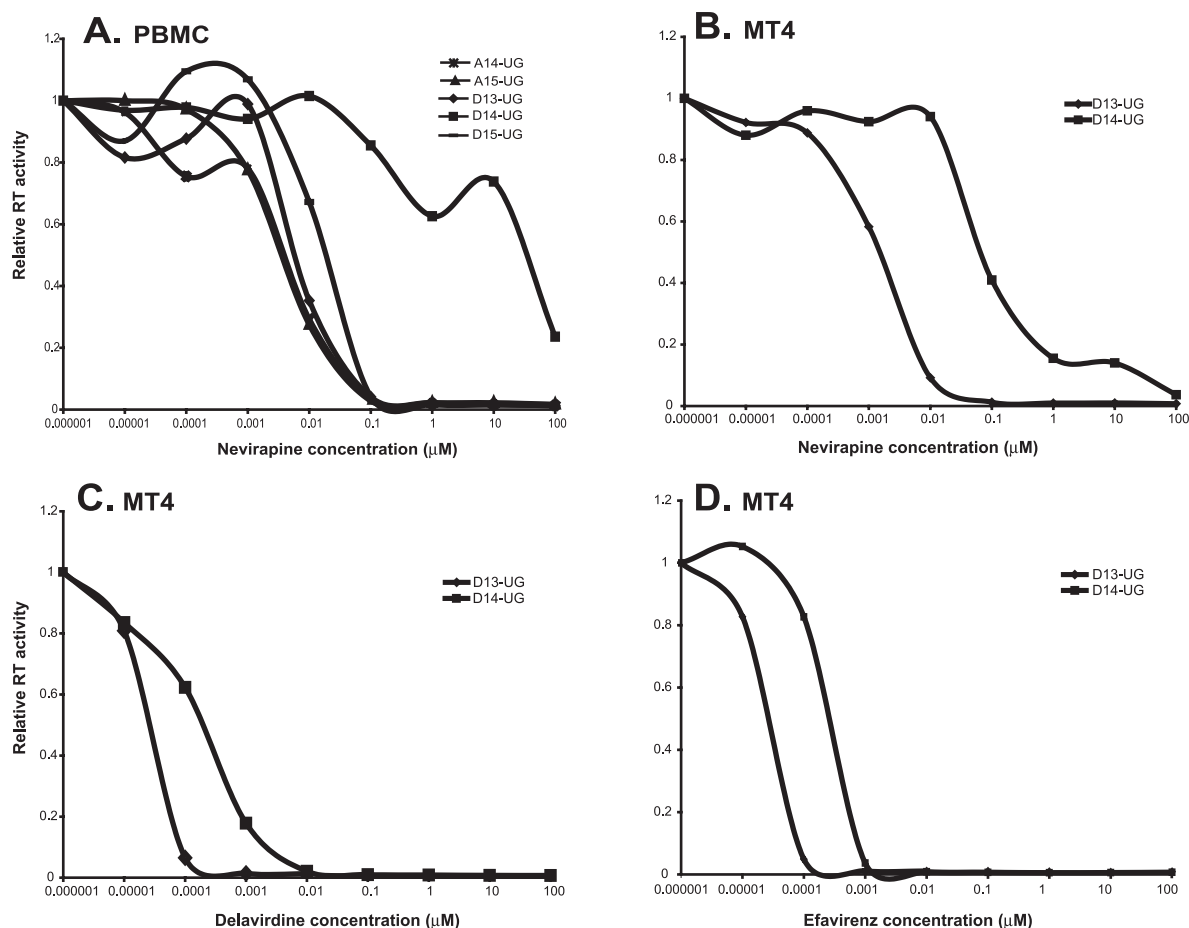


FIG. 2. Susceptibility and resistance of the subtype A and D HIV-1 isolates to nevirapine, delavirdine, and efavirenz. The graphs show the change in the relative RT activity on the y axis as a function of the drug concentration (on a log<sub>10</sub> scale) on the x axis. (A) Susceptibility of all five primary HIV-1 isolates to nevirapine in PBMC cultures. (B, C, and D) Susceptibility of both D13-UG and D14-UG to nevirapine, delavirdine, and efavirenz, respectively, in MT4 cell cultures. Error bars are not shown on the curves, but standard deviations derived from triplicate experiments were typically 10 to 20% of the value.

amino acid substitutions that are defined as secondary or compensatory mutations for ARV drug resistance (e.g., M36I in the PR of A14-UG, D14-UG, and D15-UG). Substitutions known to confer high-level ARV drug resistance (e.g., primary drug-resistant mutations) were not observed in any of the subtype A or D isolates, including the NNRTI-resistant isolate D14-UG. A polymorphism was observed at one site associated with NRTI resistance, i.e., T69T/M (also indicated by bold type in Table 3).

