

Initial Appearance of the 184Ile Variant in Lamivudine-Treated Patients Is Caused by the Mutational Bias of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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Treatment of human immunodeficiency virus type 1-infected patients with lamivudine (3TC) results in the appearance of drug-resistant virus variants with a mutation at the 184Met codon (ATG) of the reverse transcriptase (RT) gene. The 184Ile (ATA) variant appears first, but subsequently the 184Val (GTG) variant outcompetes the 184Ile variant. We demonstrated previously that the 184Val enzyme and the corresponding virus are more fit than 184Ile, thereby explaining eventual outgrowth of 184Val. In this study, we set out to determine why 184Ile is usually observed first after initiation of 3TC therapy. With a limiting dilution approach during *in vitro* selection with 3TC, we measured a significantly higher frequency of the G→A substitution toward the ATA codon (184Ile; 56%) than the A→G substitution toward GTG (184Val; 12.5%). This result indicates that the initial appearance of the 184Ile variant in patients is a consequence of the mutational bias of the RT enzyme. Interestingly, a novel 3TC-resistant variant which was generated by T→C substitution (184Thr; 28%) was also observed. The RT enzyme of the 184Thr variant was less than 10% active compared with the wild-type enzyme, and the replication capacity of this variant was severely reduced. Selection of the 184Thr variant illustrates that the limiting dilution approach allows the selection of drug-resistant variants with suboptimal fitness.

The rapid development of drug-resistant variants during antiviral treatment of human immunodeficiency virus type 1 (HIV-1)-infected patients is the primary reason for the ineffectiveness of antiviral drugs. The generation of resistant variants is a consequence of the error proneness of the HIV-1 reverse transcriptase (RT) enzyme. During viral replication, RT copies the single-stranded RNA genome into double-stranded DNA. Due to the lack of 3' exonuclease proofreading activity, incorporation of missense nucleotides occurs with relatively high frequency. The misincorporation rate of the RT enzyme ranges from 10^{-4} to 10^{-5} , depending on the nature of the template and source of the RT enzyme (2, 5, 15, 16, 23, 30). Analysis of the nucleotide substitution pattern shows that transitions are favored over transversions, even though each nucleotide can undergo only one transition versus two transversions (3, 13, 16, 17, 20, 23, 28). The misincorporation rate and the mutational bias of HIV-1 RT may shape the virus population from which drug-resistant variants evolve. It was proposed by Coffin (9) that the combination of the mutation rate and the high replication rate (10^9 viruses per day [14, 29, 36]) results in a virus population containing all variants with single-hit mutations. According to this scenario, drug-resistant variants may preexist in the virus population prior to therapy. Sequence analysis of naive patient samples confirmed the existence of drug-resistant variants in the virus population (11, 22, 27). In addition, the rapid appearance of drug-resistant variants during antiviral therapy suggests the preexistence of these variants.

Rapid selection of drug-resistant variants was observed in

patients treated with the nucleoside RT inhibitor (–)-2'-deoxy-3'-thiacytidine, also called lamivudine (3TC) (32, 34). Genetic analysis of multiple RNA samples that were isolated from patients as early as 2 weeks after initiation of treatment demonstrated a mutation in the catalytic domain of RT. The wild-type amino acid 184 methionine (Met; ATG) was replaced by either 184Ile (ATA) or 184Val (GTG). Longitudinal sampling revealed a transient appearance of the 184Ile variant, which subsequently disappeared from the viral population due to the outgrowth of the 184Val variant. Both variants can be selected *in vitro*, and site-directed mutagenesis experiments demonstrated high-level 3TC resistance (4, 8, 12, 31, 33, 34). Eventual outgrowth of the 184Val variant at the expense of 184Ile during therapy is consistent with superior RT polymerase function (1, 6, 7) and viral replication rate of the 184Val variant in primary cells (1).

Inspection of the nucleotide sequence of both 3TC-resistant variants indicates that 184Val (GTG) originates from wild-type Met (ATG) and not from the initial 184Ile variant (ATA). Both variants are generated from the wild-type ATG sequence by transitional substitutions (G→A for the 184Ile variant and A→G for the 184Val variant). In the present study, we investigated whether differences in the frequency of G→A versus A→G substitutions can explain the initial appearance of 184Ile. In order to select resistant variants without the influence of fitness differences, a limiting dilution approach was used.

