AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 17, Number 9, 2001, pp. 807–818
Mary Ann Liebert, Inc.

Selection by AZT and Rapid Replacement in the Absence of Drugs of HIV Type 1 Resistant to Multiple Nucleoside Analogs

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ABSTRACT

We studied the intrahost evolution and dynamics of a multidrug-resistant HIV-1, which contains an insertion of two amino acids (aa) and several aa changes within the reverse transcriptase (RT) gene. From an individual receiving intermittent therapy, sequences of 231 full-length molecular clones of HIV-1 RT were obtained from serum-derived viruses at 12 consecutive time points over a period of 6 years, 17 to 20 clones per time point. In the 3.5-year period prior to the first course of therapy, only wild-type (wt) viruses were found. As soon as 6 months after the start of zidovudine (AZT) monotherapy, all viruses contained an insertion of two aa between positions 68 and 69 of the RT and aa changes at positions 67 and 215, a combination conferring resistance to multiple nucleoside analogs. After termination of therapy, the insertion mutants were rapidly and completely replaced by the wt viruses. In turn, the insertion mutants replaced the wt viruses after initiation of therapy with 3TC, d4T, and saquinavir. After termination of triple therapy, the wt viruses completely replaced the mutants within 1 month, which is markedly faster than has been observed earlier for the replacement of AZT-resistant viruses. Fast replacements of the mutant virus populations after termination of therapy indicate gross competitive disadvantage of the insertion mutant in the absence of therapy, which we estimated by using several models. The insertion mutants attained high virus loads, demonstrating that virus load cannot be used as a direct measure of virus fitness.

INTRODUCTION

Several reverse transcriptase (RT) and protease inhibitors are now available for treatment of HIV-1 infection, with drug combinations being more effective than monotherapy.[1–5] A general problem in antiretroviral treatment is the development of drug-resistant virus mutants.[6–8] The rate of appearance of drug-resistant mutants and their drug susceptibility profiles are highly variable among patients and depend on multiple factors, including therapy potency, drug levels, and patient compliance.

A number of mutations within the RT and protease have been associated with resistance to antiretroviral drugs, with the list of drug-resistant mutations being constantly extended. Until recently, all described RT-resistant mutations have been amino acid changes in the RT gene. We[9] and others[10,11] have identified a new type of multidrug-resistant HIV-1 mutants, which have an insertion of two amino acids between codons 68 and 69 (or 69 and 70, depending on sequence alignment) and an amino acid change at position 215 (T215Y), which is associated with zidovudine (AZT) resistance. In most of the mutants, an amino acid change at position 67 (D67E, G, or S) is also observed.[9–16] Moreover, the insertion mutants have one or two amino acid changes just before or after the insertion, making sequence alignment and numbering of sequence positions in this region ambiguous. The insertion mutants are resistant to an unprecedentedly broad spectrum of nucleoside RT inhibitors, including AZT, 3TC, d4T, ddI, and ddC.[9–11,17,18] Site-directed

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mutagenesis experiments have confirmed the role of the insertion alone, as well as in combination with the T215Y change, in conferring reduced susceptibility to most RT nucleoside inhibitors.[10,11,17] Genotypic analyses have revealed that the insertion mutants are present in about 3% of individuals with the T215Y change, which is one of the most common drug-resistant mutations.[9,10,16,18,19] So far, the appearance of the insertion mutants could not be related to a certain therapy regimen, as those mutants were derived from patients after prolonged switching therapy.[9–11,15,16,18–20] Originally, based on treatment histories of individuals harboring the insertion mutants, it has been suggested that the insertion mutants are specifically selected by treatment regimens containing ddI or ddC alone or in combination with AZT, but not during 1–3 years of AZT monotherapy.[10]

In the present study, we analyzed the evolution of the HIV-1 RT gene in an individual treated with various antiretroviral drugs. We demonstrated that multidrug-resistant insertion mutants do arise and may be selected during AZT monotherapy. By analyzing virus population dynamics over a 6-year period in relation to availability and kind of therapy, we demonstrated substantial differences in fitness between the wild-type (wt) and mutant viruses, dependent upon the drug environment. Our results demonstrate the capacity of the insertion mutant to maintain high virus loads in the presence of drugs.

MATERIALS AND METHODS

Patient history

The patient presented in this study was visiting the Academic Medical Center, Amsterdam, for follow-up and was described in our previous study[9] as patient 2. The patient was initially treated by AZT alone, with short courses of AZT therapy in combinations with 3TC, or ddI, or RTV (Fig. 1). Since the patient has failed on this therapy, it has been terminated. Later, triple therapy with 3TC, d4T, and SQV was initiated. Plasma sample obtained from the patient during triple therapy contained high levels of HIV-1 RNA, and therefore triple therapy was terminated. Phenotypic analysis of a virus isolated from the patient during triple therapy revealed high resistance to AZT, 3TC, and d4T, and moderate resistance to ddI and ddC.[9] At this time point, the virus contained an insertion of two amino acids between codons 68 and 69 of RT, as well as amino acid changes at codons 67 and 215. To study the evolutionary changes in virus populations in relation to availability and type of therapy, we sequenced full-length RT clones from viruses obtained from plasma samples of the patient at 12 time points over a 6-year period. The history of the treatment regimens, HIV-1 RNA loads, $CD4^+$ cell counts, and sampling moments are shown in Fig. 1.