To identify the putative sites conferring NNRTI resistance in D14-UG, we first compared the RT substitutions between the NNRTI-sensitive subtype D isolates D13-UG and D15-UG

and the resistant isolate D14-UG. In the first 330 amino acid residues of RT, only seven sites (T39A, K49R, D121Y, I135L, T139V, V245T, and I257L) had different amino acids in D14-UG than in NNRTI-sensitive D13-UG and D15-UG. Of these seven sites, only three sites had unique amino acids in D14-UG (L135, V139, and T245) compared to other subtype D isolates, a finding which may explain the high level of NNRTI resistance. To identify sites in close proximity to the NNRTI binding pocket, each of these amino acid residues in D14-UG RT (i.e., substitutions from other subtype D isolates) was also mapped onto the X-ray crystal structure of a subtype B RT-nevirapine complex (Fig. 3). Only I135L and T139V in the p51

TABLE 2. Susceptibilities of subtype D HIV-1 isolates to NNRTIs and selected ARV drugs in MT4 cultures

Virus	Mean $\pm$ SD IC <sub>50</sub> (nM) of the following drug in MT4 cell cultures <sup>a</sup> :							
	AZT	3TC	EFV	NVP	DLV	IDV	LPV	RTV
D13-UG	4.6 $\pm$ 0.14	1.5 $\pm$ 0.07	0.025 $\pm$ 0.006	2.9 $\pm$ 2.2	0.024 $\pm$ 0.005	2.9 $\pm$ 0.71	0.125 $\pm$ 0.078	2.1 $\pm$ 0.71
D14-UG	0.39 $\pm$ 0.09	4 $\pm$ 0.7	0.2 $\pm$ 0.11	2,300 $\pm$ 1,270	0.32 $\pm$ 0.12	2.2 $\pm$ 0.42	0.15 $\pm$ 0.028	4.4 $\pm$ 1.8

<sup>a</sup> Data were calculated as described in Materials and Methods. Each value is the mean of three independent experiments. Abbreviations: AZT, zidovudine; 3TC, lamivudine; EFV, efavirenz; NVP, nevirapine; DLV, delavirdine; IDV, indinavir; LPV, lopinavir; and RTV, ritonavir.

TABLE 3. Phenotypic assays for drug susceptibilities and substitutions in the PR-RT coding sequence (from consensus subtype B isolate NL4-3)

Virus	Coreceptor usage	Biotype	ARV drug <sup>a</sup>	Ratio of hypersusceptible isolates to resistant isolates	Substitution(s) in PR <sup>b</sup>			Substitution(s) in RT <sup>b</sup>		
A14-UG	Dual tropic		DLV	0.2	I13V	K14R	I15I/V	K11T	K20R	V21V/I
			EFV	0.3	G16G/E	E35D	<b>M36I</b>	V35T	T39L	Q102K
			NVP	0.2	R41K	R57K	H69K	K122E	D123N	C162S
			AMP	0.3	L89M			K173L/S	Q174K	D177G
							I178I/V	V179I	T200A	
							I202V	Q207N	R211K	
							<i>V245Q</i>	E248D	R277K	
							T286A	E291D	P294T	
A15-UG	CXCR4	SI	DLV	0.4	I13V	K14R	G16E	K11T	K20R	V21I
			EFV	0.4	K20R	E35D	<b>M36I</b>	V35T	R83R/K	Q102K
			AMP	0.3	P39P/S	R41K	R57K	K122K/E	D123D/N	C162S
			NFV	0.4	H69K	I72I/T	L89M	K173A/S	D177G	V179I
							I202V	Q207A	R211R/K	
							<i>V245Q</i>	E248D	R277K	
							T286A	E291D	P294T	
D13-UG	CXCR4	SI	ZDV	0.3	T12V	I13V	R41K	V35T	E40D	V60I
			DLV	0.4	<b>I64V</b>		<b>T69T/N</b>	Q102K	K122E	
			NVP	0.2			I142I/V	C162S	D177E	
							I178M	G196G/E	T200K/M	
							E204D	Q207E	R211K	
							P243T	<i>V245K</i>	K249Q	
							A272S	L282C	<i>L283I</i>	
							T286A	E297Q		
D14-UG	CXCR4	SI	DLV	18.3	I13V	I15L	L33V	K22R	V35T	T39A
			EFV	3.7	R41K	<b>I64V</b>		E40D	K49R	V60I
			NVP	19.8				Q102K	D121Y	K122E
			AMP	0.4				<i>I135L</i>	<i>T139V</i>	C162S
			0.4				D177E	T200I	I202V	
			0.4				Q207E	R211K	<i>V245T</i>	
							P247P/A	D250E	I257L	
							A272P	R277K	L282C	
							L283I			
D15-UG	CCR5	NSI			I13V	E35E/D	<b>M36I</b>	K11K/T	K20K/R	K22K/R
					R41K	D60D/E	I62I/V	V35T	T39I/M	V60V/I
					<b>L63L/P</b>	<b>I64V</b>	H69Y	Q102K	K122E	D123D/N
					L89L/M			<i>I135I/T</i>	C162S	V179V/I
							T200T/I	I202I/V	Q207E	
							R211K	<i>V245K/Q</i>	E248E/D	
							D250D/E	A272A/P	R277R/K	
							L282C/R	<i>L283L/I</i>	T286T/A	

<sup>a</sup> Abbreviations: DLV, delavirdine; EFV, efavirenz; NVP, nevirapine; AMP, amprenavir; ZDV, zidovudine; and LPV, lopinavir.

