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Increased Multinucleoside Drug Resistance and Decreased Replicative Capacity of a Human Immunodeficiency Virus Type 1 Variant with an 8-Amino-Acid Insert in the Reverse Transcriptase

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Resistance to antiretroviral drugs is generally conferred by specific amino acid substitutions, rather than insertions or deletions, in reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1). The exception to these findings is the amino acid insertions found in the β3-β4 loop of the RT enzyme in response to treatment with nucleoside reverse transcriptase inhibitors. This insert consists most commonly of two amino acids, but we describe in detail the evolution of a variant with an 8-amino-acid (aa) insert in a patient treated with zidovudine (ZDV) and 2′-3′-dideoxycytidine (ddC). The 24-nucleotide insert is a partial duplication of local sequences but also contains a sequence segment of unknown origin. Extensive sequence analysis of longitudinal patient samples indicated that the HIV-1 population prior to the start of therapy contained not the wild-type amino acid 215T in RT but a mixture with 215D and 215C. Treatment with ZDV and subsequent ZDV-ddC combination therapy resulted in the evolution of an HIV-1 variant with a typical ZDV resistance genotype (41L, 44D, 67N, 69D, 210W, 215Y), which was slowly replaced by the insert-containing variant (41L, 44D, insert at position 69, 70R, 210W, 215Y). The latter variant demonstrated increased resistance to a wide range of drugs, indicating that the 8-aa insert augments nucleoside analogue resistance. The gain in drug resistance of the insert variant came at the expense of a reduction in replication capacity when assayed in the absence of drugs. We compared these data with the resistance and replication properties of 133 insert-containing sequences of different individuals present in the ViroLogic database and found that the size and actual sequence of the insert at position 69 influence the level of resistance to nucleoside analogues.

The clinical benefits of antiretroviral treatment are seriously limited by the emergence of drug-resistant human immunodeficiency virus type 1 (HIV-1) variants during therapy (15). HIV-1 is estimated to produce on the order of 10^{10} virions per day in an untreated individual. This, combined with an error-prone and recombination-prone replication machinery, provides this virus with an enormous capacity to adapt to a given environmental change, such as antiviral therapy. The majority of treated patients with detectable plasma viremia harbor drug-resistant viral variants. Amino acid substitution is the most common mechanism to generate resistance to a drug. For instance, high-level resistance to ZDV, the first available nucleoside analogue for the treatment of HIV-1 infection, results from combinations of amino acid substitutions at six positions (41, 67, 70, 210, 215, and 219) in the targeted reverse transcriptase (RT) enzyme. These mutations were first identified in clinical isolates and subsequently correlated with zidovudine (ZDV) resistance by site-directed mutagenesis studies. The acquisition of drug resistance may come at a price, which is reduced performance of the mutated RT enzyme and impaired virus replication capacity (RC) (1, 2, 10, 14, 20). Ongoing viral replication can result in the selection of variants with compensatory changes elsewhere in the viral genome that partially rescue the replication defect (35). Impaired replication and subsequent selection of compensatory changes have also been reported for virus variants that are resistant to protease inhibitors (11, 17, 28, 46).

Treatment with combinations of nucleoside reverse transcriptase inhibitors (NRTIs) may result in the selection of amino acid substitutions in RT that provide cross-resistance within this drug class. For instance, Q151M is a multinucleoside resistance mutation that usually coincides with changes at codons 62, 75, 77, and/or 116 (21, 40, 41). A dipeptide insertion between RT amino acids 69 and 70 has also been implicated in multinucleoside resistance (25, 32, 45). These so-called finger insertion mutations usually appear during therapy with ZDV in combination with other nucleoside analogues, and the insert is generally present in combination with some of the typical ZDV resistance mutations (12, 25, 32, 43, 45). The inserted dipeptide is most often SS or a variation thereof and is present in approximately 2% of NRTI-treated individuals (9, 42–44). The insert provides a certain level of drug resistance, but it also has a negative impact on viral replication fitness in the absence of drugs (37).

HIV-1 RT can become resistant to nucleoside analogues through two mechanisms. Increased discrimination against the unnatural deoxyribonucleoside triphosphate of the nucleoside analogue can block its incorporation and thus result in less chain termination. Alternatively, the ability to remove chain

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terminators from blocked DNA chains may be enhanced by the resistance mutations, allowing DNA synthesis to resume. The finger insertion mutations have been shown to act through the second mechanism, as they increase the ATP-dependent removal of chain terminators (8, 26, 30, 31, 33).

