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Amino Acid Substitutions at Position 190 of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Increase Susceptibility to Delavirdine and Impair Virus Replication

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Suboptimal treatment of human immunodeficiency virus type 1 (HIV-1) infection with nonnucleoside reverse transcriptase inhibitors (NNRTI) often results in the rapid selection of drug-resistant virus. Several amino acid substitutions at position 190 of reverse transcriptase (RT) have been associated with reduced susceptibility to the NNRTI, especially nevirapine (NVP) and efavirenz (EFV). In the present study, the effects of various 190 substitutions observed in viruses obtained from NNRTI-experienced patients were characterized with patient-derived HIV isolates and confirmed with a panel of isogenic viruses. Compared to wild-type HIV, which has a glycine at position 190 (G190), viruses with 190 substitutions (A, C, Q, S, V, E, or T, collectively referred to as G190X substitutions) were markedly less susceptible to NVP and EFV. In contrast, delavirdine (DLV) susceptibility of these G190X viruses increased from 3 to 300-fold (hypersusceptible) or was only slightly decreased. The replication capacity of viruses with certain 190 substitutions (C, Q, V, T, and E) was severely impaired and was correlated with reduced virion-associated RT activity and incomplete protease (PR) processing of the viral $p55^{gag}$ polyprotein. These defects were the result of inadequate $p160^{gagpol}$ incorporation into virions. Compensatory mutations within RT and PR improved replication capacity, $p55^{gag}$ processing, and RT activity, presumably through increased incorporation of $p160^{gagpol}$ into virions. We observe an inverse relationship between the degree of NVP and EFV resistance and the impairment of viral replication in viruses with substitutions at 190 in RT. These observations may have important implications for the future design and development of antiretroviral drugs that restrict the outgrowth of resistant variants with high replication capacity.

The nonnucleoside reverse transcriptase inhibitors (NNRTI) comprise a large group of compounds that are potent inhibitors of human immunodeficiency virus type 1 (HIV-1) replication (10, 29). The NNRTI are attractive agents for the treatment of HIV-1 infection because they are highly selective, potent, and relatively nontoxic. Currently, three NNRTI have received U.S. Food and Drug Administration approval for the treatment of HIV/AIDS: nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV). Suboptimal treatment with NNRTI results in the rapid emergence of drug-resistant virus. Although structurally diverse, all NNRTI drugs inhibit HIV-1 reverse transcriptase (RT) by binding to a hydrophobic pocket adjacent to the active site (21, 43, 45). Consequently, NNRTI resistance is often conferred by RT mutations that compromise susceptibility to other NNRTI, making subsequent treatments with this drug class less effective.

NNRTI resistance is associated with mutations within the NNRTI binding pocket, which includes at a minimum amino acids (aa) 100 to 110, 180 to 190, and 220 to 240 in RT. Depending on the NNRTI or whether the NNRTI is used in combination with other drugs, viruses with different NNRTI resistance mutation patterns may emerge (5, 26, 27, 34, 35, 38). In vitro selection of drug-resistant virus with several NNRTI, including quinoxalines, thiocarboxinilides, or piperazines frequently results in the emergence of HIV-1 containing RT mutations at aa 190 (17). The amino acid substitution that emerges at position 190 may depend on the degree of selective drug pressure that is applied (20). Kleim et al. show that, under strong selective pressure with the NNRTI HBY097, viruses containing a G190E mutation are selected, whereas viruses with G190A and G190S mutations emerge in response to lower drug pressure. Drug-resistant viruses containing G190 substitutions have been reported in patients treated with NVP, EFV, and MKC-442 in response to treatment (2, 3, 18, 34).

Unlike most NNRTI-resistant viruses, G190E mutant viruses exhibit severely impaired replication capacity. Other amino acid substitutions at this position, such as G190A, G190S, and G190Q, also compromise replication capacity but to much lesser extents. Several laboratories have previously reported that the impaired replication of G190E mutant viruses is due to impaired RT polymerase and RNase H activities (8, 13, 17, 28). However, the degree of impaired RT function reported in these studies varies substantially.

In this report we found that certain substitutions at position 190 of RT not only confer resistance to NVP and EFV but also cause increased susceptibility to DLV. In addition, the studies described here provide an explanation for the impaired replication of viruses containing certain G190 substitutions. A better understanding of the factors that influence the selection of G190E versus G190A mutations may aid in the design of more effective antiretroviral drugs that restrict drug-resistant outgrowth to viruses with severely impaired replication capacity.

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MATERIALS AND METHODS

NNRTI. The NNRTI drugs DLV, EFV, and NVP were generously provided by Pharmacia-Upjohn (Kalamazoo, Mich.), DuPont Pharmaceuticals (Wilmington, Del.), and Roxanne Laboratories (Redding, Conn.), respectively.

Phenotypic drug susceptibility testing. Drug susceptibility was measured by using recombinant viruses and a rapid single replication cycle assay as previously described (30). Briefly, RT-PCR was used to generate amplification products (~1.5 kb) that include the *gag* p7/p1 and p1/p6 cleavage sites, the entire protease (PR) coding region and the RT coding region from aa 1 to 305. To generate resistance test vectors (RTVs), the amplification products were cloned into an HIV-1 vector containing a luciferase expression cassette inserted in the *env* region. To test patient virus in plasma, RTVs were prepared and tested as large pools (i.e., PR or RT sequence libraries) to assess the drug susceptibility of virus populations. Individual clones from these libraries can be prepared as well. Pseudotyped viruses were produced by cotransfecting 293 (human embryonic kidney) cell cultures with RTV plasmids plus an expression plasmid encoding the Env protein of amphotropic murine leukemia virus. High-titer virus stocks were harvested 48 h after transfection and used to infect fresh 293 target cells in 96-well plates. Serial dilutions of NNRTI drugs were added to individual wells prior to infection. Approximately 72 h after infection, target cells were lysed and luciferase activity was measured to assess virus replication in the presence or absence of drugs at each concentration tested. The percent inhibition was calculated as: [1 − (luciferase activity in the presence of the drug/luciferase activity in the absence of the drug)] × 100. The data are displayed by plotting the percent inhibition of luciferase activity versus the $\log_{10}$ drug concentration. The concentration of drug required to cause a 50% inhibition in virus replication (IC_{50}) was calculated from the curve fit equation. The fold change in susceptibility is the ratio the IC_{50} of the test sample to the IC_{50} determined for a drug-sensitive reference virus that was tested in the same experiment. The fold change in susceptibility measurement in this assay has a reproducibility that is between 1.4- and 2.3-fold for replicate determinations, depending on the drug being tested (15).

Virus growth competition assays. Viral stocks of replication competent viruses were prepared by transfection of 293 cells with 5 μg of an infectious proviral clone of HIV-1. To determine the infectious virus titer, MT2 cells (30,000 cells/well in six replicates) were infected in 96-well plates with serial dilutions of each infectious virus. Cells were examined for cytopathic effect at day 4 of culture, and the 50% tissue culture infectious dose ($TCID_{50}$) was determined by the method of Reed and Muench (32). Competition assays were performed by infecting MT2 cells with two viruses in specific ratios (generally, 9:1). Thirty $TCID_{50}$ were used to infect 30,000 MT2 cells (multiplicity of infection = 0.001). After 4 h, the culture medium was removed and replaced with fresh medium. Aliquots of culture supernatants were harvested at serial time points after infection. After 3 to 4 days in culture, 25 μl of supernatant from 250 μl of culture was used to infect fresh MT2 cells. The relative proportion of the two viruses in the culture supernatants was determined at baseline and at serial time points by measuring the ratio of the peak heights in sequencing chromatograms at one or more sites that differentiated the two viruses being compared. For example, wild-type viruses have a GGA triplet coding for G at position 190 in RT. The G190Q virus has a CAA triplet encoding a glutamine at this same position. The relative proportion of C or G peaks at the first position of the 190 codon and G or A peaks at the second position of the 190 codon were determined. The sensitivity of detection of a minority peak varied from 15 to 20% at all positions tested. Artificial mixtures between 20 and 80% showed good linearity in this assay. Relative measures of replication capacity were determined by using a publicly available web-based software to calculate the selection coefficients (s) and the relative fitness (1+s [where "s" is the selection coefficient]; http://www-binf.bio.uu.nl/rdb/fitness.html) (24). Calculations were performed at all available intervals at which the culture consisted of a mixed population (20 to 80%), and the median value for s and 1+s were determined from the distribution of the calculated values.