<sup>b</sup> Bold type indicates amino acid substitutions that are defined as secondary or compensatory mutations for ARV drug resistance. Italic type indicates possible primary substitutions for drug resistance.

subunit were found in close proximity to the NNRTI binding site, while V245T in the p66 subunit was about 15Å away from this hydrophobic binding pocket.

**Impact of the I135L and V245T mutations on the drug susceptibility of recombinant HIV-1.** Based on these RT sequence and crystal structure comparisons, we suspected that L135 and T245 in D14-UG may confer resistance to nevirapine and other NNRTIs. To test this hypothesis, oligonucleotide-based site-directed mutagenesis was used to introduce the L135I or T245V mutation into the RT gene of the D14-UG PR-RT–NL4-3 chimeric strain and the I135L or V245T mutation into the D13-UG PR-RT–NL4-3 chimeric strain. Mutations conferring a putative sensitive phenotype are denoted with “S,” and those conferring a putative resistance phenotype are denoted with “R,” e.g., L135I (S) in D14-UG and I135L (R) in D13-UG. Recombinant viruses with the mutated RT

were then tested for sensitivity to ARV drugs by using the PhenoSense assay (40). As depicted in Fig. 4, the resistance of D14-UG to nevirapine was reduced from 18.3-fold (relative to that of NL4-3) to 1.8- and 2.4-fold after the introduction of the L135T (S) and T245V (S) mutations, respectively. Similar increases in delavirdine or efavirenz sensitivity (or decreases in resistance) were observed with these mutations in D14-UG. It is important to note that none of these mutations increased NNRTI sensitivity to the hypersusceptible levels observed with D13-UG or D15-UG. Conversely, D13-UG became resistant to delavirdine, efavirenz, and nevirapine when the I135L (R) mutation was used; e.g., the IC<sub>50</sub> of nevirapine (relative to that for NL4-3) increased from 0.2- to 2-fold. However, the V245T (R) mutation did not confer significant NNRTI resistance in mutated D13-UG; i.e., the IC<sub>50</sub> of nevirapine (relative to that for NL4-3) increased from 0.2- to 0.5-fold. Although each

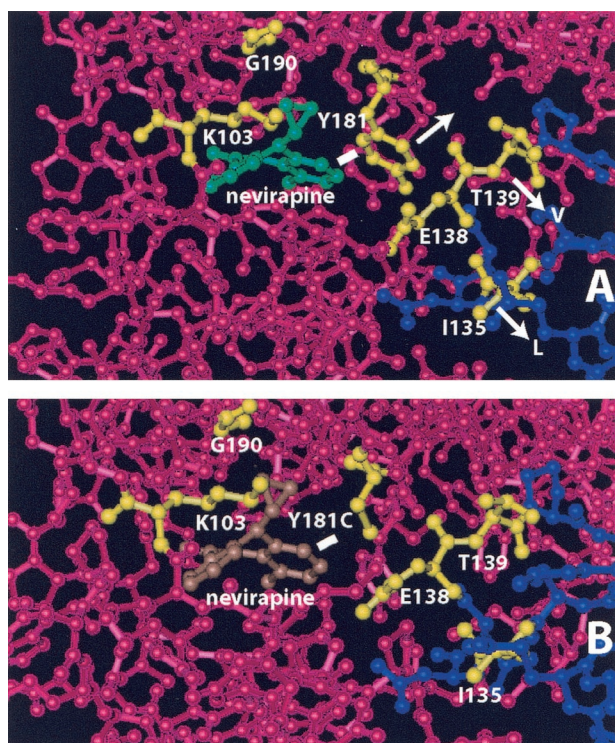


FIG. 3. Structures and positions of amino acid residues within the NNRTI binding pocket of HIV-1 RT complexed with nevirapine or efavirenz. The X-ray crystal structures of HIV-1 RT complexed with nevirapine (A), (17) and of HIV-1 Y181C RT complexed with nevirapine (B) (24) are displayed at the same perspective and size. It is important to note that the structures in panels A and B were derived from the same RTs (HXB2). The resolutions of the structures were 3.0 Å for panel B and approximately 2.4 Å for panel A. The distance between the aromatic rings for nevirapine and tyrosine 181 in panel A is approximately 1 Å (white bar). The arrows in panel A illustrate the possible movement of amino acid positions due to the T139V and T135L substitutions found in D14-UG.

mutation by itself may confer significant resistance, it is quite possible that these mutations and T139V may have a cooperative or synergistic effect on resistance to NNRTIs.