Selection of 3TC-resistant HIV-1 variants. The *in vitro* selection (Fig. 1) was initiated by infection of SupT1 cells with wild-type HXB2 virus (multiplicity of infection of 0.02). After 1 h at 37°C, the culture was split and 3TC was added in a concentration of either 1 μ M (selection A) or 10 μ M (selection B). During all further manipulations, the infected cells were maintained at the initial drug concentration. At day 3 postin-

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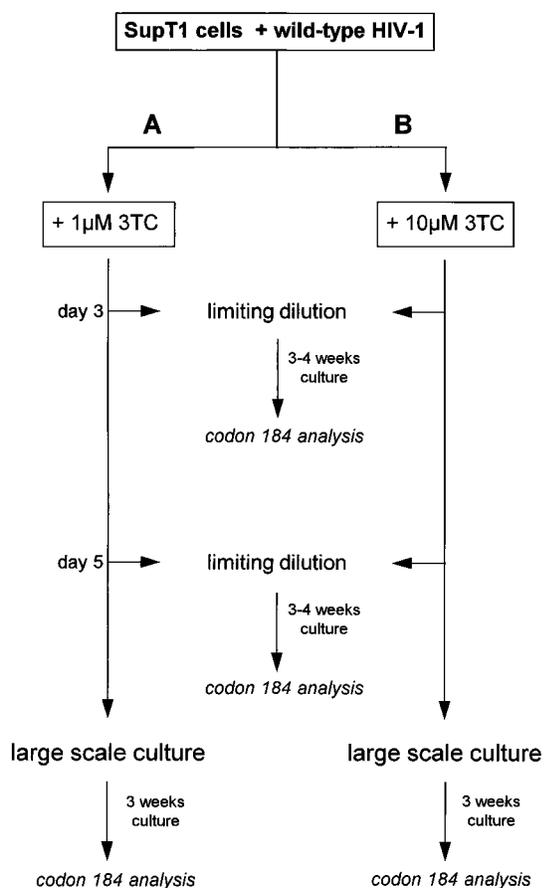


FIG. 1. A schematic presentation of the in vitro selection protocol. The selection was initiated by infection of SupT1 cells with wild-type HXB₂ virus. After infection, the drug 3TC was added at a concentration of 1 μ M (A) or 10 μ M (B). At days 3 and 5 postinfection, the cultures were titrated in fivefold dilutions. In addition, large-scale selections were performed with the remaining cultures.

fection, half of the cultures (2.5 ml) was used for titration in a 96-well plate (titrations A3 and B3). Undiluted culture (125 μ l) was placed in row A. In row B, 25 μ l of culture of row A was mixed in 100 μ l of uninfected SupT1 cells (0.2×10^6 cells per ml), and serial fivefold dilutions were pipetted in rows C to H. The remainder of the culture (2.5 ml) was supplemented with 2.5 ml of RPMI 1640 medium with the appropriate 3TC concentration. At day 5, the limiting dilution was repeated with 2.5 ml of culture (titrations A5 and B5) and 2.5 ml was maintained as large-scale culture. After 1 week, the samples corre-

sponding to individual wells were transferred to 24-well plates in order to optimize cell growth and viral replication. Cell-free supernatant and cells were harvested from individual wells when large syncytia were observed. For genotypic analysis, cells were washed with phosphate-buffered saline and incubated in 100 μ l of TETP buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0], 0.5% Tween 20 and 20 μ g of proteinase K) for 1 h at 55°C and 10 min at 95°C (9a). The complete RT gene was amplified by PCR (primer RT19, 5'-GGA CAT AAA GCT ATA GGT ACA G [positions 2453 to 2474]; primer 3'RTout, 5'-TCT ACT TGT CCA TGC ATG GCT TC [positions 4392 to 4369]) and was either directly sequenced or first cloned in the TA cloning vector (pGEM-T; Promega, Leiden, The Netherlands), followed by automated sequencing (ABI, Nieuwerkerk, The Netherlands) of individual clones. For analysis of the large-scale 3TC selections, the point mutation assay was used (18, 32).

After 3 to 4 weeks of culture, a total of 36 positive wells were obtained (Table 1), including 11 from the day 3 titration at 1 μ M 3TC (A3), 18 from the corresponding day 5 titration (A5), and 7 from the B5 titration (day 5 [10 μ M 3TC]). No positive wells were obtained in the B3 titration (day 3 [10 μ M 3TC]). It is possible that wild-type virus was able to replicate in some of the wells, in particular in the selection with the lowest 3TC concentration. Therefore, the in vitro-selected viruses were passaged onto fresh SupT1 cells in the presence of 50 μ M 3TC. Four samples, previously selected at 1 μ M 3TC, were unable to replicate at the elevated 3TC concentration. Sequence analysis of these four viruses revealed the presence of a wild-type sequence at codon 184 (Table 1).