Replication capacity testing. Viral replication capacity was measured by using a modification of the single replication cycle phenotype assay described above (30). Briefly, recombinant virus stocks were used to infect 293 cells in the absence of drug. Viruses containing PR and RT sequences derived from resistant viruses (patient-derived sequences or site-directed mutants) or drug-sensitive reference virus (NL4-3) were tested. The relative luciferase units (RLU) produced by cells infected with different viruses was monitored from 6 to 90 h postinfection. The ratio of luciferase produced by individual viruses compared to the control virus after infection was relatively constant independent of the time after infection at which the luciferase was measured. The luciferase activity was 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, or (iii) postinfection normalization based on the luciferase activity produced in transfected cells (the RTV transfected into the 293 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 the transfection luciferase levels. The replication capacity was calculated as follows: $[1 - (\text{infection } RLU_{\text{test virus}} \times CF_{TXN})/\text{infection } RLU_{\text{control virus}}] \times 100$. In this equation, CF_{TXN} is the correction factor for the transfection efficiency and is calculated by the following equation: $CF_{TXN} = \text{transfection } RLU_{\text{test virus}}/\text{transfection } RLU_{\text{control virus}}$. 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 ±0.2 log for independent measurements. Several independent studies have compared the in vitro replication capacity assay to virus replication kinetics assays and in vitro viral growth competition assays (23, 31, 33). Overall, these studies demonstrate that the rank order of individual viruses is consistent between data obtained in the single-cycle assay and data from assays employing replication-competent viruses. However, the absolute value for relative fitness determined in the assays can differ.

Genotypic analysis. The amino acid sequences of RT were derived from the DNA sequences of the RTV pools prepared as described above. Sequence analysis was performed by a thermocycling method using fluorescent dye-labeled dideoxynucleotide chain terminator chemistry (ABI, Foster City, Calif.). Sequencing reaction products were resolved by using a 96 parallel capillary gel electrophoresis system (ABI 3700). Base calling and amino acid sequence derivations were performed by using customized Sequencher software that performs automated reference comparison and reporting of amino acid changes compared to the reference sequence (GeneCodes, Ann Arbor, Mich.). RT amino acid sequences were compared to those of a reference strain of HIV-1 (NL4-3; GenBank accession number M19921).

Site-directed mutagenesis and chimeric virus construction. The megaprimer method of site directed mutagenesis (40) was used to construct a series of isogenic viruses containing single amino acid substitutions at position 190 of RT, double amino acid substitutions at positions 190 and 74, or PR and RT active-site knockout mutations (D25G in PR and D185G in RT). Mutations were introduced directly into the RTV described above as a *Apa*I/*Pin*AI fragment. For each mutant, the entire 1.5-kb *Apa*I/*Pin*AI fragment was sequenced to ensure that only the desired mutations were present in the viral vectors tested. An *Rsr*II restriction site was introduced into the RTV at aa 17 and 18 in RT, with silent mutations at each position (GAT GGC CCA was changed to GAC GGA CCG). Chimeric viruses were constructed by using the *Apa*I-*Rsr*II fragment and the *Rsr*II-*Pin*AI fragment from different parent virus clones to replace the *Apa*I-*Pin*AI fragment of the RTV.

In vitro RT measurements. Virion-associated RT elongation activity was measured by using real-time PCR to quantify the RT-dependent production of a specific cDNA product from a heteropolymeric RNA template (22). The RT-PCR takes place in a single position in a 96-well plate. The RNA template is a 3-kb transcript derived from the *gag* region of the NL4-3 molecular clone of HIV-1 and was in vitro synthesized by T7 polymerase by use of a standard transcription protocol (MegaScript T7 kit; Ambion, Inc., Austin, Tex.). Viral supernatants obtained from transfected 293 cells were lysed in a buffer containing 0.2% of NP-40, 50 mM Tris HCl (pH 8), 10 mM dithiothreitol, 10 mM $MgCl_2$, and 50 mM KCl and incubated for 30 min on ice. The lysate was diluted 1/100 with buffer without NP-40, and 5 μl was included in the RT-PCR mix (0.3 mM concentrations of the deoxynucleoside triphosphates, 1 nM concentrations of the primers [5′-TGG ACA TAA GAC AAG GAC CAA and 3′-CCA ACA AGG TTT CTG TCA TC], a 1 nM concentration of probe [AGA GCC GAG CAA GCT TCA CAG GA], RNase inhibitor [40 U], 10 ng of RNA template, 5 mM of $MgCl_2$, AmpliTaq Gold (1 U), 1× TaqMan buffer). The reaction was incubated for 2 h at 37°C for the RT step, incubated 5 min at 95°C to inactivate the viral RT and activate the AmpliTaq Gold polymerase, and subjected to 40 cycles of PCR amplification (each cycle consisted of 15 s at 95°C and 90 s at 58°C) (ABI 7700; Applied Biosystems). The RT activity was normalized by p24 antigen concentration of input viral lysates.

Western blot analysis. To study gag-pol processing, viral stocks were harvested from 293 cells 2 days after transfection. Virions were pelleted by centrifugation at 14,000 rpm for 60 min, resuspended in 100 μl of sample buffer for *gag* processing analysis or in 20 μl of sample buffer, and then heated to 100°C for 5 min. Virion proteins were separated by electrophoresis on a 12% sodium dodecyl

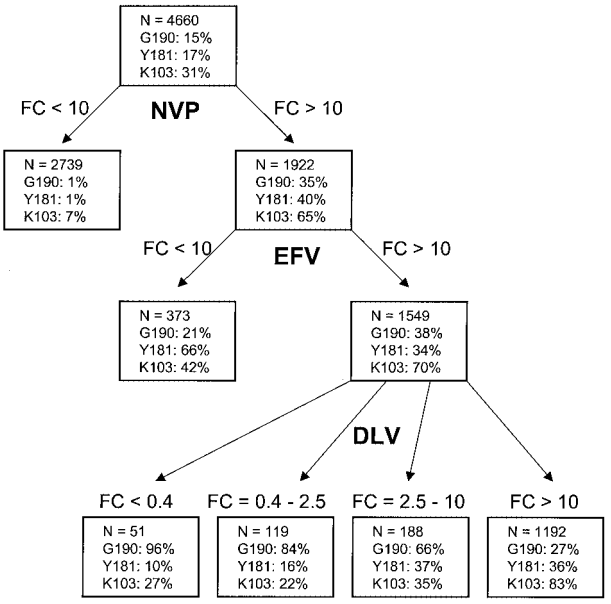


FIG. 1. Classification tree of NNRTI susceptibility. The viruses are derived from a database, including matched phenotype and genotypes, from viruses sent to a commercial testing laboratory for routine antiretroviral resistance testing. The individual entries of the database are split into successive branches based on their susceptibility to individual NNRTI. Viruses are first divided based on having a fold change (FC) in susceptibility to NVP greater or less than 10-fold compared to a wild-type reference. Successive splits are for an EFV susceptibility greater or less than 10-fold and for a DLV susceptibility <0.4-fold (hypersusceptible), between 0.4- and 2.5-fold (same as the wild type), between 2.5- and 10-fold (low-level reductions in susceptibility), and >10-fold (resistant). The prevalence of viruses with substitutions at G190, Y181, and K103 are indicated as a percentage of the total in each of the branches.

sulfate-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked by using 5% milk in TBST (0.1% Tween 20, 5 mM Tris-HCl [pH 8], 100 mM NaCl) and incubated with either a 1:1,500 dilution of rabbit anti-p24 antisera (obtained through the AIDS Research and Reference Reagent Program) or a 1:500 dilution of polyclonal antibodies against RT (American Research Products). After three washes in TBST, the membranes were incubated with 1:1,500 dilution of anti-rabbit immunoglobulin G that was horseradish peroxidase conjugated (Amersham Life Sciences) and developed by using the ECL chemiluminescence kit (Amersham Life Science) according to the manufacturer's recommendations.