**Comparison of the relative levels of fitness of NNRTI-resistant and NNRTI-sensitive HIV-1 isolates.** Pairwise competitions were performed with PBMCs and each primary HIV-1 isolate. The relative production of each isolate in a dual infection was assessed by HTAs to provide a measure of *ex vivo* fitness for that virus (i.e., relative fitness) as well as the difference in fitness between the two isolates in the dual infection. Figure 5A illustrates the difference in fitness between isolates in pairwise competitions. In general, the viruses had similar relative fitness values, as indicated by the mean relative fitness values for four competitions (with the exception of virus D15-UG, which was competed against only D13-UG, D14-UG, and D15-UG) (Fig. 5A and B). Interestingly, the NSI/R5 subtype D isolate D15-UG outcompeted two of the SI/X4 isolates (D13-UG and D14-UG) and showed fitness equal to that of A14-UG and A15-UG in PBMC competitions. This is yet another example of how an NSI/R5 isolate can replicate more efficiently than an SI/X4 isolate in PHA- and IL-2-treated PBMC cultures (34, 43). The primary NNRTI-resistant isolate D14-UG was slightly less fit than A14-UG and A15-UG and was outcompeted by D15-UG in direct competitions. D14-UG did, however, outcompete D13-UG, the least fit of all of the primary HIV-1 isolates. These results suggest that NNRTI-resistant D14-UG replicates with nearly the same efficiency as NNRTI-sensitive subtype A and D isolates. These results are summarized in the plot in Fig. 5B. With the exception of virus D13-UG, there was no significant difference in the mean relative fitness values derived from the pairwise competitions of the five viruses. To place these results into context with previous studies, note that it is common to find 10- to 100-fold differences in relative fitness values among primary, wild-type HIV-1 isolates (7).

Primary isolates were also competed against NL4-3 in PHA- and IL-2-treated PBMCs. HTAs were used to calculate the

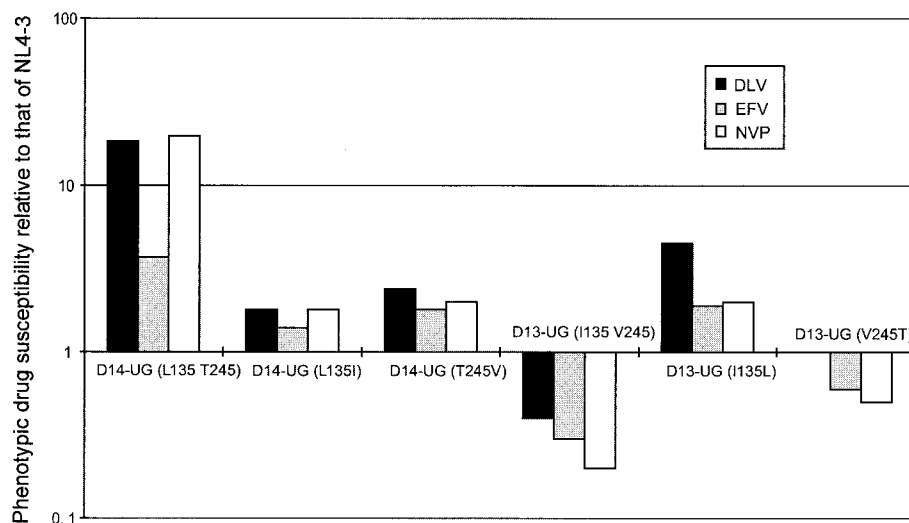


FIG. 4. NNRTI susceptibility of HIV-1 recombinants containing the subtype D wild-type and mutated PR-RT segments. The PR-RT regions of wild-type D14-UG, wild-type D13-UG, L135I D14-UG, T245V D14-UG, I135L D13-UG, and V245T D13-UG were cloned into an NL4-3 background. Susceptibility to nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV) is reported as the  $IC_{50}$  relative to that of NL4-3.

