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In Vivo Evidence for Instability of Episomal Human Immunodeficiency Virus Type 1 cDNA

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Current regimens for the management of human immunodeficiency virus type 1 (HIV-1) infection suppress plasma viremia to below detectable levels for prolonged intervals. Nevertheless, there is a rapid resumption in plasma viremia if therapy is interrupted. Attempts to characterize the extent of viral replication under conditions of potent suppression and undetectable plasma viremia have been hampered by a lack of convenient assays that can distinguish latent from ongoing viral replication. Using episomal viral cDNA as a surrogate for ongoing replication, we previously presented evidence that viral replication persists in the majority of infected individuals with a sustained aviremic status. The labile nature of viral episomes and hence their validity as surrogate markers of ongoing replication in individuals with long-term-suppressed HIV-1 infection have been analyzed in short-term in vitro experiments with conflicting results. Since these in vitro experiments do not shed light on the long-term in vivo dynamics of episomal cDNA or recapitulate the natural targets of infection in vivo, we have analyzed the dynamics of episomal cDNA turnover in vivo by following the emergence of an M184V polymorphism in plasma viral RNA, in episomal cDNA, and in proviral DNA in patients on suboptimal therapies. We demonstrate that during acquisition of drug resistance, wild-type episomal cDNAs are replaced by M184V-harboring episomes. Importantly, a complete replacement of wild-type episomes with M184V-containing episomes occurred while proviruses remained wild type. This indicates that episomal cDNAs are turned over by degradation rather than through death or tissue redistribution of the infected cell itself. Therefore, evolution of episomal viral cDNAs is a valid surrogate of ongoing viral replication in HIV-1-infected individuals.

Suppression of plasma viremia to below detectable levels can be sustained in human immunodeficiency virus type 1 (HIV-1)-infected individuals by antiretroviral regimens currently in use (14, 30). Nevertheless, replication-competent viruses persist in these individuals, and viremia rapidly resumes when antiretroviral suppression is interrupted (10, 13, 16, 28). Lymphocytes from aviremic patients harbor latent proviruses, and after in vitro activation, these lymphocytes yield replication-competent viruses (5, 9, 12, 43). The latent reservoir is believed to be established early in infection and to constitute quiescent memory CD4$^+$ T lymphocytes harboring transcriptionally silent proviruses (7, 19). The existence of a reservoir of latently infected cells is considered to be the major obstacle to eradication of HIV-1 through prolonged antiretroviral suppression (36). This has prompted strategies aimed at triggering cell cycle progression of latently infected cells and reactivation of latent proviruses in infected individuals (2, 8, 22, 23, 32).

In addition to a pool of latently infected lymphocytes, there is evidence that viral persistence in the face of potent antiretroviral suppression may be perpetuated by ongoing viral rep-

lication. For example, in patients where drug regimens do not completely block HIV-1 replication, it has been proposed that intermittent episodes of plasma viremia may contribute to the maintenance of the viral reservoir of both latently infected and productively infected cells (33, 44). Even when plasma viremia is completely suppressed, continued sequence evolution in the viral envelope gene, as well as expression of viral RNA in lymphoid tissues (11, 15, 26, 33, 45), supports the view that viral replication can persist in the face of potent antiretroviral suppression. The effectiveness of current therapeutic regimens has been monitored by using HIV-1 RNA assays which have a sensitivity to 40 to 50 copies/ml. Below this assay threshold, more-sensitive assays of HIV-1 RNA reveal that low-level plasma viremia persists in patients even after years of therapy (11, 41). While this low-level, steady-state viremia could result from the continued activation of latently infected cells, its level can be suppressed by therapy intensification, suggesting that it is a result of ongoing replication (18). The major contributor to viral rebound that occurs when therapy is interrupted is also a matter of debate. In patients with extended periods of undetectable plasma viremia, rebounding virus was genetically similar to viruses isolated from the latent reservoir, and in patients with residual viral replication, the rebound virus corresponded to minor viral variants detected in lymphoid tissues (44). In contrast, the rebounding virus in plasma was found to be ge-

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netically distinct from cell-associated viral RNA and virus recovered from resting memory CD4$^+$ T cells, suggesting that the viral rebound was from a source other than the latent reservoir (6, 20).