Genotypic analysis of the selected viruses. The genotypic analysis of the 32 remaining 3TC-resistant viruses revealed a codon 184 mutation in all samples (Table 1). The 184Ile variant (codon ATA) was present in 18 wells, and the 184Val variant (codon GTG) was observed in only four samples. Interestingly, a novel 184 variant (184Thr [codon ACG]) was detected in 9 wells. Furthermore, one 184Ile variant with an alternative Ile codon (ATT) was selected. Of the selected codon 184 variants, 56% result from a G \rightarrow A transition (184Ile). This frequency is substantially higher than that of A \rightarrow G transitions (184Val, 12.5%). The novel 184Thr mutant is generated by another type of transition (C \rightarrow T) and was found at an intermediate frequency (28%). The alternative 184Ile codon (ATT [G \rightarrow T]) was observed once, consistent with the idea that transversions occur less frequently than transitions. Analysis of both large-scale selections (1 and 10 μ M 3TC) by the point mutation assay demonstrated the presence of the ATA codon (Ile).

The limiting dilution protocol generated the 184Ile and 184Val variants but also revealed a third class of 3TC-resistant virus, 184Thr. In order to rule out the presence of other mu-

TABLE 1. Codon 184 analysis of 3TC-resistant variants

Concn of 3TC (μ M)	Titration day	ATG (Met)	ATA (Ile) G \rightarrow A ^a	GTG (Val) A \rightarrow G ^a	ACG (Thr) T \rightarrow C ^a	ATT (Ile) G \rightarrow T ^b
1	3	1	3	2	4	1
1	5	3	10	2	3	0
10	3	0	0	0	0	0
10	5	0	5	0	2	0
Total ($n = 36$)		4 ^c	18	4	9	1
Relative frequency (%)			56	12.5	28	3

^a Transitional substitution.

^b Transversional substitution.

^c These four virus variants were not able to replicate at 50 μ M 3TC.