RESULTS

Mutations at G190 in RT are associated with increased susceptibility to DLV. Certain mutations in the NNRTI binding pocket have been shown to differentially affect susceptibility to the three NNRTI. For example, the Y181C mutation confers large reductions in susceptibility to NVP and DLV but has little or no effect on EFV susceptibility (6, 34, 36). In a large database of phenotypic susceptibility data, we observed a group of viruses that display >10-fold reductions in susceptibility to NVP and EFV but remain susceptible (fold change, 0.4 to 2.5) or display increased susceptibility to DLV (fold change, <0.4) (Fig. 1). In order to examine whether a specific mutation or pattern of mutations was associated with decreased susceptibility to NVP or EFV and increased susceptibility to DLV, we queried the database containing matched phenotypes and genotypes (n = 4,660). A total of 170 viruses in this database had the specific phenotype described above (NVP and EFV, >10-fold; DLV, <2.5-fold). Interestingly, a large percentage of viruses with this phenotypic profile were found to have mutations at G190 of the RT gene (n = 149/170, i.e., 88%). In contrast, mutations at G190 were observed in only 15% of the entire database ($P < 0.0001$). We observed several different amino acid substitutions at position 190 in RT. Previously reported substitutions at 190, including A, S, Q, T, and E were observed. In addition, we identified viruses with G190C and G190V. In this database, G190A was the most common substitution present, accounting for ca. 83% of the viruses with G190 substitutions (G190S, 15%; G190E, 1%; and G190Q, G190C, G190T, and G190V, <1% of viruses with G190 substitutions). Viruses containing G190X mutations in combination with additional NNRTI mutations (e.g., at positions 100, 101, 103, and 181) often displayed reduced susceptibility to DLV. G190 mutations were seen in the database at approximately the same frequency as Y181 substitutions (15 versus 17%) and about half as frequently as K103 substitutions (31%). It is also interesting that the population of viruses with a fold change of >10 for all three NNRTI is clearly enriched for the K103 substitution and the population of viruses with NVP FC and EFV FC values of <10 is enriched for the Y181 substitution. Other mutations that increase susceptibility to DLV (e.g., P225H and F227L) have been previously described (2, 3, 11). These mutations were also observed in our database at a low frequency (2% each) and were highly enriched in the viruses with normal or increased susceptibility to DLV.

NNRTI susceptibility profiles for a representative patient-derived virus containing each of the amino acid substitutions (A, C, E, Q, S, and V) at 190 of RT are shown in Fig. 2. In all cases, viruses with substitutions at position 190 exhibited resistance to NVP and EFV ranging from 10-fold to >600-fold. 190C and 190V were associated with large increases in susceptibility to DLV (~100-fold), whereas 190A and 190S are associated with more moderate hypersusceptibility (2.5- to 10-fold). In contrast, 190Q and 190E viruses show low level reductions in susceptibility (two- to eightfold).

In order to directly determine the effect of different G190 substitutions on NNRTI susceptibility, site-directed mutagenesis was used to replace the G190 of the reference virus with each of the amino acid substitutions observed in the various patient viruses (A, C, E, Q, S, and V). Each mutant virus was evaluated for susceptibility to NVP, EFV, and DLV (Table 1). The NNRTI susceptibility determined for each of the site-directed mutants was consistent with the NNRTI phenotypic profiles of the corresponding patient viruses. The NNRTI susceptibility of the G190E and G190V viruses generated by site-directed mutagenesis could not be characterized because the replication of these viruses was severely impaired (see below). The high concordance in NNRTI susceptibility between the patient viruses and the site-directed mutants confirms that the G190 substitutions were primarily responsible for the NNRTI susceptibility profiles of the patient viruses.

Impaired replication of viruses containing RT mutations at position 190. It has been previously reported that substitutions at position 190 of RT result in viruses with lower infectivity in tissue culture (17, 20, 28). To further evaluate the impact of these RT mutations on HIV replication, the replication of each

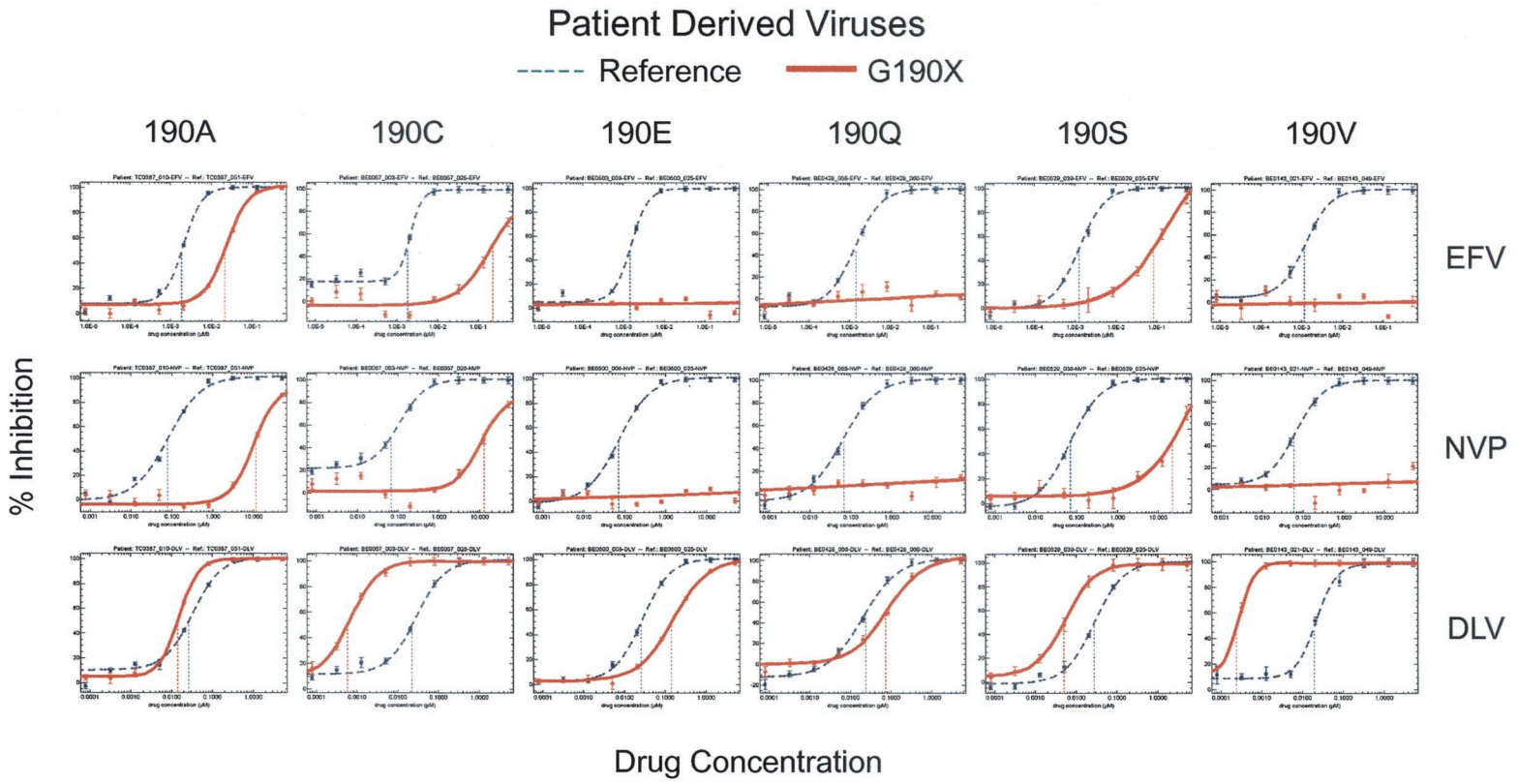


FIG. 2. NNRTI susceptibility profiles of patient viruses with G190X substitutions. Recombinant viruses containing various substitutions at amino acid position 190 of RT (190A, 190C, 190Q, 190S, 190V, and 190E) were tested for susceptibility to EFV (top panel), NVP (center panel), and DLV (bottom panel). The graphs show the percent inhibition on the *y* axis as a function of the drug concentration (on a $\log_{10}$ scale) on the *x* axis. Each graph shows the susceptibility curves for the patient virus (solid line) and the drug-sensitive reference (dashed line).