Genotypic drug resistance tests have been routinely used as a diagnostic tool since 1996 in the Academic Medical Center of the University of Amsterdam. Our database of HIV-1 genotypes presently contains more than 2,000 sequences, and finger insertion mutations were identified in eight samples. Consistent with other reports, we observed mainly dipeptide inserts. However, we also observed a unique variant with an 8-aa insert that is the topic of this study. We describe the evolution of this 8-aa virus variant in a patient on ZDV–2′-3′-dideoxycytidine (ddC) therapy. We performed assays to measure the drug resistance properties, the replication capacity, and the relative in vivo fitness of this HIV-1 variant. In addition, we analyzed the ViroLogic database that includes data on the drug resistance phenotype. This analysis indicates that the larger finger inserts provide a higher level of ZDV resistance.

MATERIALS AND METHODS

Clinical history of the patient. Patient M12286 is a 41-year-old male who was first found to be HIV-1 seropositive and having AIDS as a result of extrapulmonary tuberculosis on 3 March 1994. He began ZDV monotherapy on 3 May 1994 at a CD4 lymphocyte count of 320/mm^3, and ddC was added on 7 May 1996 at a CD4 count of 220/mm^3. In spite of the availability of the first HIV-1 protease inhibitors shortly thereafter, the patient preferred to maintain ZDV-ddC. Once the CD4 lymphocyte count in the following years had gradually declined to a nadir of 90 cells/mm^3, the patient started highly active antiretroviral therapy (HAART) with a mix of RT and protease inhibitors (lamivudine [3TC], stavudine [d4T], nelfinavir [NFV], and saquinavir) on 29 June 1999. The plasma HIV-1 RNA level prior to starting HAART was 120,000 copies/ml (NucliSense; bioMérieux, Boxtel, The Netherlands) and declined to below quantifiable levels (<40 copies/ml) within 3 months, with the CD4 count showing a rise to 290 cells/mm^3. Viral suppression was sustained, and CD4 cells gradually rose to >500/mm^3. Over the years the patient developed severe peripheral lipoatrophy. In order to be able to make an informed decision about the options to replace stavudine in his regimen by other nucleoside or nucleotide analogs, a genotypic resistance test was performed on the last sample obtained while the patient was on ZDV-ddC before commencing HAART.

Population-based sequence analysis. HIV-1 RNA was isolated from plasma by the Boom method (6). The plasma was prepared from blood samples collected in evacuated tubes with EDTA. We used 2 ml of plasma, except for samples with a high HIV-1 load (>50,000 HIV-1 RNA molecules/ml), in which case 200 μl was used as input. Purified RNA was reverse transcribed using primer 3′RT-out, and the protease and RT genes were amplified with primers 5′ PROT-I, 3′ET21, 5′ PROT-II, and 3′RT20 as described previously (5).

Clonal sequence analysis. Nested PCR fragments generated with primers 5′PROT-II and 3′RT20 (5) were ligated into the PCR2.1-TOPO TA cloning vector, and One Shot Top10 chemically competent cells (Invitrogen, Breda, The Netherlands) were used for transformation. Colonies were picked and suspended in brain heart infusion medium containing ampicillin. The *Escherichia coli* suspension was used as input for PCR amplification, and the DNA fragments were sequenced with BigDye Terminator reagent. Electrophoresis and data collection was performed as described above.

Length polymorphism PCR and fitness calculation. The PCR products obtained after amplification with the primers 5′PROT I and 3′ET21 were used as input in a nested PCR with primers R11772-2 (5′-AAAATTGGGCCTGAAAA TCC-3′; position 2295 in HIV-1 LAI) and R11772-4 (5′-TTCCCAGAAGTCT TGAGTTC-3′; position 2398 in HIV-1 LAI). The PCR product is 148 bp for the virus with the insert and 124 bp for the wild-type virus. We previously demonstrated the usefulness of such an internally controlled assay system for detecting small length polymorphisms (24). The DNA fragments were analyzed by ethidium bromide-stained gel electrophoresis on a 3% MetaPhor agarose gel (Cambrex, Rockland, Maine) with a 25-bp marker and quantified using ImageQuant TL (Amersham Biosciences Europe, Roosendaal, The Netherlands).

The change in ratio of the two RT-PCR products over time was used to calculate the fitness difference for the virus with and without an insert. The calculation of the selection coefficients was performed as described previously (13; see also http://www-binf.bio.uu.nl/~rdb/fitp.html). If changes in the viral load during the observation period are ignored, one can use the following equation that is used to estimate selection coefficients (s) in population genetics:

$$s = \frac{1}{T} \times \left[\ln\left(\frac{q_t\, p_0}{p_t\, q_0}\right) \right]$$

in which the time course of the relative frequencies p and q of two variants are considered. Plotting $\ln(p/q)$, i.e., the natural logarithm of the ratio of the two variants, as a function of the generation time (T) results in a straight line. The generation time of HIV-1 was previously calculated to be 2.6 days (34) but has recently been adjusted to 2.0 days (29). The latter value was used to calculate the fitness difference between viruses with and without an insert.