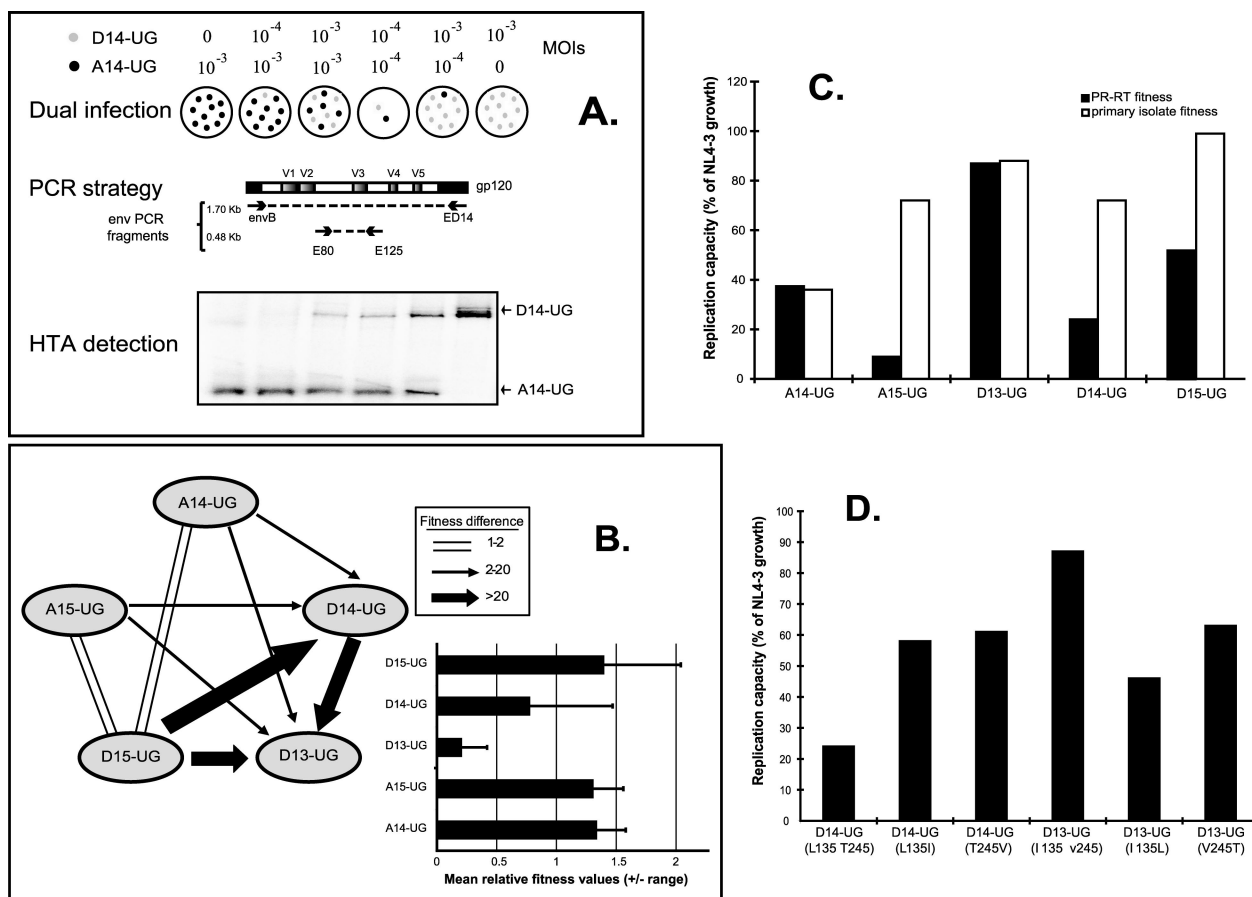


FIG. 5. Relative fitness values for primary isolates and recombinant viruses with wild or mutated PR-RT. (A) Schematic representation of the competition assays and HTAs to determine the fitness of D14-UG versus A14-UG. MOIs, multiplicities of infection. (B) Fitness values and differences derived from pairwise competitions. The mean relative fitness value and standard error for a particular isolate were obtained from four competitions against the other four HIV-1 isolates in PBMCs. An arrow indicates the “winner” of each competition; the thickness of the arrow indicates the extent of the fitness difference derived from this competition. Relative fitness values were measured as the proportion of each virus produced from the dual infection divided by the initial fraction of that virus added to the culture. (C) Competition assays with the five primary HIV-1 isolates and NL4-3 in PBMCs (white bars). Although the same relative fitness values and fitness differences were calculated, fitness was reported as a percentage of NL4-3 growth in direct competition. Black bars indicate the single-cycle replication capacity of the PR-RT- NL4-3 recombinants in MT2 cells relative to that of NL4-3. (D) Impact of specific mutations in the PR-RT regions of D13-UG and D14-UG on the replication of recombinant viruses, determined by using single-cycle infection assays with MT2 cells. Replication capacity is reported as a percentage of NL4-3 growth.

percentage of primary isolate production relative to that of NL4-3 within a competition. The replication capacity of recombinant viruses with the PR-RT region of the five HIV-1 isolates was determined as single-cycle growth by measurement of luciferase activity (see Materials and Methods). Relative PR-RT virus growth was normalized to that of NL4-3 virus growth in separate MT2 cell infections. It is important to note that fitness derived from competition with a single HIV-1 isolate (e.g., NL4-3) is less accurate than that obtained from pairwise competition as described above. Replication of the primary HIV-1 isolates relative to that of NL4-3 ranged from 36 to 99% in competition experiments performed with PBMC cultures, whereas replication of the PR-RT- NL4-3 recombinant viruses relative to that of NL4-3 ranged from 9 to 87% in single-cycle mono-infections of MT2 cells. However, results from the PR-RT- NL4-3 single-cycle growth assays did not always match those from the growth competition experiments with the primary HIV-1 isolates. For example, the replication

capacity of NNRTI-resistant D14-UG was 72% that of NL4-3 in competition experiments (Fig. 5C) but was less than 25% the growth of NL4-3 when the A14-UG PR-RT- NL4-3 virus was used in single-cycle mono-infections. Although the fitness of the primary A14-UG and D13-UG isolates were nearly identical to that of their recombinant PR-RT counterparts, the fitness values for the A15-UG, D14-UG, and D15-UG PR-RT regions in the recombinant viruses were at least threefold lower than that observed with the parental primary HIV-1 isolate. These results suggest that other regions in the HIV-1 genome or steps in the replication cycle may compensate for poor PR-RT fitness, especially if the intrinsic NNRTI resistance mutations have a negative impact on the fitness of the complete virus.