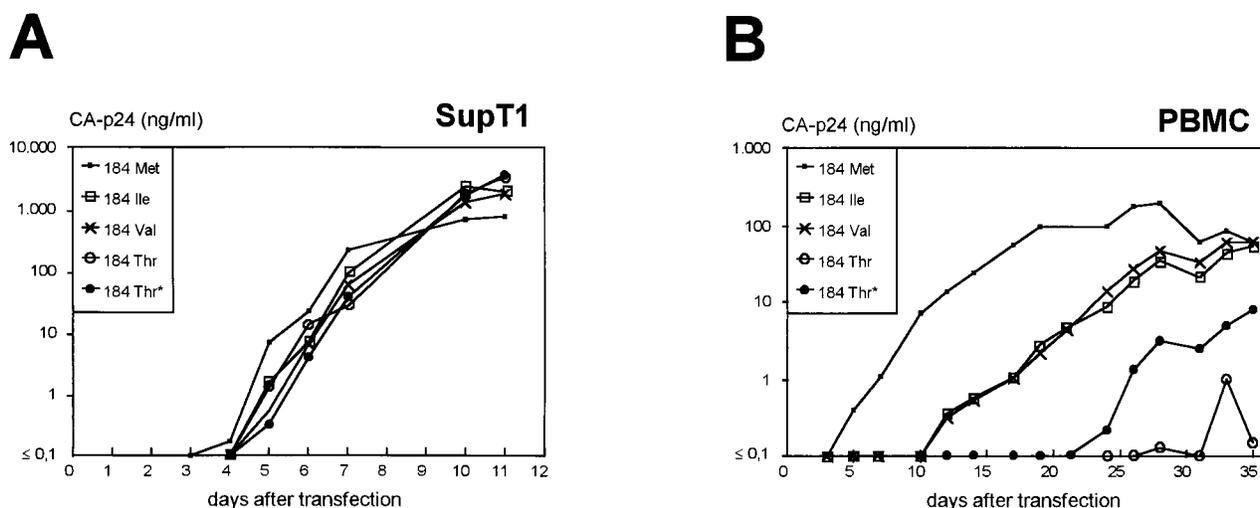


FIG. 2. Replication kinetics of codon 184 HIV-1 variants in SupT1 cells (A) or PBMCs (B). Viral replication was monitored by CA-p24 antigen production and measured by enzyme-linked immunoassaying as described previously (24, 26). Similar replication kinetics were observed in several independent experiments. SupT1 cells (5×10^6) were transfected with $1 \mu\text{g}$ of the HIV-1 molecular clone, and 0.5×10^6 SupT1 cells were added posttransfection in a final volume of 5 ml (10). The PBMC transfection was performed with $10 \mu\text{g}$ of proviral DNA and 5×10^6 phytohemagglutinin stimulated PBMCs which were prepared by Ficoll-Paque (Pharmacia Biotech, Roosendahl, The Netherlands) density gradient centrifugation of heparinized blood from HIV-1-seronegative individuals. After the transfection, 2×10^6 fresh PBMCs in RPMI medium supplemented with 10% fetal calf serum, gentamicin ($10 \mu\text{g}/\text{ml}$), and 5% Lymphocult interleukin-2 (Biotest, Zaventem, Belgium) were added. Once a week, half of the culture suspension was replaced by 2×10^6 fresh phytohemagglutinin-stimulated PBMCs.

tations in this RT protein that may contribute to 3TC resistance, we sequenced the first 220 codons of all nine 184Thr variants. This region was chosen because most of the RT mutations that cause resistance to nucleoside analogs are located within this region. Although some substitutions in individual clones were observed, no particular mutation was consistently observed. In particular, the E89G mutation that was implicated in 3TC resistance (12) was not present in any of the 184Thr variants, suggesting that the 184Thr mutation is solely responsible for the 3TC-resistant phenotype. The 3TC susceptibility of the 184Thr variant was similar to that of the other 3TC-resistant 184 variants (50% inhibitory concentration, $>400 \mu\text{M}$ 3TC [data not shown]).

Functional analysis of the 184Thr variant. Previous *in vitro* studies demonstrated reduced replication of amino acid 184 variants (1, 35). To study the effect of the 184Thr mutation on viral fitness, molecular clones were generated by cloning in the HXB2 background of either a complete RT gene (184Thr*) or a small segment (184Thr) encompassing the 184Thr mutation. Both variants have the 184Thr mutation, but 184Thr* contains one additional amino acid change at position 376 (Thr376Ala). The 184Thr viruses were compared with wild type (184Met) and the other 3TC-resistant 184 variants (184Val and 184Ile) in replication assays in the SupT1 T-cell line and in peripheral blood mononuclear cells (PBMCs) (Fig. 2A and B). We previously reported a reduction in replicative capacity of the 184Ile and 184Val variants compared with the wild-type 184Met in PBMCs, but not in a transformed T-cell line (1). Similarly, the 184Thr variants replicated efficiently in the transformed SupT1 cells but demonstrated a dramatic replication defect in PBMCs. The observation that no replication defect is apparent in SupT1 cells explains the selection of 184Thr variants in the limiting dilution protocol with SupT1 cells.

We also measured the RT activity of the 184Thr variant in a poly(rA) · oligo(dT) assay (Fig. 3). Both virion-associated RT enzymes (184Thr and 184Thr*) and the *Escherichia coli*-expressed 184Thr enzyme were used. The 184Thr samples demonstrated reduced polymerase activity (Fig. 3A and B; 8 to

9%). The polymerase activities of the 184Ile and 184Val enzymes, either virion associated (28 and 45%, respectively) or *E. coli* expressed (38 and 74%, respectively), are comparable with the activities reported for these variants (1, 6, 7, 35).

The amino acid 184-mutated RT enzymes have been reported to exhibit reduced processivity (1, 6). Similar processivity assays were performed with the 184Thr variants. The 184Thr variant, either isolated from virion particles or expressed as recombinant protein in *E. coli*, demonstrated a dramatic defect in the ability to synthesize long cDNA products (data not shown). The observed trend of processivity (Met>Val>Ile>Thr) mirrors the ranking order of replication and RT activity spectrum of the 184 variants.

In this study, we demonstrate that the mutational spectrum of the RT enzyme influences the selection process of drug-resistant HIV-1 variants upon antiviral therapy. Treatment of HIV-1-infected patients with the nucleoside analog 3TC results in the initial and transient appearance of the 184Ile variant, followed by outgrowth of the 184Val variant (32, 34).