Recombinant virus drug susceptibility and replication capacity assay. Drug susceptibility assays using stored plasma samples were performed using the PhenoSenseHIV assay (36) (ViroLogic, South San Francisco, Calif.). The cutoffs were as follows: 4.5 (abacavir [ABC]), 1.7 (dideoxyinosine [ddI], d4T, ddC), 3.5 (3TC), 1.4 (tenofovir [TFV]), 1.9 (ZDV), 2.5 (delavirdine, efavirenz, nevirapine). Replication capacity was measured by a modification of this assay (3). Following a single infection cycle in HEK 293 cells, luciferase activity was measured after 72 h and compared to that of a reference virus based on the NL4-3 strain (arbitrarily set at 100%). Three replicates were performed for each mutant. The replication capacity was normalized using a square-root transformation.

Nucleotide sequence accession numbers. The GenBank sequence accession numbers for the RT sequences are AY877303 to AY877315.

RESULTS

Two evolutionary paths to multidrug resistance. Clinical and virological data on patient M12286 suggested failure of antiretroviral therapy and the emergence of a drug-resistant HIV-1 variant in 1998–1999 during ZDV-ddC chemotherapy (Fig. 1). Peripheral blood was taken from the patient on 22 June 1999, and the polymerase gene segment of the plasma viral RNA was analyzed by sequencing. The deduced RT amino acid sequence was aligned with a subtype B consensus sequence. Several well-known ZDV resistance mutations and a remarkable change were noticed. The standard mutations include M41L, E44D, K70R, L210W, and T215Y. The RT variant also had a unique 8-aa insert in the β3-β4 loop (Fig. 2), and we therefore decided to perform a more detailed genotypic analysis of longitudinal samples during initial ZDV monotherapy and subsequent ZDV-ddC combination therapy.

We selected seven earlier samples for viral genotyping as indicated in the clinical scheme shown in Fig. 1. This includes one pretreatment sample (3 May 1994), one sample taken during ZDV monotherapy (21 March 1995), and five samples taken during ZDV-ddC therapy (9 April 1997, 11 June 1997, 17 September 1997, 10 December 1997, and 6 January 1999). To build an evolution scheme, the RT gene of the virus population was PCR amplified, and the sequence was determined. Several samples appeared to consist of a heterogeneous virus population yielding a mixed sequence. Those PCR-products were cloned, and multiple individual clones were sequenced.

The pretreatment RT sample (3 May 1994) does not contain the wild-type 215T residue but instead a mixed virus population with mostly 215D and also 215C variants. This finding mimics a previous observation in another patient that was infected by a ZDV-resistant virus variant (13, 18, 19). It is likely that patient M12286 was also infected by a ZDV-resistant HIV-1 variant. Most ZDV resistance mutations can easily revert to the wild-type codon, but this is much more difficult for the 215Y codon because it requires two point mutations. Nev-

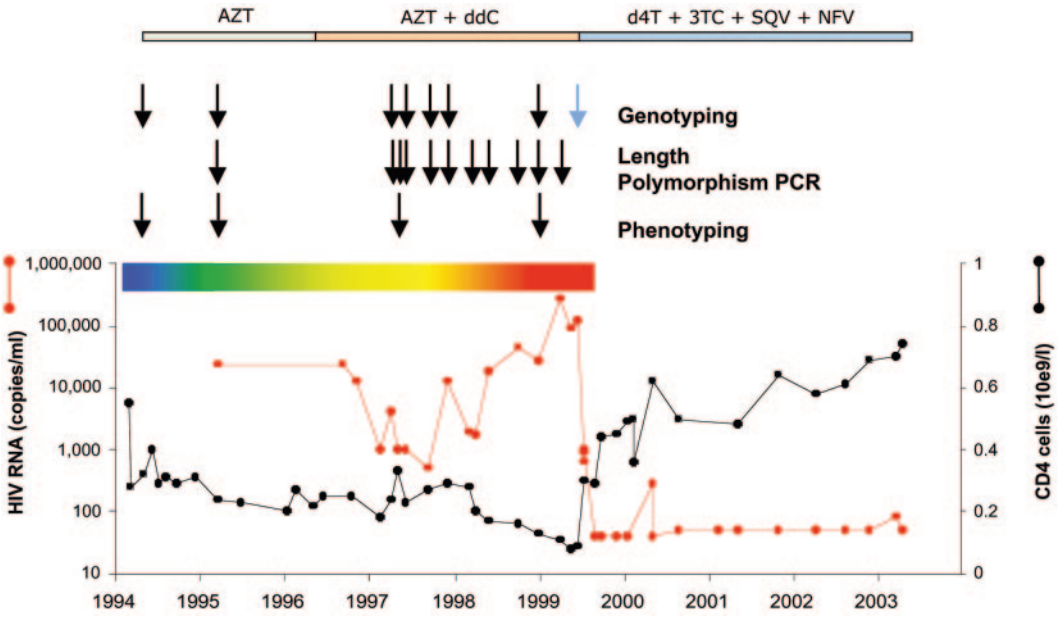

FIG. 1. Therapy and virological and clinical markers of patient M12286. The drug regimen is indicated on top. Samples used for genotyping, phenotyping, and length polymorphism PCR are marked by arrows. The blue arrow indicates the sample used for the initial genotyping, and the color bar shows the continuous evolution of different HIV-1 variants; see also Fig. 3. AZT, zidovudine; SQV, saquinavir.