The replication capacity of recombinant viruses with mutated PR-RT was also investigated with the single-cycle assay. Both the L135T (S) and the T245V (S) mutations increased the replication capacity of D14-UG from 24 to >50%, whereas

TABLE 4. Polymorphisms at RT amino acid positions associated with NNRTI resistance

Subtype	% Specific amino acid(s) at the following position for the indicated subtype (no. of sequences examined):					
	103		135		139	
	NNRTI naive <sup>a</sup>	NNRTI treated <sup>b</sup>	NNRTI naive <sup>a</sup>	NNRTI treated <sup>b</sup>	NNRTI naive <sup>a</sup>	NNRTI treated <sup>b</sup>
A	100 K (204)	91 K, 8 N, <3 Q (96)	56 I, 42 T, <3 R/L (200)	52 T, 41 I, 3 V, <3 R/M/L (96)	99 T, <3 A/K (200)	98 T, <3 A (96)
B	97 K, <3 R/N/Q/T/E (2,998)	51 K, 45 N, <3 R/S/T (1,155)	61 I, 25 T, 9 V, <3 L/M/R/K/A/N (2,973)	57 I, 27 T, 10 V, 3 L, <3 M/K/R (1,156)	99 G, <3 R/E (2,982)	100 G (1,160)
C	100 K (410)	85 K, 15 N (13)	70 I, 13 V, 12 T, 5 R, <3 M (407)	91 I, 9 V (11)	97 T, 3 A (406)	100 T (10)
D	100 K (177)	80 K, 18 N, <3 E/D (74)	74 I, 18 T, 6 V, <3 L/M (173)	67 I, 25 T, <3 V/R/M (73)	91 T, 6 A, <3 S/V (172)	96 T, 4 A (74)
Total no. of sequences examined <sup>c</sup>	3,731	1,310	3,695	1,308	3,702	1,312

<sup>a</sup> Perinatal transmission cohort,  $n = 93$  (R. Troyer and E. J. Arts, unpublished data).

<sup>b</sup> Perinatal transmission cohort,  $n = 44$  (Troyer and Arts, unpublished).

<sup>c</sup>  $n = 93$  (Troyer and Arts, unpublished).

<sup>d</sup> Perinatal transmission cohort,  $n = 44$  (Troyer and Arts, unpublished).

<sup>e</sup> All remaining sequences (not listed in footnotes *a* through *d*) were obtained from the Stanford HIV Drug Resistance Database (46).

I135L (R) and V245T (R) decreased the replication capacity of D13-UG from 87 to 46 and 63%, respectively. As discussed below, it is quite possible that the NNRTI-resistant L135 and T245 residues emerged in the RT coding region of D14-UG only when other regions of the genome or steps in the replication cycle compensated for lower RT activity. Alternatively, wild-type RT activity may not have been observed with recombinant viruses because the entire RT coding sequences of these diverse subtype A and D isolates (amino acid residues 330 to 560) were not cloned into NL4-3. In other words, the subtype A or D-subtype B NL4-3-RT chimeras in these recombinants may have had lower activity than any of the parental RTs.

**Polymorphisms at RT sites associated with NNRTI resistance.** Polymorphisms associated with NNRTI resistance were examined by using the predicted RT amino acid sequences of subtype A, B, C, and D isolates (Table 4). Over 3,000 RT sequences were obtained from the Stanford HIV Drug Resistance Database (46), NNRTI treatment-naive and NNRTI-treated adult cohorts in Uganda (47), a perinatal mother-to-infant transmission cohort from Uganda, and the ViroLogic sequence database. Analyses of these sequences clearly indicated that the L135 polymorphism in any subtype was extremely rare (<1%) (Table 4). With the exception of D14-UG, L135 was not linked to T245 in any of the RT sequences. There did appear to be some subtype-specific polymorphisms that may be associated with NNRTI resistance. Both T245 and I283 were found at much higher frequencies in the NNRTI treatment-naive subtype D RT sequences than in the RT sequences of any other subtype. The frequency of T135 (41%) was much higher in subtype A than in the other subtypes. The I135T substitution in subtype D RT was associated with low-level NNRTI resistance (14). Interestingly, the percentage of I135 (56%) was higher than that of T135 (42%) in the subtype A isolates from treatment-naive patients, in contrast to the percentage of each in the NNRTI-treated subtype A-infected population (I135, 41%; T135, 52%). This change at position 135

just bordered on significance based on chi-square distributions ( $P < 0.05$ ). However, the frequency of amino acids at any other position identified in Table 4 showed no significant difference in the presence or absence of NNRTI treatment. Finally, the frequency and number of amino acid polymorphisms at sites 135, 139, 245, and 283 were much higher than those observed at the typical sites associated with high-level NNRTI resistance, i.e., 103, 181, and 190.