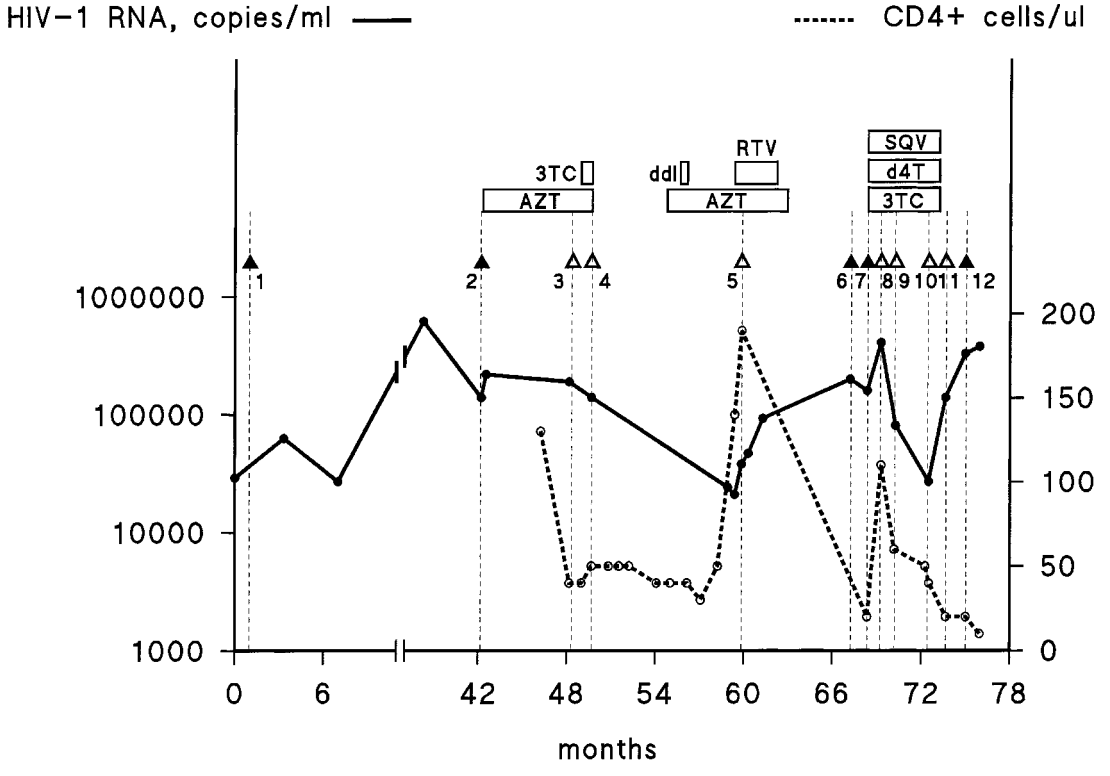


FIG. 1. HIV-1 serum RNA copy numbers (closed circles, continuous line), $CD4^+$ cell counts (open circles, dashed line), treatment history, and sampling moments (triangles) for the study individual. Closed and open triangles show the presence of the wt virus and the insertion mutant, respectively.

RNA isolation, amplification, and cloning

RNA was isolated from 200 μl of serum. HIV-1 RNA copy numbers were determined in all samples (NucliSens assay, Organon Teknika, Boxtel, The Netherlands). cDNA was obtained in an RT reaction by using the antisense primer 3′-RT-OUT (5′-CTACTTGTCCATGCATGGCTTCT, HxB2 positions 4391–4369). A fragment of 1906 nucleotides encompassing the whole RT gene was amplified by using the sense primer 5′-P66-OUT (5′-ACCTACACCTGTCAACATAAT, 2485–2505) and the antisense primer 3′-RT-OUT. The *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany) was used for all amplification reactions.

A nested PCR was performed with primers 5′-ET27 (5′-GACTCAGATTGGTTGCACTTTAAATTTTCC, 2521–2550) and 3′-RT22 (5′-AGGTTAAAATCACTAGCCATTGCTCTCC, 4284–4311). One microliter of the product (~20 ng/μl) was ligated into the pCR 2.1-TOPO vector (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Two microliters of the cloning reactions was transfected into TOP10 cells (Invitrogen, San Diego, CA).

From each plate, 30 colonies were picked up and tested for an insert with a PCR using primer set II: 5′-SP6-C-seq (5′-GATTTAGGTGACACTATAGGGTATACTGCATTTACCATACC, 2926–2946) and 3′-T7-D-seq (5′-TAATACGACTCACTATAGGGGCTAGCTGCCCCATCTACATA, 3889–3869). The products from positive clones (called fragment II) were used for sequencing.

For each positive clone, two other fragments, I and III, were generated with nested PCR by using primers 5′-ET27 (5′-CAGTCAGATTGGTTGCACTTTAAATTTTCC, 2521–2550) and 3′-END-PROT-T7 (5′-TAATACGACTCACTATAGGGGGAATATTGCTGGTGATCCTTTCCA, 3029–3005), for fragment I, and 5′-SP6-D-SEQ (5′-GATTTAGGTGACACTATAGGGGGAAAATTGGAATTGGGCAA, 3331–3350) and 3′-E-seq-T7 (5′-TAATACGACTCACTATAGGGTGCTCTCCAATTACTGTGAT, 4291–4272), for fragment III.

Sequencing and sequence analysis

For each of the 12 serum samples (Fig. 1), 17–20 full-length RT clones were sequenced. For each clone, three overlapping PCR fragments were sequenced. The final sequences were 1680 nucleotides in length. The PCR fragments were sequenced from the noncoding strand for fragments I and III with the T7 primer (5′-TAATACGACTCACTATAGGG), and from the coding strand for fragment II with the SP6 primer (5′-GATTTAGGTGACACTATAGG) by using the ABI Prism BigDye termina-

TABLE 1. SEQUENCE CHARACTERISTICS OF HIV-1 RT CLONES AT AMINO ACID POSITIONS 66–71 AND 215 AT EACH TIME POINT IN RELATION TO THERAPY EVENTS

		Sequence characteristics of the RT clones			
Time points	*Therapy event*	*Number of clones*	*Positions 66–71*	*Number of clones*	*Position 215*
HIV B consensus			K D S . . T K W[a]		T
1 and 2	Before therapy	38/38	- - - . . - - -	38/38	–
3	162 days of AZT monotherapy	10/19	- N - S S G A -	19/19	Y
		5/19	- S - S S G A -		
		3/19	- T - S S G A -		
		1/19	- N - S S G T -		
4	208 days of AZT, 20 days of 3TC	19/20	- S - S S G A -	20/20	Y
		1/20	- T - S S G A -		
5	146 days of the second AZT course, 26 days of RTV	20/20	- S - S S G A -	20/20	Y
6	133 days after termination of AZT	20/20	- - - . . - - -	20/20	–
7	First day of 3TC, d4T, and SQV	20/20	- - - . . - - -	20/20	–
8	28 days of 3TC, d4T, and SQV	9/19	- S - S S G T -	19/19	Y
		8/19	- S - S S G A -		
		1/19	- S - S S D T -		
		1/19	- S - G S D T -		
9	55 days of 3TC, d4T, and SQV	18/19	- S - S S G T -	19/19	Y
		1/19	- S - S R G T -		
10	126 days of 3TC, d4T, and SQV	18/19	- S - S S G T -	19/19	Y
		1/19	- N - S S G A -		
11	21 days after termination of 3TC, d4T, and SQV	20/20	- S - S S G T -	20/20	Y
12	62 days after 3TC, d4T, and SQV	17/17	- - - . . - - -	17/17	–

[a]In the sequence alignment, dashes indicate amino acid identity with the consensus, and dots indicate the absence of aa at this position.

tor cycle sequencing kit. The sequence products were analyzed on an automatic sequencer (ABI Prism DNA sequencer model 377XL).