ertheless, there is selective pressure on the 215Y variant to optimize its replication capacity in the absence of ZDV, and this leads to the generation of codon 215 variants that are only one mutation away from the 215Y codon (215D and 215C). This feature also explains a second unusual phenomenon seen in patient M12286: the extremely rapid appearance of the 215Y mutant upon start of ZDV therapy (Fig. 3). The 215Y mutation is usually one of the last mutations in the multistep evolution to full ZDV resistance (7). However, 215Y appears together with 41L as the first mutations to occur during ZDV monotherapy in this exceptional patient.

The sequences of several time points were used to build the evolution scheme (Fig. 3). Starting with the pretherapy sample, we observed the steady accumulation of regular ZDV resistance mutations. At this time, two evolution routes become apparent. One evolution route eventually acquires up to six standard resistance-associated mutations, but an alternative route yields the unique 8-aa insert variant that gradually outcompetes the other variant to become the dominant virus species.

All sequences from 9 April 1997 and later time points have four resistance-associated mutations in common: 41L, 44D, 210W, and 215Y. The two evolution routes add different mutations to this backbone, either 67N plus 69D or the 8-aa insert plus 70R (Fig. 3). An alternative explanation would be that the insert variant evolved from the 67N-plus-69D variant that dominates the virus population just before the insert appears (11 June 1997). However, inspection of the actual nucleotide sequence of the insert (see below) strongly argues against this scenario. Thus, it is likely that both the 8-aa insert variant and the 67N-plus-69D variant were generated from the same precursor, as indicated in Fig. 3.

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                63    69       70   75
                .     .        .    .
May 3    1994   IKKKDST--------KWRKLV

June 22 1999    IKKKDSTSTGKKDSTRWRKLV


                63                      69                                  70                  75
May 3    1994   ATA.AAG.AAA.AAA.GAC.AGT.ACT---------------------------------AAA.TGG.AGA.AAA.TTA.GTA

June 22 1999    ATA.AAG.AAA.AAA.GAC.AGT.ACT.TCC.ACA.GGG.AAA.AAA.GAC.AGT.ACT.AGA.TGG.AGA.AAA.TTA.GTA

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FIG. 2. Sequence of the 8-aa insertion in RT. The amino acid and nucleotide sequences of positions 63 to 75 are shown for time points 3 May 1994 and 22 June 1999. A duplication of 16 nucleotides is indicated by the arrow.