## DISCUSSION

The rapid expansion of new HIV-1 subtypes in the developed world and the increase in ARV drug treatment in the developing world pose new challenges in HIV-1 disease management. To date, limited information is available on the efficacy of ARV drugs and the emergence of drug resistance among genotypically diverse non-subtype B viral subtypes (1, 2, 13, 15, 30–32, 39, 53). HIV has a high mutation rate; thus, drug-resistant strains can rapidly emerge under the pressure of ARV drugs. Mutations associated with resistance are classified as primary or secondary. Primary mutations usually decrease susceptibility to specific drugs but are extremely rare in the absence of treatment. However, these primary ARV drug resistance mutations can be found at low frequencies in intrapatient HIV-1 quasispecies (40). Typically, poor fitness due to limited replication capacity prevents the emergence and dominance of these mutations in the quasispecies or the interpatient HIV-1 population (7). Continual drug pressure in an individual or even in the human population will select not only for primary drug resistance mutations but also for secondary or compensatory mutations that will improve replication capacity (7, 27, 35, 42). Finally, divergent evolution and differential selective pressure even in the absence of deliberate interventions (e.g., ARV drug treatment) may force HIV-1 isolates through a different but rare evolutionary pathway and fitness landscape (22, 36). Fortunately, the success of current ARV

TABLE 4—Continued

% Specific amino acid(s) at the following position for the indicated subtype (no. of sequences examined):							
181		190		245		283	
NNRTI naive <sup>a</sup>	NNRTI treated <sup>b</sup>	NNRTI naive <sup>a</sup>	NNRTI treated <sup>b</sup>	NNRTI naive <sup>c</sup>	NNRTI treated <sup>d</sup>	NNRTI naive <sup>c</sup>	NNRTI treated <sup>d</sup>
99 Y, <3 C (200)	94 Y, 6 C (96)	98 G, <3 A (96)	61 Q, 14 E, 10 K, 9 V, <3 T/M/I/L/N (162)	58 Q, 19 E, 8 K, 5 M, 4 V, 4 T, <3 N (74)	98 L, <3 I/E (162)	90 L, 10 I (76)	
99 Y, <3 N (2,990)	100 Y (1,157)	99 G, <3 A/R/S/E (2,990)	83 G, 14 A, <3 S/E/Q/R (1,155)	68 V, 10 E, 9 M, 5 K, 3 T, <3 Q/I/L/A/S/N/D/R (1,645)	67 V, 12 E, 7 K, 6 M, 3 T, <3 Q/R/I/L (826)	96 L, 4 I, <3 P (876)	94 L, 6 I (604)
100 Y (404)	100 Y (11)	100 G (404)	97 G, 3 A (11)	78 Q, 9 K, 4 V, 3 L, <3 E/H/N (297)	80 Q, 20 K (5)	97 L, <3 I (278)	100 L (6)
99 Y, <3 N (172)	92 Y, 8 C (74)	100 G (172)	93 G, 7 A (74)	44 K, 17 Q, 16 E, 12 T, 4 M, <3 N/H/L/I/V (108)	67 K, 11 E, 11 Q, 4 R, <3 M/T/N (46)	73 L, 27 I (111)	66 C, 21 L, 13 I (53)
3,708	1,310	3,718	1,308	2,212	951	1,427	739

drug therapies (with the exception of possibly entry inhibitors) (20, 51) may not be significantly compromised by the diversity in the HIV-1 population.

Our study is one of the few that have found innate high-level HIV-1 resistance to NNRTIs and identified at least two of the responsible mutations in the RT coding sequence, I135L and V245T. The primary subtype D isolate, D14-UG, was highly resistant to nevirapine and cross-resistant to delavirdine and efavirenz, while the other two subtype D isolates (D13-UG and D15-UG) and two subtype A isolates (A14-UG and A15-UG) were still sensitive. Recombinant viruses containing the PR-RT coding regions of these primary isolates showed the same pattern of resistance to NNRTIs as D14-UG. Both of the “sensitive” L135I and T245V mutations in the D14-UG RT increased susceptibility to NNRTIs but not to the level of the hypersusceptible D13-UG RT. On the other hand, the “resistant” I135L mutation in the D13-UG RT significantly decreased NNRTI susceptibility; the decrease caused by V245T was much smaller. It has been reported that L135 in combination with I283 in subtype B RT can confer low-level resistance to NNRTIs (~5-fold) (21)—but significantly less than that observed in 135L- and 245T-containing subtype D RT (>1,000-fold). Various amino acid substitutions at position 135 or 283 in subtype B isolates could not independently confer NNRTI resistance. In our study, both NNRTI-resistant D14-UG and NNRTI-sensitive D13-UG RTs as well as most subtype D RTs harbored I283 as the wild-type residue (Table 4). In contrast, I283 was very rare in subtype A RTs.