The sequences were aligned manually. Phylogenetic analysis was performed by using MEGA software (neighbor-joining method), based on nucleotide distances calculated according to the Kimura two-parameter distance estimation method as well as synonymous distances.[21,22] The sequences are deposited in the GenBank, accession numbers AF331192–AF331422. To characterize intrasample sequence heterogeneity, time point consensus sequences were calculated as the mean of all clones obtained at this time point, and the Kimura two-parameter distances of all individual sequences to the time point consensus were calculated. Statistical comparison of intrasample heterogeneity was performed by using the *t* test.

RESULTS

Virus evolution and dynamics of virus populations in relation to therapy

To study the evolution of HIV-1 populations in relation to availability and type of therapy, 231 full-length RT clones (1680 nucleotides in length) were sequenced from viruses obtained at 12 time points over a 6-year period, 17–20 clones per time point. During the observation period, the individual received AZT alone or in combination with 3TC, or ddI, or RTV, triple therapy by 3TC, d4T, and SQV, or no antiretroviral therapy. The treatment history and sampling moments are shown (Fig. 1). Numbers of clones sequenced at each time point and sequence heterogeneity observed among clones at amino acid positions 66–71 and 215 are shown (Table 1). The consensus sequences of virus populations that became selected during each treatment regimen as well as in the absence of therapy are shown (Fig. 2).

The amino acid sequences of RT clones obtained from the individual at two time points before the first course of AZT monotherapy contained no insertions and were identical to the subtype B consensus in this region (66-KDSTKW-71, Fig. 2 and Table 1). All clones had a Thr at position 215, which is also characteristic of wt virus.

As soon as 162 days after the start of AZT monotherapy (time point 3), all obtained viruses contained an insertion of two amino acids between codons 68 and 69, amino acid changes at positions 67, 69, and 70, as well as an amino acid change T215Y (Table 1). Sequence heterogeneity was observed at position 67: the wt codon GAC (D) was changed into AAC (N, 11/19 clones; one nucleotide substitution), ACC, or AGC (T, 3/19 clones, or S, 5/19 clones, respectively; both changes require two nucleotide substitutions). All but two (115/117) of the insertion mutants obtained later in infection had S at this position, suggesting a selection for this amino acid. Therefore, sequence heterogeneity observed at position 67 at time point 3 is likely a reflection of the following evolutionary pathway: GAC → AAC → AGC.

Since, besides the insertion, there were amino acid changes at positions 69 and 70 (Table 1), the evolutionary pathway of these sequence changes could not be reconstructed. These changes could be a result of an SS insertion between codons 68 and 69 and amino acid changes at positions 69 and 70 (TK → GA), or of amino acid changes TK → SS at these positions with an insertion of GA after position 70, or more complex evolutionary processes (e.g., deletion of TK, insertion of SSGA). Besides the insertion and amino acid changes at positions 67 and 215, there were two other amino acid differences between the consensus sequences of the wt and mutant populations—L329I and R350K (Fig. 2). At both of these positions, the consensus sequences of the insertion mutants were identical to the subtype B consensus (Fig. 2). Moreover, at both of these positions extensive sequence variation was observed among the wt as well as mutant populations during the observation period (not shown). Therefore, these differences between the wt and mutant consensus sequences are unlikely to be related to the drug-resistant phenotype or to reflect compensatory changes. Rather, they could be a result of sampling effects. A number of other amino acid changes was observed in individual sequences among both the wt and mutant population, reflecting the high mutation rate of HIV-1 (Fig. 3). The means and ranges of intrasample sequence distances found in our study were similar to the levels of virus heterogeneity that are generally observed in infected patients.[23]

The insertion mutants were the only viruses found in the individual during ongoing therapy by AZT alone (Fig. 1, time point 3) or in combination with 3TC (time point 4) or RTV (time point 5). The mutant virus population in serum was com-

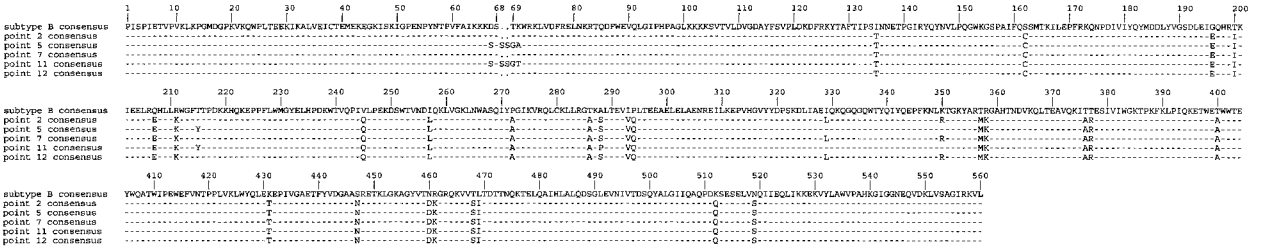


FIG. 2. Consensus sequences of the RT gene of HIV-1 populations that were selected at the end of each treatment regimen as well as in the absence of antiretroviral therapy. For each time point, consensus sequences were calculated as the arithmetic mean of all individual clones obtained at this time point. The HIV-1 subtype B consensus is shown for comparison. Dashes indicate amino acid identity with the subtype B consensus; dots are introduced to facilitate alignment. Amino acid positions are numbered starting from the first amino acid of the RT protein.