TABLE 1. Fold change in susceptibility to NNRTI for recombinant viruses containing substitutions at G190 (patient-derived viruses) and genetically engineered viruses containing single amino acid changes at G190 of RT (site-directed mutants)[a]

Mutation in RT	Phenotypic susceptibility to NNRTIs (fold change)					
	Site-directed mutants			Patient-derived viruses		
	DLV	NVP	EFV	DLV	NVP	EFV
190A	0.4	96	7	0.5	134	11
190C	0.01	170	170	0.02	183	111
190Q	6	>600	>270	2.7	>600	270
190S	0.3	191	56	0.18	299	65
190V (+L74V)	0.002	600	>270	0.01	>600	>270
190E	ND	ND	ND	5.6	>600	>270
190T	0.04	356	150	NA	NA	NA

[a] Changes in NNRTI susceptibility are expressed as the fold change in IC_{50} based on the IC_{50} of a drug-susceptible reference virus (IC_{50} of sample/IC_{50} of reference). ND, not determined; NA, not applicable.

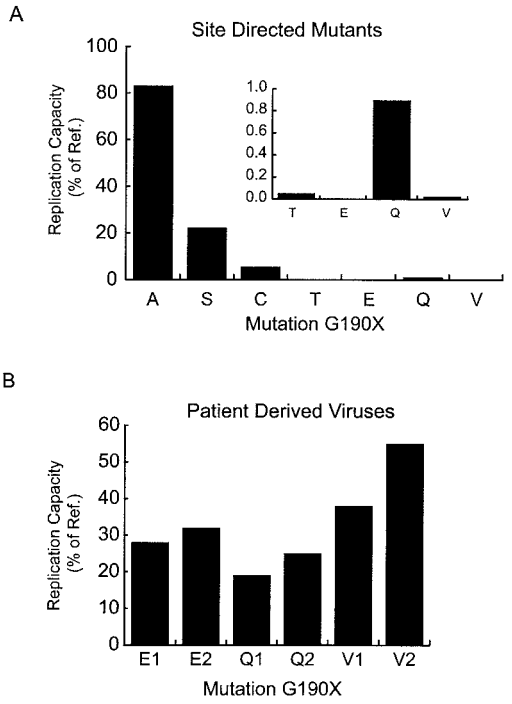


FIG. 3. Replication capacity of recombinant viruses with substitutions at aa 190 in RT. The replication capacity is expressed as a percentage of the reference virus (100%) tested in the same experiment. (A) Site-directed mutant virus clones. The inset plot represents viruses with low replication capacities (190E, 190Q, and 190V) with a more sensitive scale. (B) Recombinant virus pools containing patient-derived PR and RT segments. Recombinant patient-derived viruses with substitutions at 190E (E1 and E2), 190Q (Q1 and Q2), and 190V (V1 and V2) are shown.

site-directed mutant virus was characterized by using the single-cycle replication assay as described above. Viruses containing different G190 substitutions displayed a wide range of replication capacities (<0.01 to 83%). The replication capacity of the G190A virus was only slightly diminished (83%) compared to the G190 reference virus, whereas the G190S and G190C viruses were significantly more impaired (21 and 5%, respectively) (Fig. 3A). Viruses with the G190Q, G190T, and G190V substitutions were severely affected (0.9, 0.05, and 0.01%, respectively). Replication of the G190E virus was too low to measure with this assay (<0.01%).

Although the replication capacities of the site-directed mutant viruses containing the G190E, G190Q, and G190V substitutions were extremely low or undetectable, patient-derived viruses containing these substitutions were able to replicate at substantially higher levels. The replication capacity of patient-derived viruses containing 190E, 190Q, and 190V mutations ranged from 20 to 60% (Fig. 3B). These data suggest that other amino acid differences in the patient viruses may compensate for the low replication capacity resulting from the G190X mutations. Compensatory amino acid substitutions can partially restore the impaired replication capacity of viruses containing drug resistance mutations (25). In particular, it has been reported that specific mutations at positions 74 and 75 of RT, which have been associated with NRTI resistance, partially compensate for mutations at position 190 (8, 19). Consistent with these earlier observations, all of the viruses carrying the most deleterious mutations (G190E, G190Q, G190V, and G190T) in our database also carry either L74 or V75 mutations and the majority have the L74V substitution.

To measure the effect of an L74V mutation on the replication capacity of G190X mutant viruses, L74V+G190X double mutants were generated by site-directed mutagenesis and tested for replication capacity using the single-cycle replication assay. The L74V mutation alone had no measurable effect on the replication capacity of the reference virus or on the 190A virus (Fig. 4). However, L74V increased the replication capacity of viruses with 190S, 190C, 190Q, 190T, and 190V mutations (Fig. 4). Replication of a virus with G190E+L74V mutations still could not be measured in this system. As stated above, susceptibility of the G190V single mutant could not be determined due to low replication capacity. However, the G190V+L74V double mutant had significantly higher replication capacity, which allowed an accurate measure of NNRTI susceptibility (see Table 1). The susceptibility pattern was consistent with patient-derived viruses containing G190V substitutions.

The increased replication capacity observed on G190X viruses with the L74V mutation was relatively small compared to the respective patient-derived viruses. An analysis of the RT and PR sequences of patient-derived viruses containing G190X mutations revealed numerous mutations and/or polymorphisms in these regions (see Fig. 5A). To map the position of putative compensatory mutations in the patient-derived segment, chimeric viruses containing either the patient-derived RT segment or the Gag-PR segment in the NL4-3 reference backbone were prepared (see Fig. 5). The replication capacity of recombinant viruses containing only the RT region from the patient viruses was lower than that of recombinant viruses containing the entire Gag-PR-RT fragment but higher than that of the site-directed mutant viruses carrying the respective 190 substitution. This observation suggests that amino acid substitutions in the Gag-PR segment may also play a role in the improved replication capacity of patient viruses containing G190V or G190E mutations. Recombinant viruses carrying patient-derived Gag-PR regions alone exhibited significantly higher replication capacities than recombinant viruses with the

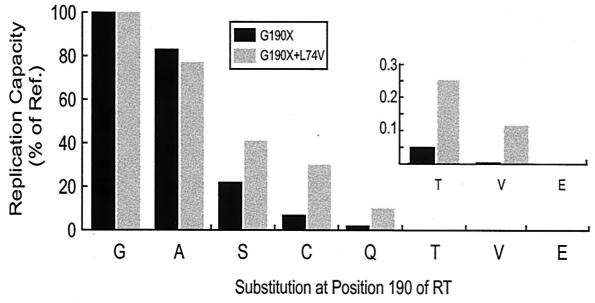


FIG. 4. Effect of L74V mutations on replication capacity in viruses containing 190 mutations. Recombinant viruses containing mutated RT sequences were derived by site-directed mutagenesis of the reference virus with amino acid substitutions at position 190 with or without the L74V mutation. Replication capacity was measured in a single replication cycle assay and is expressed as a percentage of the G190 reference virus. Solid bars represent the relative replication capacity of G190X viruses. Gray bars represent the relative replication capacity of the L74V+G190X viruses.

entire Gag-PR-RT fragment. However, the Gag-PR segment was not sufficient to rescue a virus with a site-directed G190V or G190E mutation in RT, suggesting that the compensatory Gag-PR mutations must be in the context of compensatory mutations in the RT (Fig. 5C). Taken together, these observations demonstrate that compensatory mutations in both the RT and Gag-PR regions can contribute to the increased replication capacity observed in the patient viruses with 190E and 190V mutations.