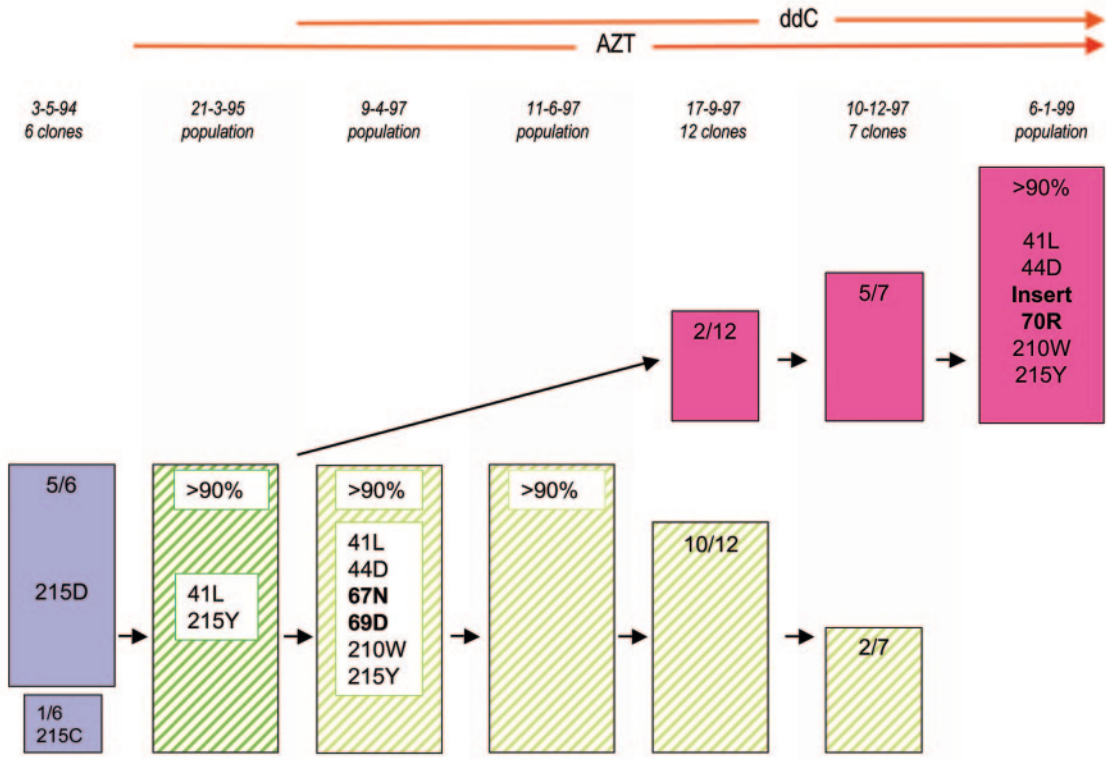

FIG. 3. Evolution scheme of RT drug resistance mutations. The sizes of the colored boxes represent the population sizes of the distinct viral genotypes. The arrows indicate the two evolution pathways. AZT, zidovudine.

Probing the history of the 8-aa insert. Inspection of the nucleotide sequence of HIV-1 variants with and without the 8-aa insert indicates that a large part of the 24-nucleotide insert is due to duplication of local RNA sequences during reverse transcription (Fig. 2). Interestingly, the duplicated sequence contains the wild-type codons at positions 67 and 69, indicating that the duplication occurred before the 67N and 69D mutations were selected in the virus population. This is why the second evolution route is shown to originate in 1995 in Fig. 3. The insert also contains an 8-nucleotide stretch of nonviral origin. To accurately probe the history of the insert, we analyzed multiple patient samples around the time that the insert was acquired (Fig. 1). We designed an RT-PCR assay that amplifies the RT finger region and quantitates the relative amounts of virus with or without the insert. This analysis indicated that the complete 24-nucleotide insert appeared at once (the first positive sample was from September 1997) (Fig. 4A). The first PCR product with an insert (17 September 1997) was also cloned and sequenced. The insert sequence was identical to the sequence obtained on 22 June 1999, indicating that the insert sequence did not evolve over time. Combined with the observation that there are no intermediates among 12 clones, these observations support the idea that the 24-nucleotide insert was generated in a single step.

Evolution of viral fitness. The evolution scheme indicates the slow but steady outgrowth of the insert variant over the substitution variant during ZDV-ddC combination therapy. To obtain an accurate fitness determination, we used the data obtained with the length polymorphism assay described above. We quantified the two DNA bands in the mixed samples, and the ratio shows a linear increase over time (Fig. 4B). We calculated that the virus with the insert at position 69 (69-insert) is 1.9% more fit under therapy than the virus without insert (legend, Fig. 4).

Phenotypic drug resistance testing. We selected four plasma samples for a detailed analysis of the drug resistance phenotype (Fig. 1); one pretherapy sample (3 May 1994), one sample taken during ZDV monotherapy (21 March 1995), and two samples taken during ZDV-ddC combination therapy (7 May 1997 and 6 January 1999). In terms of RT mutations, these samples represent the 215D, 41L/215Y, 41L/44D/67N/69D/210W/215Y, and 41L/44D/69insert/70R/210W/215Y virus variants. Resistance test vectors were constructed and analyzed as described in Materials and Methods. The experiments were performed in triplicate in order to accurately quantitate subtle differences in drug susceptibility among these HIV-1 variants. The phenotypic drug susceptibility profiles of the four patient samples are shown in Table 1. The pretherapy sample was drug sensitive as expected, but all subsequent samples exhibited significant drug resistance. The 1995 sample (41L, 215Y) showed moderate reductions in susceptibility to ZDV (71.4-fold), TFV (2.4-fold), and d4T (1.8-fold). The subsequent samples showed profoundly increased levels of ZDV resistance (1,050- and >2,000-fold, respectively), and both variants exhibited multinucleoside resistance phenotypes against ABC, ddI, 3TC, d4T, TFV, and ddC. Most notably, the insert variant from 1999, in contrast to the 1997 variant without the insert,