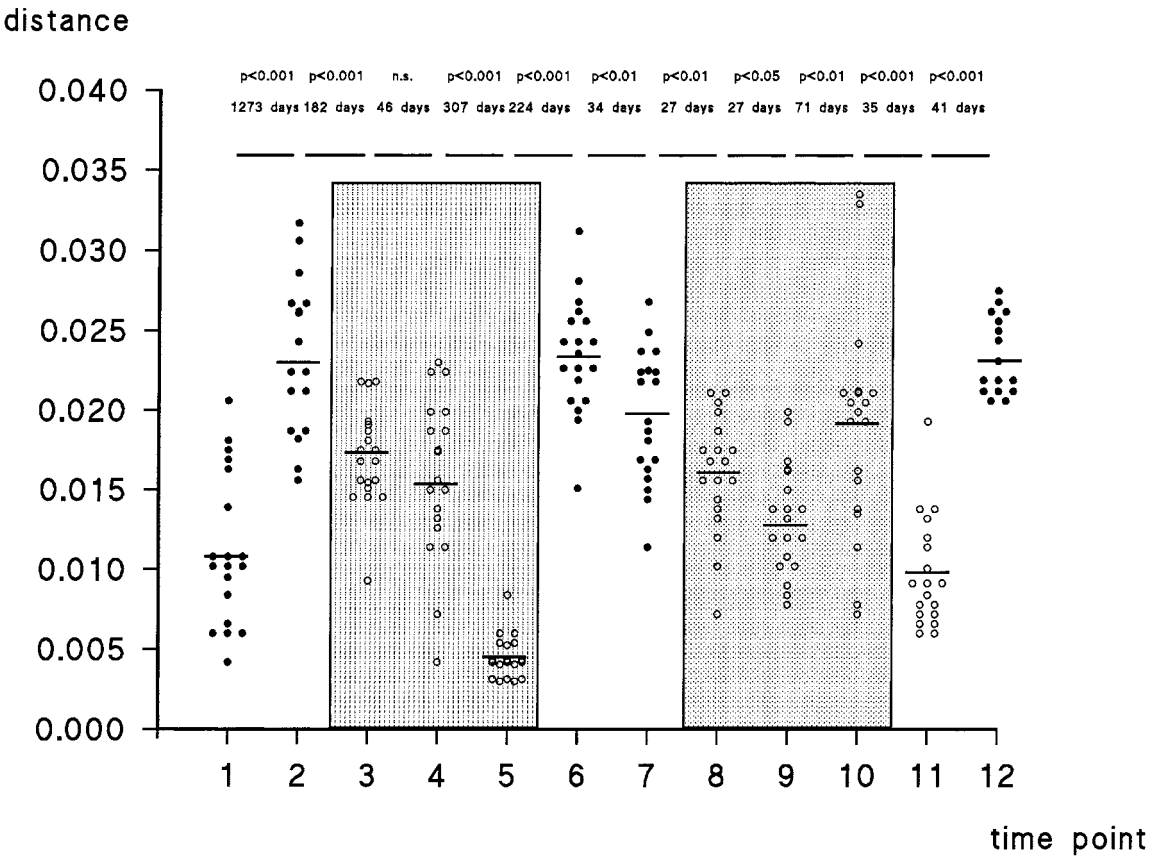


FIG. 3. Intrasample nucleotide sequence heterogeneity of HIV-1 populations at each time point. For each time point, a consensus sequence was calculated as the mean of all clones present at this time point. Circles show genetic distances of each clone to the time point consensus; horizontal lines show the mean distances. Closed and open circles show the presence of the wt virus and the insertion mutant, respectively. *p*-Values for the comparisons of genetic distances and time intervals between consecutive time points are shown. Therapy periods are boxed.

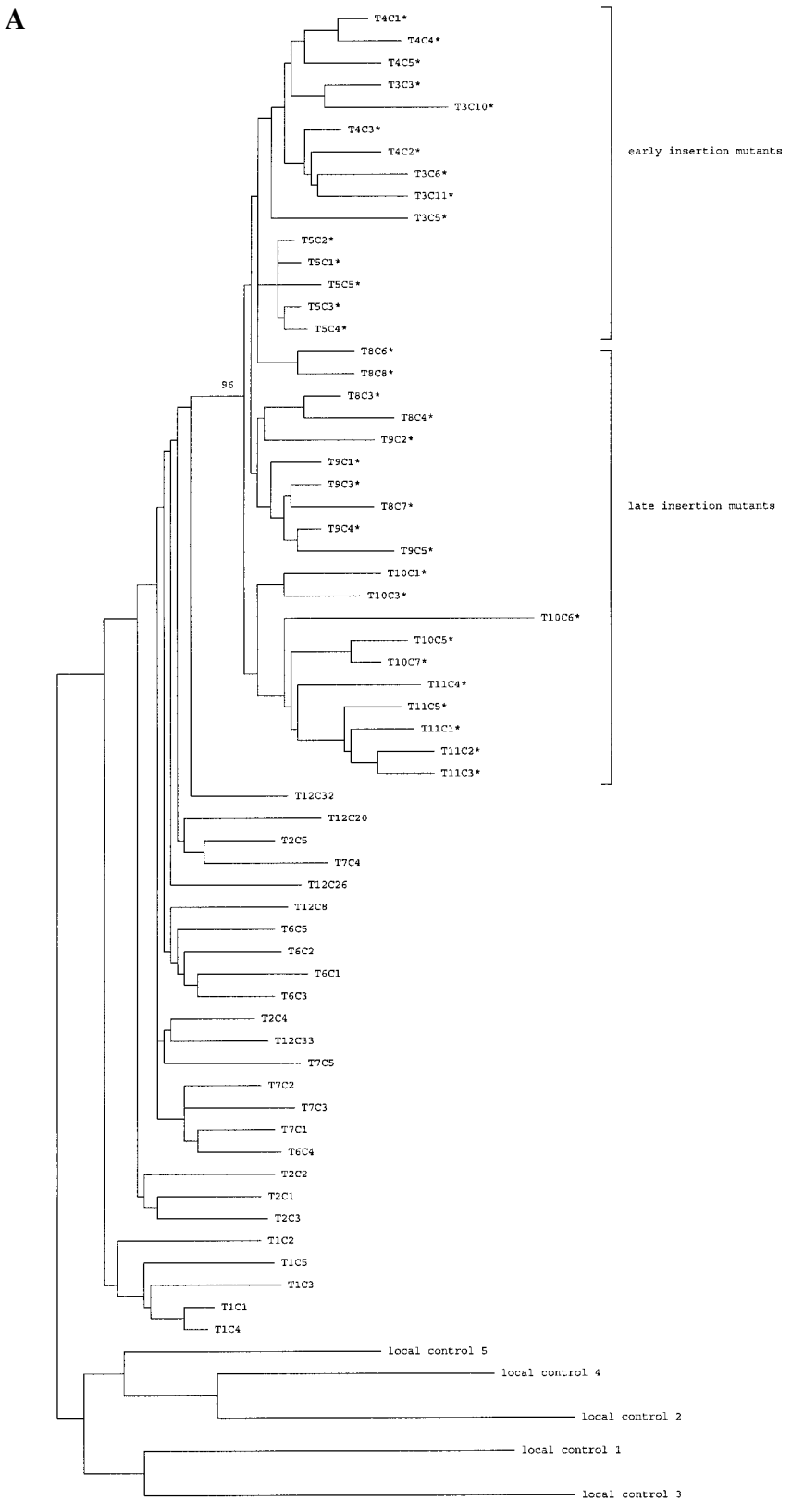
pletely replaced by the wt viruses 133 days after the termination of therapy (time point 6). Wt viruses were present in serum until the beginning of triple therapy (time point 7, Table 1 and Fig. 2).