Replication capacity of viruses with G190 substitutions in growth competition experiments. To confirm that in vitro replication capacity measurements derived from the single-cycle recombinant virus assay were consistent with data generated in conventional competition assays, we prepared several of the viruses described in the present study as replication-competent viral stocks and competed them against each other in replication assays. The virus stocks were prepared as described above and used at 9:1 ratios (low RC virus:high RC virus) to infect MT2 cells. Four competition experiments were performed. Figure 6 shows the percentages of wild-type and mutant virus present in the cultures at various time points during the competition, as well as the median (1+s) values determined for each set of competition assays. The relative fitness of the viruses (190G > 190S > 190C > 190Q) is clear from the time to reversion in each of the cultures. The increase in the fitness of the 190Q+74V compared to the 190Q alone virus is also clearly demonstrated by the rapid replacement of the single mutant by the double mutant in the culture. The relative fitness determined in the replication competent growth competition assays is consistent with the replication capacity determined by using the single-round recombinant virus assay.

RT and PR activity of viruses containing RT mutations at position 190. Virion-associated RT elongation activity was examined to further investigate the impaired replication of viruses containing 190 mutations. The virion-associated RT activity measured in viruses carrying G190E, G190V, G190T, and G190Q substitutions was nearly undetectable. In contrast, the RT activity of viruses with G190C, G190S, and G190A were 5, 15, and 65%, respectively, compared to the G190 reference virus (Fig. 7A). Overall, there was a strong correlation between virion-associated RT activity and replication capacity (compare Fig. 7A with Fig. 3A).

To rule out defects in PR activity associated with viruses carrying G190X mutations, $p55^{gag}$ processing in virions was evaluated by Western blot analysis. Normal processing by HIV-1 PR results in the complete conversion of $p55^{gag}$ to the matrix ($p17^{MA}$), capsid ($p24^{CA}$), nucleocapsid ($p17^{NC}$), p6, p2, and p1 proteins. By using an anti-p24CA antibody for analysis, incomplete processing is evident by the presence of unprocessed $p55^{gag}$ protein and the accumulation of a p41MA-CA intermediate cleavage product. Unexpectedly, in nearly all cases, viruses containing amino acid substitutions at position 190 of RT exhibited partial $p55^{gag}$ processing (Fig. 7B). The extent of $p55^{gag}$ processing correlated strongly with viral replication capacity. The G190 reference virus processed $p55^{gag}$ completely, and processing in the G190A virus was only slightly impaired. The G190C and G190S viruses displayed partial processing, whereas processing in the G190E, G190Q, G190T, and G190V was severely impaired. These results suggested that the low replication capacity of viruses carrying a single mutation at position 190 of RT could be associated with a defect in PR activity.

Since the HIV-1 PR is also responsible for the conversion of $p160^{gagpol}$ into the mature PR ($p10^{PR}$), RT ($p66/p51^{RT}$), and integrase ($p33^{IN}$) proteins, it is possible that the decreased RT activity associated with G190X mutants is a result of incomplete maturation of $p160^{gagpol}$. To evaluate qualitative or quantitative differences in virion-associated RT, the amount of mature RT protein present in virions was evaluated by using a polyclonal antibody directed against the RT heterodimer p66-p51. Using the G190 virus as a reference, reduced p66-$p51^{RT}$ levels were observed in the site-directed mutant viruses containing amino acid substitutions at position 190 (Fig. 7C). The G190A virus contained slightly less RT than the G190 reference virus. Lesser amounts of RT were detected in the G190C and G190S viruses, whereas RT was nearly undetectable in the G190E, G190Q, G190T, and G190V mutant viruses. Not surprisingly, the amount of virion-associated RT protein that was detected correlated very well with virion-associated RT activity and replication capacity.

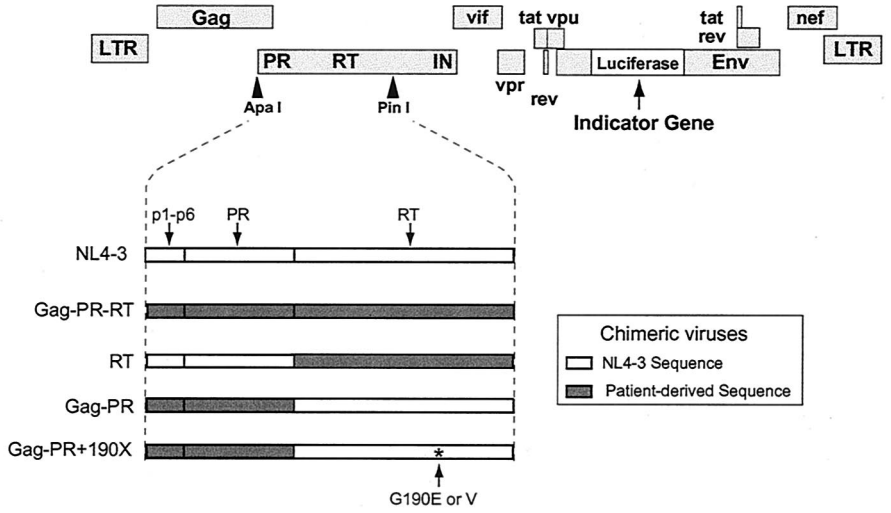
There are several possible explanations for the reduced levels of mature RT proteins found in the G190X virions. The $p160^{gagpol}$ precursor may be degraded in the cytoplasm and unavailable for virion incorporation, the $p160^{gagpol}$ precursor may be stable but not efficiently incorporated during assembly, or the $p160^{gagpol}$ precursor may be incorporated but yet not appropriately processed. Depending on which explanation is operable, $p160^{gagpol}$ precursor might be expected to accumulate either in the cytoplasm or the virions produced from cells transfected with G190X vector DNA. The amount of virion- and cell-associated $p160^{gagpol}$ was examined. In these experiments, a PR active-site mutant (*D25G*) was used as a reference for the production and accumulation of the unprocessed $p160^{gagpol}$ polyprotein. $p160^{gagpol}$ was detected in transfected cells and in virions with the D25G mutation, however, the polyprotein was not detected in the G190 reference and G190X mutant viruses (Fig. 7D). Taken together with the p66/p51 Western blot data, these results suggest that insuffi-

A

PR	I15V, G57R, L63P, I72S, V77I
RT	K20R, V35I, I50V, V60I, K65R, S68R, K73L, V75A, K101Q, Q102R, K122E, D123N, I135V, Q151M, C162S, I178V, M184V, G190V, I195T, G196E, K219E, V276I, R277K, Q278E, A288S, V293I, E297R

PR	L10I, I15V, K20R, E35D, M36I, N37D, I54V, G57K, Q58E, I62V, L63P, A71V, V82T, I84V, I85V, L90M
RT	K20R, M41L, K43Q, E44D, V60I, D67N, L74V, Q102R, V118I, K122E, I135V, C162Y, I178F, G190E, G196R, Q197K, T200A, Q207N, L210W, R211K, T215Y, K219N, E224N, A272P, R277K, T286A, V293I, E297K

B



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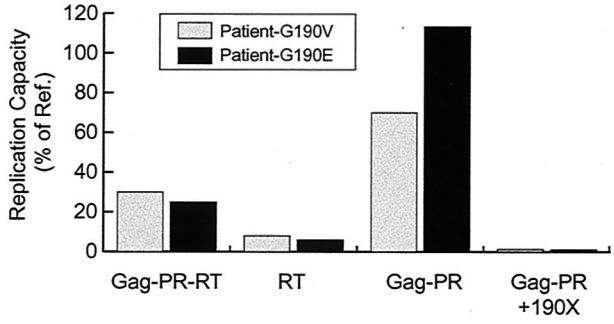

FIG. 5. Compensatory mutations improve replication of a patient-derived virus with G190V or G190E substitutions. (A) Amino acid sequences of PR and RT (aa 1 to 305) are shown for clones derived from two patient isolates carrying the mutation G190V or G190E. The sequence is reported as the differences from laboratory reference strain NL4-3. (B) Schematic representation of viruses carrying Gag p1 to p6, PR, and RT sequences from NL4-3 (open bars) or from the individual patient virus clones (shaded bars). Recombinant viruses were constructed by exchanging Gag-PR and/or RT sequences of the NL4-3 reference virus with the corresponding sequences from patient isolates containing either the G190V or the G190E mutation. (C) Replication capacity of chimeric viruses. Shaded bars indicate recombinant viruses obtained from patient isolates carrying the mutation G190V, and the solid bars indicate viruses obtained from patient isolates carrying G190E.

cient incorporation of $p160^{gagpol}$ precursor into virions could be the cause of low levels of PR and RT.