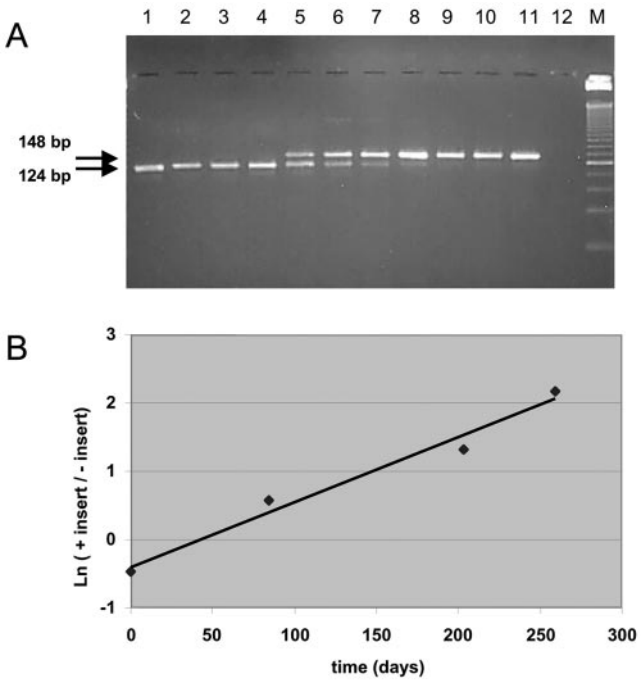

FIG. 4. Monitoring of the appearance of the 8-aa insert. (A) Length polymorphism PCR. Lanes 1 to 11 represent samples collected on the following dates: 21 March 1995, 9 April 1997, 7 May 1997, 11 June 1997, 17 September 1997, 10 December 1997, 8 April 1998, 3 June 1998, 7 October 1998, 6 January 1999, and 7 April 1999. Lane 12 shows the negative PCR control, and lane M shows a 25-bp molecular weight marker (Invitrogen). The PCR products were analyzed on 3% Metaphor agarose gels (Cambrex) and stained with ethidium bromide. (B) By using the quantification of the PCR products, the relative abundance of the virus with and without an insert was plotted in time according to the equation used for estimating selection coefficients in population genetics ($t = 0$ for the sample in which the insert was first detected on 17 September 1997). With an HIV-1 generation time of 2.0 days, the fitness difference between the virus with and without an insert is 1.9% (the slope of the regression line, as calculated using the formula $y = 0.0095x - 0.3979$, multiplied by 2.0; $R^2 = 0.9761$).

showed a trend towards increased resistance to ZDV and ddC, explaining its outgrowth in the presence of ZDV and ddC.

The single-cycle infection assay allows quantitation of the viral RC, which was assayed in the absence of antivirals in three independent experiments (Table 1). The increase in drug resistance between 1997 and 1999 was accompanied by an apparent partial loss of RC (average decrease of 1.3-fold), although the difference in measured RC between samples was not statistically significant.

TABLE 2. RT finger inserts in the ViroLogic database

Size of insert (no. of amino acids)	Amino acids at 69[a] position	No. of sequences with indicated insert[b]
1	NN	1
1	SN	2
1	ST	1
1	TD	2
1	TN	3
1	TT	18
2	AEA	2
2	AEG	1
2	ASA	1
2	AVA	1
2	AVG	2
2	SCA	1
2	SCG	3
2	SEA	2
2	SES	4
2	SQS	1
2	SSA	4
2	SSG	10
2	SSS	24
2	SST	6
2	STT	1
2	SVG	6
2	SVS	1
2	SVT	3
5	DRKGSE	1
8	TSTGKKDST	1

[a] The amino acids between positions 68 and 70 are shown. Thus, 2 aa are shown for a 1-aa insert, 3 aa for a 2-aa insert, etc.
[b] Of the 134 total samples with insertion mutations, only those with no heterogeneity in the sequence ($n = 102$) are shown.

A larger insert correlates with increased ZDV resistance. We analyzed the ViroLogic database of phenotype, genotype, and RC data from over 20,000 clinical samples for the incidence and phenotypes of inserts around position 69 (Table 2). All insertions in the finger domain were considered to occur after the threonine at position 69, even if optimal nucleotide sequence alignment indicated that the insertion occurred elsewhere (e.g., between positions 68 and 69). A total of 134 sequences from different individuals were found to contain an insert: 28 had a 1-aa insert, 104 had a 2-aa insert, one sample had a 5-aa insert, and another had the 8-aa insertion of this study. The samples had insertions of various sequence and obviously also differed with respect to the presence of other drug resistance-associated mutations. Nevertheless, the large

TABLE 1. Drug resistance increases during antiviral therapy of patient M12286

Sample date	Fold resistance to indicated drug										RC[b] (%)
	ABC	ddI	3TC	d4T	TFV	ddC	ZDV	DLV	EFV	NVP	
3 May 1994	0.8	1.1	1.1	1.0	0.8	0.9	1.1	0.8	0.8	0.8	165
21 March 1995	2.0	1.2	1.5	1.8	2.4	0.9	71.4	0.6	0.7	0.8	149
7 May 1997	5.5	2.0	7.5	3.4	4.6	1.8	1,050	0.4	0.6	0.5	115
6 January 1999[c]	7.1	2.3	8.8	5.1	5.1	2.4	>2,000	0.3	0.4	0.4	83

[a] EFV, efavirenz; NVP, nevirapine; DLV, delavirdine.
[b] RC compared to wild-type control.
[c] Sample with 8-aa insert.