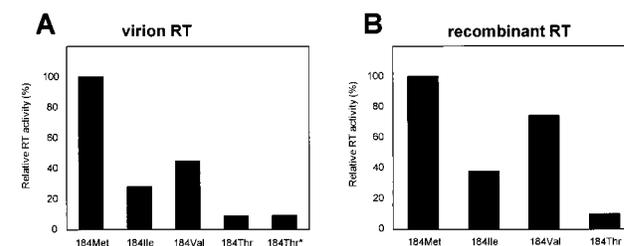


FIG. 3. Reduced RT enzyme activity of the codon 184 variants. RT activity was assayed by a poly(rA) · oligo(dT) assay as described by Back et al. (1). The RT assay was performed either with virion-derived RT (A) or *E. coli*-expressed RT (B) (11a). The wild-type and 184 variant RT enzymes were released from virions by Nonidet P-40 treatment (0.5% final concentration), and equal amounts of enzyme on the basis of CA-p24 levels were used. The concentrations of *E. coli*-expressed RT enzymes were determined on Coomassie-stained protein gels. The RT activities of the RT variants were quantitated on a PhosphorImager, and the activity of wild-type RT (184Met) was set to 100%.

Replacement of the 184Ile variant by the 184Val variant correlates with a superior fitness of the latter virus (1). A similar fitness difference was observed in the presence of 3TC, as shown in sensitive competition experiments (reference 21 and data not shown). With the limiting dilution approach, we now document a significantly higher frequency of G→A mutation leading to 184Ile compared with the A→G mutation resulting in 184Val. This indicates that the initial appearance of the 184Ile variant during 3TC therapy is a consequence of the mutation bias of the RT enzyme.

Based on the high turnover rate of the virus in HIV-positive individuals and the high mutation rate of the RT enzyme, Coffin (9) calculated that variants resulting from single-nucleotide substitutions should be produced on a daily basis. Thus, drug-resistant variants may preexist in the virus population before the start of antiviral therapy. Indeed, mutations associated with reduced sensitivity to RT inhibitors and protease inhibitors have been identified in naive patients (11, 22, 27). Because both 3TC-resistant variants (184Ile and 184Val) are generated by a single point mutation, it is likely that both variants are present in untreated patients. Our data suggest that their relative concentrations in the pretherapy virus pool are determined in part by the mutational bias of the RT enzyme.

During in vivo and regular large-scale in vitro selections, several drug-resistant variants will emerge in parallel and competition will result in outgrowth of the fittest variant. This competition is excluded in the limiting dilution protocol, thereby allowing selection of suboptimal variants. In this study, we report a novel 3TC-resistant variant (184Thr) with reduced RT enzyme activity. Thus, the limiting dilution approach can identify additional drug-resistant markers that may help in structure-function studies of the RT enzyme and its interaction with inhibitors. Another factor that can influence the selection of drug-resistant variants is the cell type used. For instance, the 184Thr variant does not replicate efficiently in primary cells but demonstrated efficient replication in the SupT1 cell line. Previously, we suggested that the difference in intracellular deoxynucleoside triphosphate levels may explain this cell-type-dependent replication (1). Consistent with this idea, a previous study (31) reported the selection of the 184Val variant in primary cells, whereas both 184Ile and 184Val variants were selected in T-cell lines (4, 12, 31, 33).

All 3TC-resistant viruses analyzed in this study contain a substitution of the amino acid 184Met, either to Val, Ile, or Thr. These results suggest that replacement of residue 184Met is the only way to escape from the selective pressure imposed by 3TC. This is particularly striking in comparison with other RT inhibitors such as nevirapine or BHAP that trigger alternative escape routes with multiple amino acid substitutions (25). The absolute necessity of substituting amino acid 184 in order to obtain the 3TC-resistant phenotype is illustrated by the selection of the suboptimal 184Thr variant. Apparently, no other amino acid substitutions can generate functional RT variants that are 3TC resistant.

In a previous study, we suggested that the substitution pattern observed in selection of drug-resistant variants can provide information on viral fitness requirements (19). We hypothesized that virus variants resulting from difficult substitutions (e.g., transversions) are selected only if these variants exhibit improved fitness over variants that can be generated from the master sequence by relatively easy substitutions (e.g., transitions). In this study, we measured nucleotide substitution frequencies at RT codon 184 (Table 1), and the results are consistent with the hypothesis. Of the 32 selected viruses, 31 variants were made by transitional substitu-

tions and only one transversion (G→T) was observed. In fact, of the 19 Ile variants obtained, 18 were generated by transition (G→A) and only 1 was generated by transversion (G→T). Furthermore, a trend is observed that some transitions occur more frequently than others (G→A>T→C>A→G>C→T). This four- to fivefold difference in frequency of the G→A versus A→G mutation at codon 184 explains the initial appearance of the Ile variant in 3TC-treated patients. If drug-resistant variants are present prior to therapy (9), we show that their prevalence is determined by the mutational bias of the HIV-1 RT enzyme.

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