Patient-derived viruses carrying the mutations G190E, G190Q, and G190V displayed replication capacities up to 500-fold higher than the site-directed mutants carrying only the respective mutation at position 190. Therefore, we analyzed RT and PR activities and the amount of RT protein in two patient isolates for each of these three mutant viruses. Both the

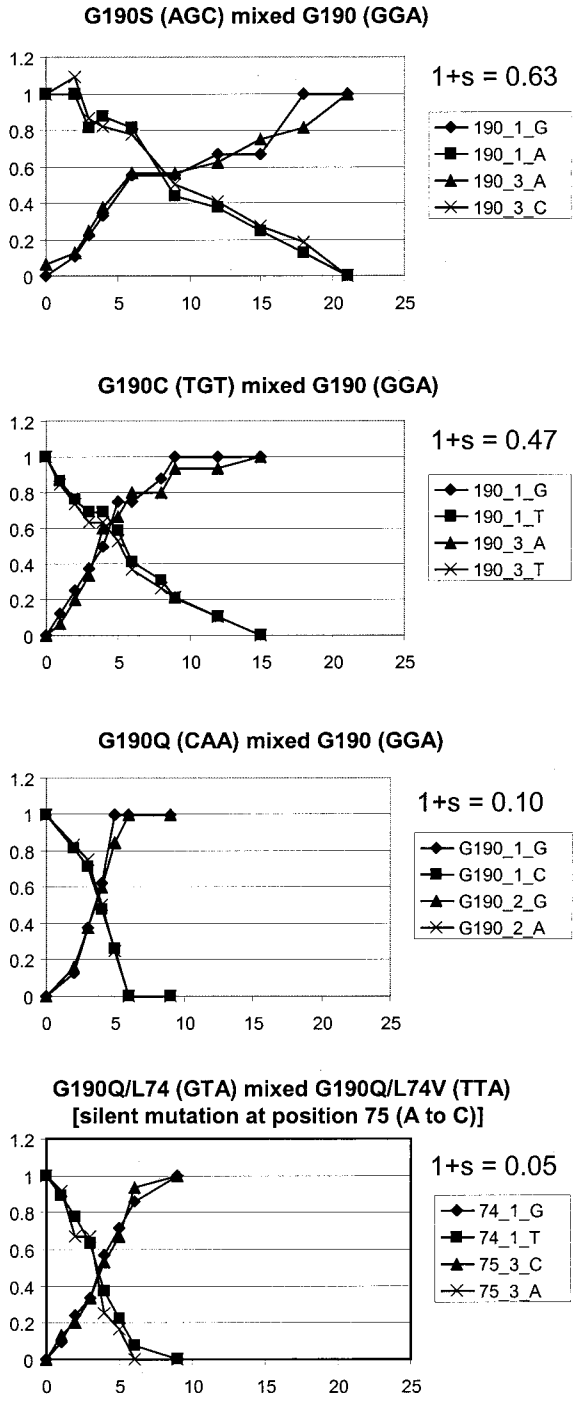

FIG. 6. Virus growth competition assays. Virus stocks were mixed at 9:1 ratios and used to infect MT-2 cells, and the percentages of the two viruses present at various passages after infection were determined by quantifying the relative peak heights on sequencing chromatograms. The percentage of allele present at intervals after infection was plotted for each competition assay. The relative fitness (1+s) for each pair ("s" is the selection coefficient) was calculated as described in Materials and Methods.

RT elongation activity and the amount of virion-associated RT protein were higher than the respective site-directed mutant (Fig. 8A and B). In addition, Western blot analysis with $p24^{CA}$ antibody indicated that patient derived viruses accumulated less unprocessed $p55^{gag}$, less $p41^{MA\text{-}CA}$ intermediate, and more of the fully processed $p24^{CA}$ protein than the corresponding site-directed mutant viruses (Fig. 8C). Moreover, consistent with the observed increases in replication capacity (Fig. 4A), the addition of an L74V mutation also improved $p55^{gag}$ processing of both G190Q and G190V mutant viruses, whereas it was not able to rescue processing of the G190E mutant (Fig. 8D).

Hypersusceptibility to DLV is associated with increased susceptibility of RT in vitro. The susceptibility of RT enzymes derived from the G190 reference virus and three patient virus clones carrying G190C, G190V, and G190Q substitutions was evaluated with the RT elongation activity assay in the presence of different concentrations of DLV (Fig. 9). The IC_{50}s determined for the RTs were consistent with the IC_{50}s determined for the viruses. The reference G190 enzyme had an IC_{50} for DLV of 0.5 μM. The enzymes from viruses with substitutions at G190C and G190V had IC_{50}s of 0.07 and 0.14 μM, respectively, eight- and fourfold lower than the wild-type enzyme. In contrast, the enzyme from the G190Q virus, was 10-fold less susceptible to the drug than the G190 reference virus enzyme (IC_{50} = 5 μM). All three patient-derived viruses show reduced levels of RT compared to the G190 reference virus. There is a very good correlation between the inhibition of the RT in vitro and the inhibition of the virus replication in culture. However, the level of DLV susceptibility does not show a perfect correlation with the replication capacity measure or the level of incorporation of RT into particles. In particular, the G190Q virus shows low replication capacity and gag-pol incorporation yet is not hypersusceptible to DLV. These results suggest that the increased DLV susceptibility observed can be the result of both stronger interaction with the RT enzyme and less incorporation of the protein into the virions.

DISCUSSION

We describe here an NNRTI-phenotypic profile observed in certain recombinant viruses derived from patient isolates. The profile, characterized by high-level resistance to NVP and EFV and hypersusceptibility to DLV, is caused by specific substitutions at position 190 of RT. A wide variety of amino acids were identified at this position (X = A, S, C, Q, V, T, and E), and most of them are also associated with impaired viral replication. Decreased infectivity of the G190X mutant viruses is associated with decreased RT protein and low RT and PR activities in the virion; this finding is most likely due to defective incorporation of $p160^{gagpol}$ into the virions. However, hypersusceptibility to DLV imparted by G190 substitutions could be related to both, a stronger interaction with the RT enzyme and decreased incorporation of the $p160^{gagpol}$ polyprotein into the virion.

Previous studies have demonstrated that resistance to NNRTI drugs, specifically quinoxilanes, carboxanilides, and piperizines, is conferred by several amino acid substitutions at position 190 of HIV-1 RT (2, 4, 16, 18, 28). In these studies, drug-resistant viruses were selected in vitro by serial cultivation of HIV-1 in the presence of increasing drug concentrations. These drugs selected different amino acid substitutions under high or low selective pressure. G190E was often selected at high drug concentrations, whereas G190A was frequently se-