TABLE 3. The size of the 69-insert correlates with the level of ZDV resistance

Size of insert (no. of amino acids)	No. of sequences	Mean fold resistance to indicated drug							Mean RC[a] (%)
		ABC	ddI	3TC	d4T	TFV	ddC	ZDV	
1	28	7.3	2.3	89	4.4	3.4	2.3	333	50
2	104	24	4.1	100	14	10	4.7	1,222	34
5	1	19	3.2	12	6.5	13	3.1	>2,000	21
8	1	7.1	2.3	8.8	5.1	5.1	2.4	>2,000	83

[a] Mean RC compared to wild-type control.

number of 1- and 2-aa inserts allows for a comparison of the drug resistance phenotypes and RC of these groups (Table 3). An interesting correlation between the size of the insert and the level of ZDV resistance was observed. An average 333-fold resistance was observed for the 1-aa inserts (28 cases), but 1,222-fold resistance was measured for the 2-aa inserts (104 cases). Furthermore, maximal (>2,000-fold) ZDV resistance was measured for the two variants with larger inserts of 5 and 8 aa. Resistance to 3TC does not follow this trend, as a similar level of resistance is apparent for the 1- and 2-aa groups (89- and 100-fold, respectively). However, these data are confounded by the variable presence of the M184V mutation, which confers a greater loss of susceptibility to 3TC than do insertions at position 69. A two- to threefold increase in resistance was seen for d4T, ddI, ddC, ABC, and TFV comparing the 1- and 2-aa groups (e.g., from 4.4- to 14.0-fold resistance against d4T). There was also a trend towards lower RC in samples with 2-aa inserts than in those with 1-aa inserts (34 versus 50% mean RC).

The sequence of the insert has a modest effect on the resistance phenotype. Inspection of the actual amino acid sequence of the insert confirmed that certain sequences are preferred. It is important to realize that this pattern does not necessarily reflect selection at the protein level, because most inserts are made by duplication of preexisting sequences, thus strongly biasing certain insert sequences. The most frequent 1- and 2-aa inserts are T (18 of 27 samples with 1-aa inserts) and SS (24 of 73 samples with 2-aa inserts), respectively. To test whether the insert sequence also affects the drug resistance phenotype, we compared data from individual 1- and 2-aa sequences that were observed at least three times (Table 4). Modest differences in mean ZDV resistance were apparent, ranging from 231-fold for the T insert to 805-fold for the N insert, and from 730-fold for the SS insert to the maximal >2,000-fold for the CG insert.

DISCUSSION

After the initial success of ZDV-ddC therapy on the viral load in patient M12286, we observed a steady increase in the viral load in 1998 and early 1999 (Fig. 1). Superposition of the genotypic evolution (Fig. 1) indicates that this increase in viral load coincides with the appearance and eventual outgrowth of the 8-aa insert variant. The drug resistance phenotype provides a plausible explanation for this, as the insert-containing variant is slightly more resistant to ZDV and ddC than the variant lacking the insertion. Interestingly, we also noted a trend towards reduction in virus replication capacity coincident with the appearance of the insertion. Thus, it is likely that the 8-aa insert has a negative impact on the activity of the RT enzyme and consequently on virus replication in the absence of drug, but more studies with mutant RT enzymes and molecular HIV-1 clones are needed to confirm these results.

Low fitness of drug-resistant strains can result in overgrowth by the original wild-type virus if drugs are discontinued after resistance-associated virological failure. This process could have occurred in patient M12286 more recently because he stopped treatment with ZDV and ddC on 29 June 1999. However, the patient switched to an HAART regimen with two RT (d4T and 3TC) and two protease (saquinavir and NFV) drugs, which successfully reduced the viral load to less than 50 copies/ml. Treatment was able to suppress the viral load up to 28 April 2003, and a steady but slow recovery of the number of CD4-positive cells has been observed (Fig. 1). Inspection of the drug resistance properties (Table 1) indicates that samples with or without inserts have a multinucleoside resistance phe-