As early as 28 days after the initiation of triple therapy by d4T, 3TC, and SQV, the wt virus population was completely replaced by the mutant population (time point 8, Table 1). At this time point, sequence heterogeneity within the region of insertion was found among clones. The virus variant containing an SSGA insertion, which was the major insertion variant in earlier samples (time points 3–5, 58/59 clones), was found in only 8 of 19 clones sequenced at time point 8 (Table 1). Previously unseen virus variant, containing an SSGT insertion and an D67S amino acid change, was found in 9 of 19 clones. An A to T amino acid change within the insertion was caused by a single nucleotide substitution (GCA → ACA). Two other clones contained insertions of SSDT or GSDT, which have not been found earlier or later in infection (Table 1).

The SSGT variant, which was still in the minority at time point 8 (9/19 clones), was subsequently selected for. It was the major variant 55 and 126 days after the initiation of triple therapy (time points 9 and 10, 18/19 clones at both time points) and was the only variant observed in the first sample after the termination of triple therapy (time point 11, 20/20 clones). Sixty-two days after the termination of triple therapy, the insertion mutant population was replaced by the wt viruses (Table 1 and Fig. 2, time point 12).

Virus population heterogeneity

To characterize the intrasample heterogeneity of virus populations, we calculated genetic distances of sequences obtained at each time point to the respective time point consensus (Fig. 3). At all time points (except for time point 1), the mean heterogeneity of the wt virus populations was higher than that of the mutant populations. After the initial (between time points 1 and 2) increase, genetic heterogeneity of the wt viruses stabilized. In contrast, the intrasample heterogeneity was decreasing during both periods when the insertion mutants were found in the individual (Fig. 3, time points 3–5 and 8–11). During AZT therapy, a decreasing intrasample sequence heterogeneity coincided with the addition of new drugs to the treatment regimen (after the start of 3TC therapy, time point 4, and after the start of RTV therapy, time point 5). During the reappearance of the insertion mutants, their intrasample heterogeneity was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T4C1*
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early insertion mutants
T5C2*
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T5C5*
T5C3*
T5C4*
T8C6*
T8C8*
96
T8C3*
T8C4*
T9C2*
T9C1*
T9C3*
T8C7*
late insertion mutants
T9C4*
T9C5*
T10C1*
T10C3*
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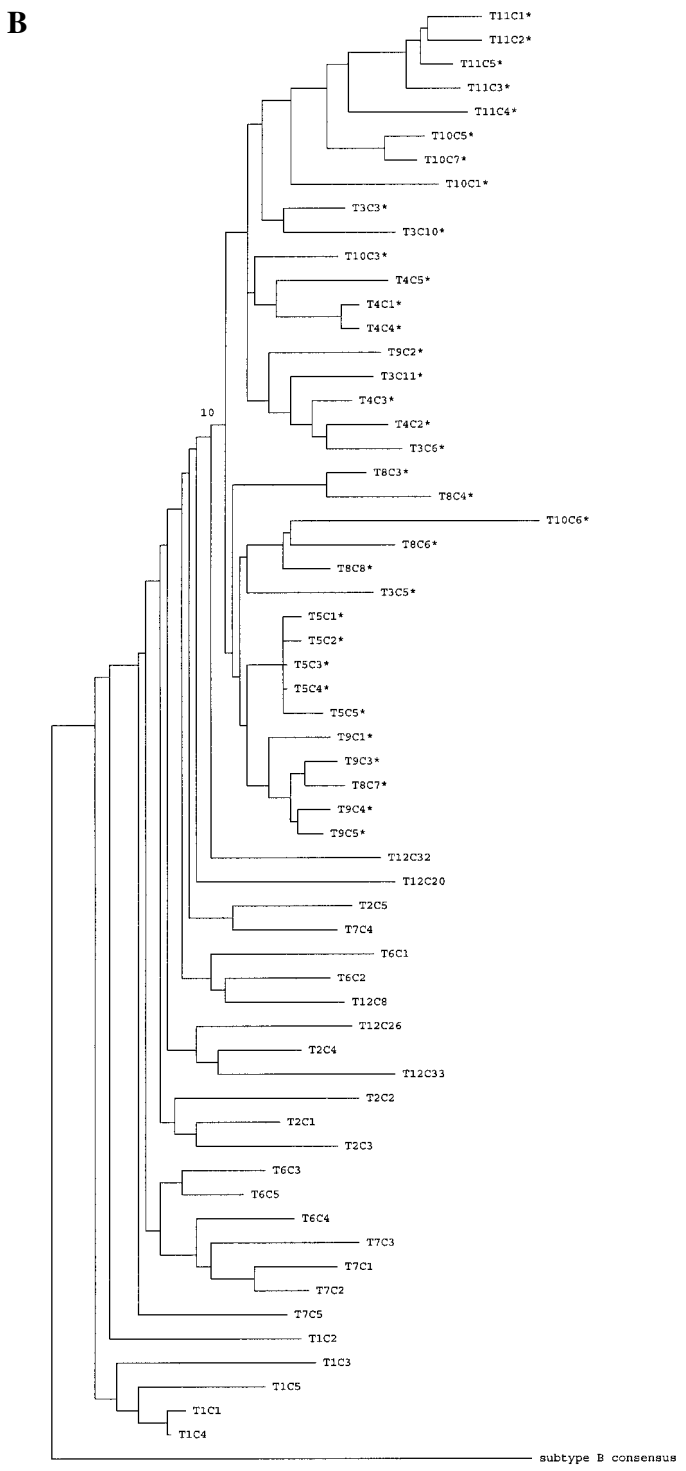

FIG. 4. Phylogenetic analysis of virus RT sequences obtained from the study individual, based on the Kimura two parameter distances (**A**) and synonymous distances (**B**). Since the trees that include all sequences are too large to be presented, phylogenetic analysis of selected sequences is shown. Similar results were obtained when all sequences were included in the analysis. Sequences are labeled by their sampling moments (T??) and clone numbers (C??). The insertion mutants are marked by an asterisk (*). Bootstrap values for the insertion mutant cluster are shown (100 replications). For (**A**), sequences from participants of the Amsterdam cohort of homosexual men are included as local controls. For (**B**), the subtype B consensus sequence is used to root the tree.

relatively stable during triple therapy and decreased markedly after its termination (Fig. 3, time point 10).