Sites 135, 139, 245, and 283 in the RT gene of all subtypes were much more polymorphic than the other RT sites most commonly associated with NNRTI resistance, e.g., 103, 181, and 190. The amino acid residues (L135 and V139) possibly responsible for high-level NNRTI resistance were found at extremely low frequencies (<1%) and were not linked with the possible compensatory sites, T245 and I283. Interestingly, NNRTI treatment of subtype A-infected patients appears to have resulted in an increase in T135 over the I135 polymorphism. T135 may be associated with low-level NNRTI resistance (21). These analyses of the RT sequences from both NNRTI treatment-naïve and NNRTI-treated populations sug-

gest that polymorphisms associated with NNRTI resistance are found at higher frequencies in subtype A and D isolates than in isolates of other subtypes. This observation may have significant consequences for treatment strategies as well as programs for the prevention of perinatal transmission in eastern Africa, e.g., Uganda, Kenya, and Tanzania (1, 2, 13, 15, 18, 30–32, 47, 53). However, this discovery of intrinsic NNRTI resistance in a subtype D HIV-1 isolate does not imply that the vast majority of isolates of subtype D or any other subtype are less sensitive to NNRTIs. In fact, it appears that subtype A and D isolates may be hypersusceptible to NNRTIs. To date, treatment with two NRTIs and an NNRTI has been quite effective in maintaining low to undetectable viral loads and is the preferred ARV drug treatment regimen in Uganda (32).

The primary D14-UG isolate displayed higher levels of resistance to both nevirapine and delavirdine than to efavirenz, suggesting an NNRTI resistance pattern similar to that conferred by Y181C (17, 24, 33, 37, 45). A careful examination of the X-ray crystal structure of RT complexed with nevirapine suggests that specific substitutions at positions 135, 138, and 139 in the p51 subunit may be synonymous with the Y181C substitution in p66 (Fig. 3) (24, 33, 45, 49). Previous mechanism studies suggested that the E138K mutation conferred NNRTI resistance through the p51 subunit of RT (12, 29). It is thought that the Y181C substitution may remove the aromatic ring stacking with nevirapine and actually open the binding pocket, thus destabilizing nevirapine binding (Fig. 3) (45). The small changes imposed by I135L, E138K, and T139V substitutions in RT may increase the distance or rotate the phenyl ring in Y181 relative to nevirapine. In the structure presented in Fig. 3 and structures not shown, nevirapine and delavirdine bind throughout the NNRTI pocket, whereas efavirenz makes closer contact with one end of the pocket, i.e., in close juxtaposition to position 103 and distal to position 181. The K103N substitution likely changes the NNRTI binding pocket but from the face opposite that of position 181 (33, 45, 49). Thus, positions 135, 138, and 139 in p51 are likely to have minimal effects on efavirenz binding to RT (structure not shown). Finally, the role of RT site 245 in NNRTI resistance is unclear, since its position in both the p66 and the p51 subunits is distal

to the NNRTI binding pocket. Residues at both positions 245 and 283 may act as secondary mutations by providing the proper structure for RT function. It is important to note that the mechanism of action of positions 135 and 138 was modeled on a subtype B RT structure (12, 29) that shares about 90 to 95% amino acid identity with the RTs of subtype D isolates.

As described above, the very rare appearance of L135 or T245 in the RT of any HIV-1 strain suggests that these sites may be important for RT function and activity. Indeed, the introduction of the resistance-inducing I135L and V245T substitutions in the D13-UG RT not only reduced susceptibility to NNRTIs but also decreased RT activity. Furthermore, the D14-UG RT with both resistant residues was less fit than the NNRTI-sensitive D13-UG RT. These results suggest that counterparts of the primary isolate would display a decrease in relative replication efficiency. In contrast, D14-UG was as fit as D13-UG and other subtype A and D isolates, implying that reduced RT activity may not affect the overall replication efficiency of the primary isolate. Ball et al. previously showed that the replication efficiency of drug-naïve, "wild-type" primary HIV-1 isolates is governed by the efficiency of host cell entry and not other intracellular steps in the replication cycle (7). This is not to suggest that other steps do not play a role in replication efficiency (i.e., fitness). However, minor deficiencies in RT activity, such as that seen with D14-UG, may be compensated for by increasing the efficiency of other steps in the replication cycle, e.g., entry (42). In effect, the Wright fitness landscape may fit this hypothesis (22, 54). The epistatic interactions between different parts of a system, in this case, between RT function and different steps in the replication cycle, must compensate for each other to maintain a fit state. Thus, a detrimental mutation, such as that at position 135, would be rare and must be rapidly compensated for to ensure survival.

The rare occurrence of 135L in RT and the possible impairment of fitness in the absence of compensatory mutations suggest that this intrinsically NNRTI-resistant strain may not have a significant impact on ARV drug treatment in HIV-infected individuals. In countries such as Uganda, over 90% of ARV drug-treated individuals (over 5,000) are receiving NNRTIs in first-line treatment regimens. In addition, government programs and international research and aid organizations are now providing nevirapine to thousands of HIV-infected pregnant women at the time of delivery and to newborns. In the future, this NNRTI selective pressure may have an impact on the evolution of subtype A and D HIV-1 isolates. Furthermore, there have not been comprehensive screens (i) to test the sensitivity of non-subtype B strains to ARV drug inhibition and (ii) to determine whether other unknown substitutions not found in subtype B confer ARV drug resistance. Indeed, several studies have now identified rare or new ARV drug resistance mutations that have emerged in non-subtype B-infected patients during drug selective pressure (13, 14).

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