TABLE 4. The sequence of the 69-insert influences the resistance phenotype

Amino acids at position 69	No. of sequences	Mean fold resistance to indicated drug							Mean RC[a] (%)
		ABC	ddI	3TC	d4T	TFV	ddC	ZDV	
TT	18	6.5	2.1	102	3.5	2.7	2.2	231	46
TN	3	8.1	2.6	70	4.9	4.1	2.2	805	105
SVG	6	27	5.1	46	20	22	4.5	2,031	56
SCG	3	38	7.3	82	43	19	9.5	2,400	50
SSS	24	16	3.0	97	9.0	5.7	3.7	1,221	23
SVT	3	35	5.2	139	16	7.4	5.3	1,013	23
SES	4	44	6.5	116	23	13	7.5	1,826	5.0
SSA	4	12	2.6	104	6.4	3.6	3.6	730	16
SSG	10	28	4.2	124	15	9.1	5.1	1,084	48
SST	6	22	2.8	136	5.3	3.7	2.8	1,079	28

[a] Mean RC compared to wild-type control.

notype. The insert variant exhibits 8.8-fold resistance to 3TC and 5.1-fold resistance to d4T. It is therefore unlikely that outgrowth of the archived original virus will occur in this patient. Instead, one would expect additional mutations in the RT enzyme that boost the 3TC-d4T resistance level, but inhibition by the two protease drugs seems to have precluded such an evolutionary escape thus far. Based on the results of the resistance test, the patient's treating physician decided to replace d4T by ZDV, as this would be expected to be less detrimental with respect to further progression of peripheral lipoatrophy, while being equivalent with respect to the maintenance of any decreased viral fitness based on the mutations which had been detected. NFV was replaced by lopinavir/ritonavir in view of suboptimal NFV plasma concentrations. At the last visit in April 2004, the patient was in stable condition, the plasma HIV-1 RNA remained suppressed at <50 copies/ml, and the CD4 count was >500/mm^3.

We observed an unusual biphasic evolution route of HIV-1 in patient M12286. First, a regular ZDV variant appears with the 67N and 69D mutations. Subsequently, the insert variant appears, and this virus slowly overtakes the population. There is a precedent for such a biphasic evolution of drug-resistant HIV-1 variants in the case of the nucleoside analogue 3TC (22). This is due to differences in mutational frequency, which determines the initial outcome, and differences in replication fitness, which determines the eventual outcome of evolution. Treatment with 3TC triggers the evolution of resistant HIV-1 variants with a single amino acid substitution in the catalytic core: the YMDD motif is changed either to YIDD (M184I) or YVDD (M184V). Some patients initially develop the I variant, which is outcompeted over time by the V variant (16, 39). This pattern is not due to sequential evolution (M→I→V) because both the I and V codons are made directly from the M precursor. A detailed analysis revealed that M184I is made first because it requires an easier type of mutation (G to A) than the M184V variant (A to G) (4, 22, 23). Eventual outgrowth of V over I is due to its increased replication capacity (2). We think that a similar two-step evolution scenario accounts for the pattern observed in M12286. The amino acid insert is likely to be a rare mutational event, which explains its delayed appearance. However, this variant is more fit in vivo in the presence of ZDV and ddC. The in vitro experiments confirm an increase in drug resistance but also a modest reduction in replication capacity in the absence of drugs.

We observed the acquisition of an 8-aa insert in the finger domain of the HIV-1 RT enzyme. This example adds to the growing list of larger finger inserts in the RT protein, including a 5-aa duplication of envelope gene sequences (27) and an 11-aa insert of unknown origin (38). Part of the 24-nucleotide insert is likely caused by duplication of local RNA sequences during reverse transcription, but an 8-nucleotide stretch is not of viral origin. We performed a BLAST search in the expressed sequence tag bank with a 21-nucleotide fragment consisting of the 8-nucleotide part of the insert that is not a duplication of local HIV-1 sequences. Several close matches with human genome sequences, including a cDNA clone from the Jurkat T-cell line (accession number BX362453), were scored. This candidate donor sequence is expressed in the right cell type for recombination to occur with HIV-1, and it has a perfect match with the 8-nucleotide insert as well as the flanking sequences. It seems possible that the insert was acquired in two steps, the initial duplication of 16 nucleotides that caused a shift in the open reading frame and a subsequent insertion of 8 additional nucleotides of nonviral origin. Alternatively, the 24-nucleotide segment could have been inserted in one mutagenic event.

The large number of insert variants present within the ViroLogic database allowed us to test for the effects of various types of insertions at position 69 of RT. Even though this analysis includes many different viral backbones with many additional drug resistance mutations, the large number of insert variants allowed us to cautiously suggest some trends. Most inserts contain a 2-aa insertion (80%), while a minority have 1-aa (~20%) and, rarely, larger (>2-aa) inserts. This distribution with 2 aa as the most common insert is consistent with a previous survey (32). Interestingly, we observed a direct correlation between the size of the insert and the level of resistance to all NRTIs except 3TC. We also noticed a modest effect of the insert sequence on the drug resistance phenotype. It is important to verify these findings with molecularly cloned virus variants that differ exclusively in the sequence of the RT insert.

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