Phylogenetic relationships between the insertion mutants and the wt viruses

Phylogenetic analysis based on nucleotide distances revealed separation of virus clones into two clusters according to presence or absence of the insertion (Fig. 4A). Yet, the wt and mutant sequences clustered together and separately from local controls (sequences from participants of the Amsterdam cohort studies of homosexual men, Fig. 4A). This observation strongly suggests that the appearance of the insertion mutants is the result of the intrahost evolution of the original wt virus population, and not that of superinfection. Moreover, insertion mutants present in the individual during triple therapy (time points 8–11) were phylogenetically closely related to the early virus mutant population (time points 3–5). To additionally support this observation, we analyzed synonymous differences among virus sequences[21,22] (Fig. 4B). Since synonymous substitutions do not change amino acids (and, therefore, virus phenotype), their analysis could not be biased by convergent evolution under selective pressure of antiretroviral drugs (when, for instance, a T215 amino acid change arises independently in two different virus populations, artificially making them more similar in a phylogenetic analysis). Our analysis of synonymous distances resulted in the bootstrap value for the mutant sequence cluster dropping from 96 to 10 (Fig. 4A versus 4B). Moreover, early (time points 3–5) and late (time points 8–11) insertion mutants did not form separate clusters (Fig. 4B). This finding is evidence for the reappearance of the insertion mutants being related to an expansion of the original mutant (still present in long-lived cells and/or body compartments other than serum) rather than to a new insertion. Our observation that each time the wt or mutant virus populations reappeared in the individual, they were heterogeneous (Fig. 3, time points 6, 8, and 12), suggests that their reservoirs are extensive.

Relative fitness of the insertion mutant

The fast replacement of the wt virus by the insertion mutant during therapy and the fast reversion to the wt after termination of therapy (Table 1 and Fig. 1) suggest substantial fitness differences. We developed two models to estimate the relative fitness of the insertion mutant from the virus dynamics. Since the insertion in the RT is expected to influence the replication rate of the virus, but not its half life, we write

$$\frac{dW}{dt} = rW - \delta W \text{ and } \frac{dM}{dt} = r(1+s)M - \delta M \qquad (1)$$

for the dynamics of wt virus W and mutant virus M. The r is the (wt) replication rate, $1/\delta$ is the generation time, and s is the classical coefficient of selection. Because this model combines the dynamics of productively infected cells and free virions, the generation time should be about 2 days.[24,25] The same model was used before for estimating selection coefficients.[26,27]

For estimating the relative fitness $(1 + s)$, write the solutions $W(t) = W(0)e^{(r-\delta)t}$ and $M(t) = M(0)e^{[r(1+s)-\delta]t}$, to see that s is computed from the logarithms of the ratios M/W at time 0 and time t:

$$s = \frac{1}{rt}\left(\ln\frac{M(t)}{W(t)} - \ln\frac{M(0)}{W(0)}\right) \qquad (2)$$

$p = M/(W + M)$ may be defined as the frequency of the mutant genotype and Eq. (2) may be rewritten into

$$s = \frac{1}{rt}\left(\ln\frac{p(t)}{1-p(t)} - \ln\frac{p(0)}{1-p(0)}\right) \qquad (3)$$

Thus, for estimating the relative fitness, we need to know the ratio of the genotype frequencies and the total replication rt.[27] Both are not provided by the data however.

First consider the reversion to wt virus between time points 11 and 12, i.e., 21 and 62 days after termination of triple therapy. At time point 11, 20/20 sequences were mutants, and 41 days later 17/17 sequences were wt. Since $1 - p = 0$ and $p = 0$ at time points 11 and 12, respectively, we cannot employ Eq. (3). Additionally, not knowing the true frequencies of wt virus, we cannot estimate its replication rate r. Similar problems apply to the data during treatment. At time points 7 and 8, 20/20 sequences were wt and 19/19 sequences had the insertion, respectively.

Previous publications estimated that the doubling time of wt HIV-1 following therapy interruption is about 1.6 days[28] to 2.0 days.[29] In terms of Eq. (1), an average of 1.8 days means that $r - \delta = \ln 2/1.8 \approx 0.4$/day. Knowing that $\delta \approx 0.5$,[24,25] we estimate that between time points 11 and 12 $r = 0.9$/day. Considering a "worst case scenario" for the genotype frequencies by assuming that the next sequence to be sampled would be of the other type, a conservative estimate of the ratio (M/W [or $p/(1-p)$]) at time points 11 and 12 is 20 and 1/17, respectively. The corresponding s becomes

$$s = \frac{\ln(1/17) - \ln 20}{0.9 \times 41} = -0.16 \qquad (4)$$

Thus, the relative fitness of the insertion mutant should be less than 84%, compared to the wt virus. Since the replication rate r during treatment is not known, it is not possible to use a similar approach for time points 7 and 8.

In the Appendix, we resolve these problems by developing a model for calculating the likelihood of any combination of fitness difference r and initial frequency $p(0)$. This gives a minimal estimate for the s. We compute the smallest selection coefficient that is obtained with 95% probability. The Appendix develops from the model given in Eq. (1) by changing the interpretation of W being the loser and M being the winner. The selection coefficient should therefore be positive. We estimated an upper bound for the replication rate r of the loser by the expansion of the total virus load. Between time points 11 and 12, the total virus load expands 2.36-fold in 41 days, and between time points 7 and 8, the virus load expands 2.56-fold in 27 days. Solving $\ln 2.36 = (r - \delta) \times 41$ and $\ln 2.56 = (r - \delta) \times 27$ with $\delta = 0.5$/day,[25] we obtain $r = 0.52$/day for the replication rate of the mutant in the absence of therapy, and $r = 0.53$/day for the replication rate of the wild-type virus during therapy. Note that the upper bound $r = 0.52$/day estimate for the replication rate of the insertion mutant in the absence of therapy is nevertheless smaller than the $r(1 - s) = 0.9 \times 0.84 = 0.76$/day estimate used above, meaning that the true doubling time during this interval is longer than 1.6 days.