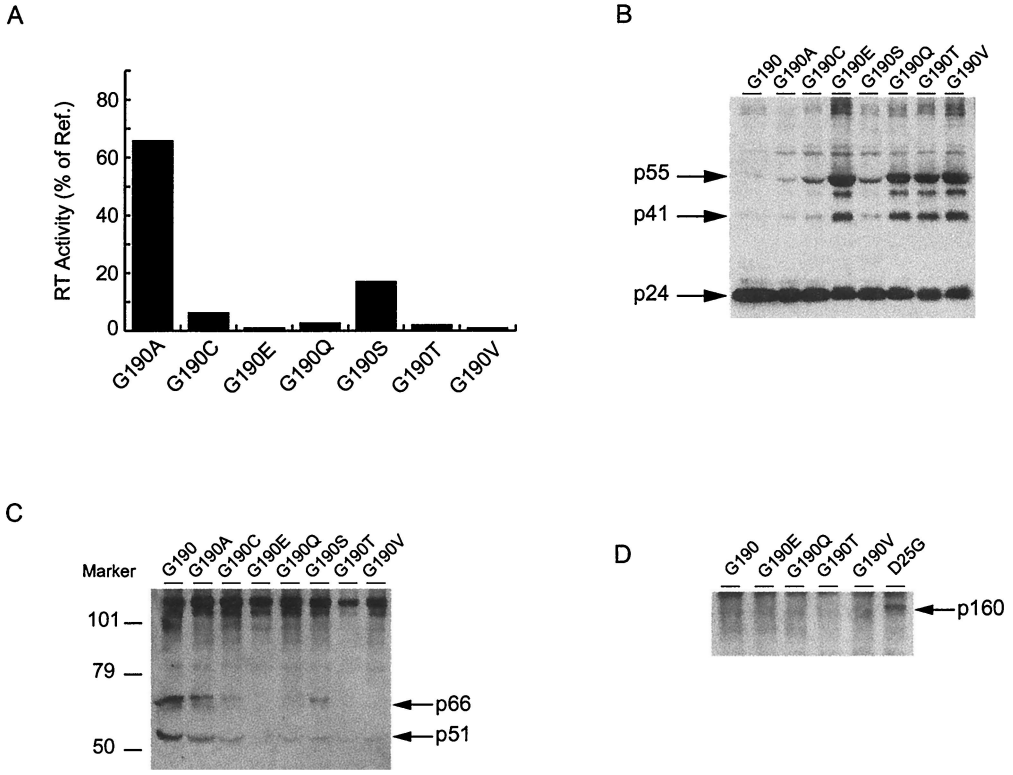

FIG. 7. RT activity and processing of $p55^{gag}$ and $p160^{gagpol}$ polyproteins in viruses containing 190 substitutions. Viral stocks were produced by transient transfection of 293 cell cultures and normalized by p24 levels prior to analysis. The same stocks were used for all of the analyses shown in this figure. (A) Virion-associated RT activity measured in viruses with G190A, G190C, G190E, G190Q, G190S, G190T, and G190V is indicated as a percentage of the reference G190 virus. (B) Processing of virion-associated $p55^{gag}$. Western blot analysis with anti-p24 antibodies. The mobility of the viral protein $p55^{gag}$ (p55), the intermediate $p17^{MA}$-$p24^{CA}$ (p41), and the fully processed $p24^{CA}$ (p24) are indicated with arrows. (C) Processing of virion associated $p160^{gagpol}$. Western blot analysis with anti-RT antibodies. Arrows indicate the position of the p66 and p51 RT proteins. (D) Virion incorporation of Pr160gagpol. Western blot analysis with polyclonal anti-RT antibodies. Arrow indicates the position of Pr160gagpol polyprotein. D25G is a virus with a mutation in the active site of PR.

lected at lower drug concentrations. Several studies have demonstrated that NNRTI treatment in a clinical setting also selects for viruses containing G190X mutations (2, 3, 37). In the present study, we present data from a large database containing matched HIV-1 genotypes and phenotypes of viruses derived from patients in clinical practice. In this data set, viruses with substitutions at G190 were present in 15% of the data set as a whole. Viruses carrying G190E, G190Q, G190C, G190T, and G190V mutations were significantly less prevalent than those carrying G190A and G190S mutations. The striking disparity between the number of G190A and G190E viruses that emerges in vivo can, at least in part, be explained by the difference in replication capacity of viruses carrying these mutations. Interestingly, we observed an inverse correlation between resistance to NVP and EFV and the replication capacity in viruses containing substitutions at 190 in RT (compare Fig. 2 with Fig. 3). Previous studies have demonstrated that certain NNRTI resistance mutations (e.g., P236L, Y181C, V179D, and V106A) are associated with a decreased replicative capacity of HIV-1 (1, 14). In these studies, the phenotype was shown to be linked to abnormalities in viral RNase H activity. This could be through altered folding of the RT protein or direct repositioning of the RNA-DNA duplex through the RNase H domain such that the RNase H cleavage events would be less efficient than that of the wild-type RT (39). It is important to note that we did not evaluate RNase H activity in these experiments, and the RNase H domain of RT was not included in the patient derived fragments cloned into the RTVs. Therefore, we cannot exclude the possibility that additional compensatory mutations were present in the patient viruses that we did not capture in our test sequences.

Olmstead et al. reported that G190E viruses replicate less efficiently than G190 wild-type virus in cell culture (28). In addition, the recombinant G190E RT protein displayed lower RT and RNase H activities in vitro (8, 13, 17, 28). In the present study, we found that viruses with substitutions at position 190 displayed a wide variety of replication capacities ($<$0.01 to 83%), depending on the amino acid present in that position. Interestingly, patient-derived viruses carrying an amino acid substitution at position 190 in the context of certain other mutations or polymorphisms replicated at significantly higher levels than the respective site-directed mutant, suggesting that compensatory mutations can partially mitigate the deleterious effect of certain 190 substitutions. This compensatory effect was complex and included mutations in the RT and in the PR regions (Fig. 5). Bleiber et al. (7) conducted similar studies on the role of mutations in the PR and RT of patient-derived HIV-1 on infectivity, replication, RT activity, and pro-