Figure 5 depicts a contour plot of the likelihood that the data are explained by any combination of the fitness difference *rs* [see Eq. (1)] and $p(0)$ (i.e., the initial frequency of the winner). From Fig. 5A, where the mutant is the winner between time points 7 and 8, we read that the minimal value on the 95% lower confidence level is $rs \approx 0.185$, which is attained around an initial frequency of the insertion mutant of $p(0) = 0.075$. Because we estimated $r \leq 0.53$/day for the replication rate of the wt virus during therapy, we estimate $s \geq 0.35$. The relative fitness of the insertion mutant during treatment should therefore be 135% at least.

From Fig. 5B, in which the wt is the winner between time points 11 and 12, we read a minimal value of $rs \approx 0.12$ at the 95% confidence limit, which is attained around an initial frequency of the wt virus of $p(0) \approx 0.072$. Because we estimated $r \leq 0.52$/day for the replication rate of the mutant in the absence of therapy, we estimated $s > 0.23$. The relative fitness of the insertion mutant in the absence of therapy should therefore be less than $100/1.23 = 81\%$, close to the fitness of 84% estimated above.

DISCUSSION

Recently, several groups have reported a new type of drug-resistant HIV-1 mutants, which have an insertion of two amino acids between codons 68 and 69 in combination with amino acid changes at position 215 and, in most cases, at position 67.[9–11,13,20] Frequencies of the insertion mutants in treated populations were remarkably consistent in several studies: about 3% of individuals with the T215Y change.[9,10,18,19] So far, the appearance of the insertion mutants could not be related directly to particular therapy regimens,[9,13,15,20] although it has been suggested that selection by ddI and/or ddC, and not by AZT monotherapy, is likely to play a role.[10]

In the present study, we established that multidrug-resistant insertion mutants do arise and can be selected for by AZT monotherapy. In addition to previous studies, in which extensive genetic variation of the insertion mutants among individuals (interhost heterogeneity) has been noted, we provide data on the intrahost evolution of this mutant. Generation of a so complex mutant requires multiple evolutionary events, which we were able to trace in our study. Previously, three sequence patterns have been found in insertion mutants: (1) an insertion of two amino acids between codons 68 and 69 together with amino acid changes at adjacent positions, (2) a T215Y change, and (3) amino acid changes at position 67 (in the majority of mutants). In our study, the earliest observed insertion mutant (at time point 3) already had all three sequence changes. While the T215Y amino acid change was conserved in all insertion clones over time, we observed the evolution of the other two sequence patterns. As the result of a single nucleotide substitution, the wt D at position 67 was changed into N in the majority of early insertion mutants (Table 1). A second nucleotide substitution within the same codon resulted in the second amino acid change ($D \rightarrow N \rightarrow S$), which was rapidly selected for. Based on these data, we suggest that the D67N mutant is less fit and represents an intermediate variant during the 67D to 67S

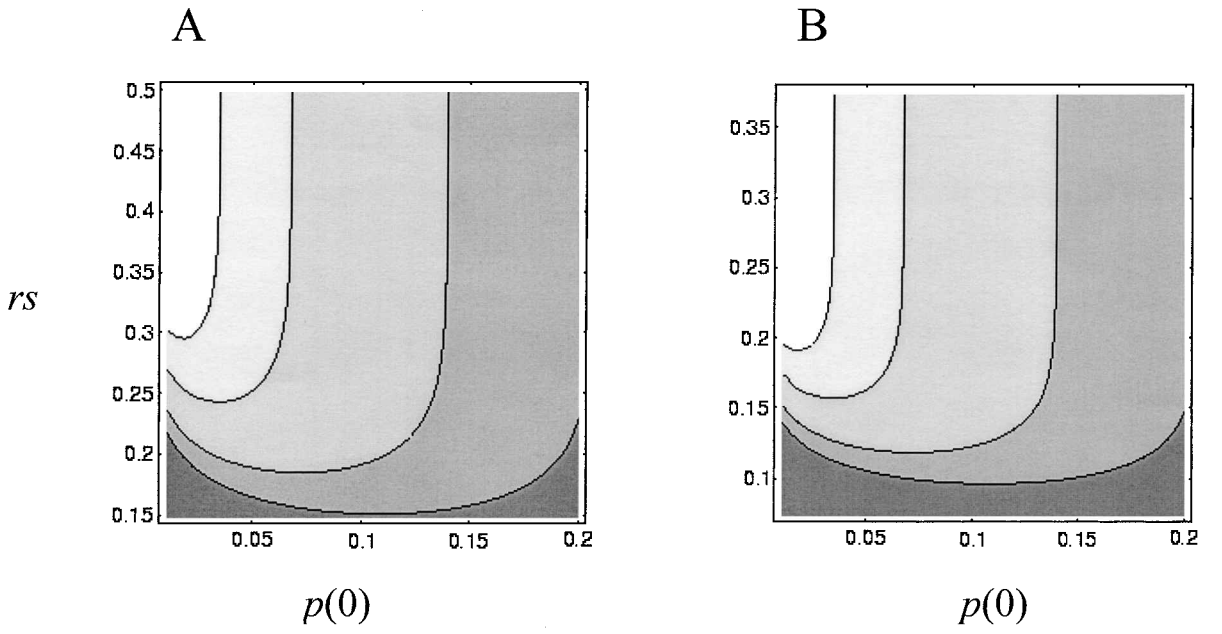

FIG. 5. The likelihood λ as a function of the fitness difference *rs* and the initial frequency $p(0)$ of the winning variant. (**A**) The expansion of the insertion mutant during therapy between time points 7 and 8; $p(0)$ is the unobserved initial frequency of the mutant virus. (**B**) The expansion of the wild-type virus after therapy between time points 11 and 12; $p(0)$ is the unobserved initial frequency of the wt virus. Shaded contours (from light to dark) depict $\lambda = 0.5$, 0.25, 0.05, and 0.01. The 95% confidence limit for each pair [*rs*, $p(0)$] corresponds to the contour line at $\lambda = 0.05$ (see the Appendix).

evolution. Within the insertion region, the SSGA mutant, which was present during the first therapy regimens (AZT alone or in combination with 3TC or RTV), was also found to be one of the two major virus subpopulations after a month of triple therapy (time point 8; Table 1). Subsequently, the second major subpopulation, containing SSGT, was selected for, suggesting its competitive advantage.

Our observation that the earliest insertion mutant (time point 3) already had all three sequence patterns that have previously been described in patients after years of antiretroviral therapy suggests that these changes may be cooperative and essential for maintaining the RT function. In the 3D structure of the RT protein, position 215 is located close to the insertion region.[30,31]

During both periods when the insertion mutants were present in the individual (Fig. 3), a decreasing intrasample virus heterogeneity was observed. This trend was especially prominent during the first period. It is likely that addition of 3TC and RTV to the treatment regimen during this period resulted in population bottlenecks.[32] Successive selection of particular viruses from a variety of insertion mutants (competitive exclusion, Table 1) may have also contributed to a decrease of virus heterogeneity between time points 3 and 5. This phenomenon was also observed in the second period (time points 8–11), when suppression of the wt virus by triple therapy originally (time points 8–10) resulted in reemergence of various competing insertion mutants. Of them, only one variant was finally selected (time point 11, Table 1). The observation that this variant (with an SSGT insertion), although being found at earlier time points, became the only virus variant present after termination of triple therapy may indicate its higher fitness relative to other insertion mutants. Our finding that the intrasample heterogeneity was higher for the wt population than for the mutant emphasizes the importance of selection pressure by drugs and possible benefits of therapy even if it is not achieving maximal virus suppression.

Termination of antiretroviral therapy in patients with drug-resistant mutants generally leads to the restoration of the wt virus population, which is interpreted as a consequence of competitive disadvantages of drug-resistant mutants in the absence of drugs. Impaired fitness has earlier been demonstrated in cell cultures for various drug-resistant mutants.[33–39] We demonstrated a gross competitive disadvantage of the insertion mutant compared to the wt virus in the absence of drug pressure. This conclusion is based on our observation that after termination of triple therapy, the mutant population was replaced by the wt viruses within a month. Since *in vivo* fitness estimates for viruses resistant to single RT inhibitors are not available (except for the 215 mutants[26,40]), we cannot directly compare our fitness data with those for other drug-resistant viruses. Earlier, for AZT-resistant mutants, dynamics of restoration of the wt virus population after the termination of AZT monotherapy was described.[41–43] Considering the fact that it takes from half a year to several years for the wt viruses to replace AZT-resistant mutants, and that the restoration of the wt genotype is generally incomplete,[42] we suggest that the fitness decrease of AZT-resistant mutants is less than that of the multidrug-resistant insertion mutants. For an AZT-resistant, 215Y mutant, the fitness reduction in the absence of drugs has been estimated to be between 10% and 25%,[26,40] compared to the fitness loss of at least 16% for the insertion mutant, demonstrated in the present study.

The HIV-1 RNA load in plasma hardly changed when, following termination of triple therapy, the mutant population was replaced by the wt viruses (Fig. 1). This may seem surprising, since lower fitness of a virus strain, which results in the preferential elimination of this virus in competition with another strain, therefore cannot be equated to lower steady-state virus load. Besides virus fitness, other factors shaping intrahost environment, including characteristics of the immune response, target cell availability, virus tropism, and the presence or absence of competitor viruses, may all be crucial for maintenance of steady-state virus load.[44] Therefore, it seems to be incorrect to directly compare fitness of two HIV-1 strains based on their loads in the host(s) without considering peculiarities and dynamics of the intrahost environment.

APPENDIX

The sampled sequences at time points 7, 8, 11, and 12 contained either the wt viruses or the insertion mutants only. It is therefore impossible to calculate the conventional ratios of genotype frequencies. It is possible, however, to compute a lower confidence limit on the fitness difference rs and to obtain an upper bound estimate for the replication rate r. It will be convenient to relabel Eq. (1) such that we always consider the outgrowth of a "winner" that was initially present at an unknown minority frequency $p(0)$. Thus, the growth rate of the winner is $[r(1+s) - \delta]$/day, and that of the loser is $(r - \delta)$/day. Since the loser completely disappears over the observation interval, an upper bound of the loser's growth rate can be obtained from the expansion of the total viral load over the interval. In the text, we thus estimated for the replication rate of the wt virus between time points 7 and 8 that $r < 0.52$/day, whereas that of the mutant between time points 11 and 12 should be $r < 0.53$/day.

As a function of the initial frequency $p(0)$ and the replication rate r, the frequency $p(t)$ of the winner at the end of an observation interval of t days is given[26] by

$$p(t) = \frac{p(0)e^{rst}}{1 - p(0) + p(0)e^{rst}} \tag{5}$$

The absence of the minority species in all samples allows the confidence level for any pair of parameters $[rs, p(0)]$ to be estimated by computing the likelihood λ of the data by the binomial statistics

$$\lambda = [1 - p(0)]^{n(0)} p(t)^{n(t)} \tag{6}$$

where $n(t)$ is the number of sequences samples at time t, and $p(t)$ is provided by Eq. (5). The confidence limits then correspond to contour lines at level $1 - \lambda$ $[rs, p(0)]$. The likelihood λ $[rs, p(0)]$ is plotted as a gray-scale coded contour plot in Fig. 5.

By our simplified notation, it may seem that we have assumed exponential growth over both observation intervals. A recent paper,[27] however, demonstrated that the parameter r may be replaced by an arbitrary function $r(t)$, allowing for nonexponential growth. That study showed that $r(t)$ can be estimated by the total viral expansion over the observation interval. Since

we used the same expansion to compute the replication rate r, identical results would be obtained if the growth were nonexponential.

ACKNOWLEDGMENTS

We thank Margreet Bakker for processing clinical samples, Suzanne Jurriaans for HIV-1 RNA quantitation, Lia van der Hoek for providing sequences from the Amsterdam Cohort of homosexual men, and André Noest and Stan Marée for developing the model outlined in the Appendix. The work of R.dB. and André Noest is financially supported by the Dutch AIDS foundation (PccO Grant 1317).

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