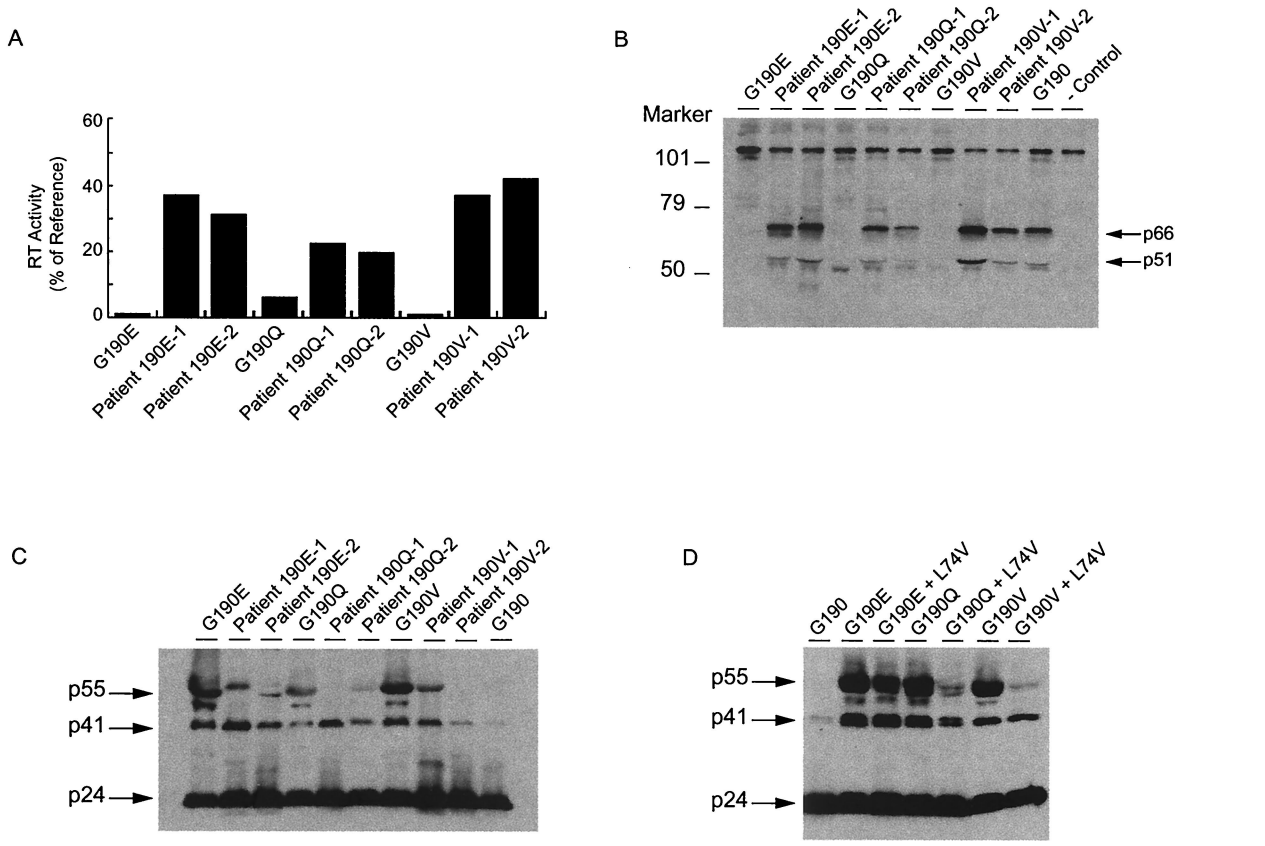

FIG. 8. RT activity and processing of $p55^{gag}$ and $p160^{gagpol}$ polyproteins in patient-derived virus pools containing mutations at position 190 of RT. Viral stocks were produced by transient transfection of 293 cell cultures and normalized by p24 levels prior to analysis. The same stocks were used for all of the analyses shown in this figure. (A) Virion-associated RT activity of viruses containing PR and RT sequences from patient isolates with G190E (patient 190E-1 and patient 190E-2), G190Q (patient 190Q-1 and patient 190Q-2), and G190V (patient 190V-1 and patient 190V-2) is compared to the RT activity of the site-directed mutants G190E, G190Q, and G190V. (B) Processing of virion associated $p160^{gagpol}$. Western blot analysis with anti-RT antibodies. The mobility of molecular weight markers is indicated by the numbers on the left. (C) Processing of virion associated $p55^{gag}$. Western blot analysis with anti-p24 antibodies. The mobility of the viral protein $p55^{gag}$ (p55), the intermediate $p17^{MA}$-$p24^{CA}$ (p41) and the fully processed $p24^{CA}$ (p24) are indicated with arrows. (D) Processing of virion associated $p55^{gag}$ with site-directed mutants G190E, G190Q, and G190V with or without a second mutation at position 74 of RT (L74V).

tein maturation in recombinant viruses in vitro. Consistent with our findings, these authors reported complex interactions between PR and RT sequences in their assay and also correlated the presence of certain mutated RTs with impaired fitness and specifically with decreased packaging of $p160^{gagpol}$ into virions. Carron de la Carriere et al. (9) reported that recombinant viruses carrying resistant PR sequences from certain patient viruses showed a reduction in the amount of packaged RT and virion-associated RT activity. In these viruses, the addition of RT mutations could partially rescue the defect caused by the patient-derived PR sequences. Kleim et al. reported previously that continued exposure to NNRTI can select for a secondary mutation either at position L74 or at position V75 that appeared several passages after the G190X mutation (19). These mutations had previously been associated with ddI and ddC resistance. However, in this case they appeared in the absence of NRTI pressure and are clearly linked to the G190 mutation. In addition, studies with recombinant proteins demonstrated that the double mutants G190E+L74V and G190E+V75I displayed RT and RNase H activities very similar to the wild-type enzyme (8). In the current study, the double mutant viruses G190X+L74V in most cases replicated better that the single 190X mutants. However, the replication capacities of these viruses were still much lower than the reference G190 virus. The high replication levels of patient-derived recombinant viruses carrying the most deleterious substitutions at position 190 (E, V, or Q) must be achieved by the accumulation of multiple secondary mutations that may be distributed throughout the Gag p1-p6, PR, and RT sequences, as well as in other regions of the genome not captured in the assay used in the present study. The L74V mutation was associated with an increase in the replication capacity of the G190Q virus in both the single-cycle replication capacity assay and the virus growth competition assay; however, the magnitude of the effect was much greater in the growth competition assay than in the single-cycle replication capacity assay. In the single-cycle replication capacity assay we saw no effect of the L74V mutation alone on replication capacity. In contrast to these results, Sharma and Crumpacker (41, 42) reported that the L74V mutation caused decreased replication when tested in a virus growth competition assay. It is likely that the virus growth competition assays are more sensitive to subtle changes

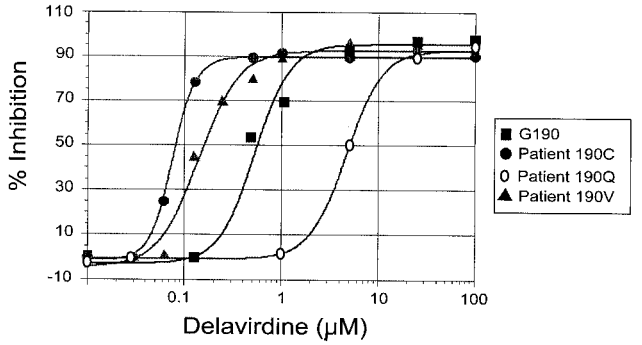

FIG. 9. In vitro measurements of susceptibility of RT to DLV. Virion-associated RT activity was measured in the presence of different concentrations of DLV and was expressed as the percentage of inhibition with respect to the activity measured in the absence of drug. The RT enzymes were obtained from three patient-derived virus clones (patient 190C, patient 190Q, and patient 190V) and from the reference G190 virus.

in replication capacity than the single-cycle assay due to the potential amplification of subtle differences over many cycles. In addition, very low replication capacities measured in the single-cycle replication capacity assay may not correlate with equally low replication in growth-dependent assays (31, 33).

Previous studies of recombinant RT proteins carrying G190E, G190Q, G190S, or G190V substitutions used RT expressed in *Escherichia coli* with different experimental systems (8, 13, 28). The enzymatic activity observed with the 190E enzyme varied from 4 to 60% of the wild-type G190 enzyme. It was proposed that these discrepancies could be explained either by different degrees of purification of the recombinant proteins or by the presence of improperly folded RT enzyme. Recombinant RT proteins with 190V, 190Q, and 190S mutations were reported to display slight or no reductions in RT activity compared to the wild-type G190 enzyme (8, 17). In contrast, the results presented here indicate that the virion-associated RT activity of site-directed mutants with 190V, 190Q, and 190S mutations are much lower than that in wild-type virions (from 1 to 15%). This observation suggests that there is not a direct correlation between the in vitro activity of the recombinant protein and the virion associated RT activity. It is important to note that the amount of mature RT protein present in virions with G190X mutations explains this lack of correlation. The results of the present study indicate that reductions in virion-associated RT activity are probably quantitative in nature, although our present studies do not rule out that there are qualitative differences as well. Viruses with the lowest replication capacity lacked detectable levels of p66/p51 RT. Western blot analysis demonstrated that the absence of detectable RT protein in the virion was not associated with accumulation of the $p160^{gagpol}$ polyprotein in either the virions or the transfected cells. In addition, viruses with G190X mutations also exhibited poor PR processing of $p55^{gag}$ consistent with reduced virion incorporation of $p160^{gagpol}$ and low PR levels. The data generated here are in agreement with a previous report describing a G190E virus that exhibited reduced replication capacity and RT activity and had low levels of p66/p51 RT protein (28). Based on the available data, aberrant folding of $p160^{gagpol}$ possibly leading to premature degradation and poor incorporation into virions is a reasonable explanation for the impaired replication capacity of G190X viruses. In agreement with this interpretation, it has been reported that residues 190 and 191 are important for RT protein stability (44). Interestingly, the increased incorporation of Pol protein and the improved processing of $p55^{gag}$ observed with the viruses carrying the double mutation 190X+L74V (Fig. 8) suggests that position 74 could directly affect protein stability.

Viruses with G190A, G190C, G190S, G190V, and G190T substitutions displayed 3- to 300-fold increases in susceptibility to DLV (Fig. 2 and Table 1). There are several possible biochemical explanations for this phenotype. It is plausible that the decreased amount of RT protein is limiting for viral replication, resulting in a virus that is more sensitive to RT inhibitors. Alternatively, the mutation at position 190 could result in tighter binding of DLV, reducing the concentration of drug necessary for viral inhibition. The susceptibility of the viruses with G190X substitutions to other RT inhibitors, such as the ones belonging to the NRTI family (AZT, 3TC, ddI, ddC, and ABC), indicated that the IC_{50}s were up to twofold lower than the IC_{50}s for the G190 reference virus (data not shown). This observation suggests that, even though the low amount of RT protein could moderately increase the susceptibility to RTIs, the more profound effect of the G190X substitution on DLV susceptibility is specific for this drug. More importantly, the IC_{50}s of DLV of viral RTs carrying G190C or G190V mutations were between four- and eightfold lower than that of the G190 enzyme, favoring the possibility of a tighter binding to the drug. In support of this interpretation, structural models have suggested that the side chain of the amino acid at position 190 could alter the size and shape of the hydrophobic pocket, resulting in a change in the binding affinity to NNRTI (12, 43).

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