Efforts to characterize the extent of ongoing viral replication in suppressed individuals and the source of the rebounding virus following interruption of therapy have been hampered by a lack of convenient surrogates of ongoing viral replication. We previously provided evidence that viral replication persists in the majority of aviremic individuals on the basis that episomal forms of viral cDNA could be detected in these individuals (35). Viral episomes containing one or two long terminal repeats (1- or 2-LTR-containing episomes) are formed after completion of viral cDNA synthesis and translocation of the viral genome to the host cell nucleus, where recombination and direct ligation lead to the formation of episomes containing one and two LTRs, respectively. In addition, we and others reported that 2-LTR-containing episomal cDNAs are labile and, as such, their presence is indicative of recent infection events (27, 29, 35). However, other studies indicate that episomal viral cDNAs are intrinsically stable in vitro, at least in experiments extending over a few days (3, 4, 31). Short-term in vitro studies examining stability of episomal viral cDNA are limited in their ability to gauge the dynamics of episomal cDNA in patients who have been aviremic for months to years. Furthermore, the cell cycle status of the infected cell appears to impact the stability of extrachromasomal linear viral DNA (38), and stability of episomal viral cDNA in cell lines and activated lymphocytes in vitro may not reflect episomal cDNA dynamics in natural targets of HIV-1 infection in vivo. Therefore, to gain information on the in vivo dynamics of episomal viral cDNA, we have monitored the emergence of drug resistance mutations in episomal viral cDNA and in proviral DNA in patients on suboptimal therapies. We show that episomal viral cDNAs acquire drug resistance mutations, while proviruses remain wild type. This provides evidence that episomal viral cDNA is labile in vivo. As such, episomal viral cDNAs are valid surrogates of ongoing viral replication in HIV-1-infected individuals.

This study (NWCS225) was conducted using archival patient samples that had originally been collected under AIDS Clinical Trials Group protocol no. 306 (24). As part of AIDS Clinical Trials Group protocol 306, peripheral blood mononuclear cells were obtained from treatment-naive individuals at various intervals after initiation of dual nucleoside analog therapy. The presence of drug-resistant viruses in plasma was determined at week 24 post-therapy initiation. Plasma HIV-1 RNA measurements (branched-DNA assay, HIV-1 quantiplex version 2.0; Chiron Corp.) was available on most patients for weeks 2, 4, 8, 12, 16, 20, 24, 28, 36, 44, and 48 post-therapy initiation. We followed the emergence of drug resistance mutations in episomal and proviral DNA in 11 patients who had received zidovudine (ZDV) plus lamivudine (3TC). Under this regimen, resistance to 3TC, which is conferred by a methionine-to-valine substitution at codon 184 (M184V) of reverse transcriptase (RT), rapidly develops, such that by week 24 post-therapy initiation, wild-type HIV-1 in plasma is replaced by 3TC-resistant virus. We developed a PCR strategy which allowed specific amplification of RT sequences within episomal cDNAs as well as proviral sequences (Fig. 1). Episomal viral cDNA and pro-

viral DNA were amplified from cellular DNA of archived peripheral blood mononuclear cells that had been obtained at various intervals post-therapy initiation (Fig. 1; Table 1). Proviruses were amplified either from total cellular DNA, using an HIV-1-specific primer and a primer specific for Alu sequences within chromosomal DNA (21), or from enriched chromosomal DNA using HIV-1-specific primers (Fig. 1). Amplification of episomal sequences was achieved in two rounds of PCR amplification. In the first round, sequences encompassing a portion of Nef, LTR circle junction sequences, Gag, RT, and integrase were amplified, followed by a second round of nested PCR using RT-specific primers (Fig. 1; Table 1). In some cases, 2-LTR circles were specifically amplified with a primer which spans the unique junction formed by ligation of 5′ and 3′ LTR sequences in the 2-LTR circle (Fig. 1). Second-round products were generated in the linear phase of amplification and directly sequenced. In this amplification strategy, second-round amplicons were not generated if the first-round amplification with episome-specific primers was omitted. Therefore, the internal second-round amplicons were derived directly from episomal sequences rather than from proviral or linear extrachromosomal sequences. Where plasma was available, genomic viral RNA was amplified and genotyped for the presence of the M184V polymorphism.

The emergence of the M184V polymorphism in episomal (1- and 2-LTR circles) and proviral DNA for eight patients is shown on Fig. 2. In each case, plasma viruses obtained around week 24 post-therapy initiation harbored the M184V polymorphism. In four cases (038, 169, 268, and 270) where plasma was available from earlier intervals, the M184V mutation was detected in genomic plasma viral RNA by 8 weeks post-therapy initiation. Despite the emergence of viruses harboring 3TC resistance mutations, viral load remained suppressed, most likely through the presence of ZDV. While the M184V mutation confers 3TC resistance, it restores ZDV susceptibility in HIV-1 variants carrying ZDV resistance mutations, and ZDV resistance mutations are less frequent in viruses from patients receiving ZCV plus 3TC than in those from patients receiving ZDV monotherapy (25, 40). Previous studies have demonstrated that the majority of patients on ZDV-3TC combinations develop M184V mutations by 12 weeks post-therapy initiation (25), which illustrates the rapid replacement of wild-type viruses by drug-resistant variants under suboptimal therapies. In most subjects (038, 073, 115, 169, 179, 268, 270, and 278), wild-type episomes were replaced by episomes containing the M184V mutation by 24 weeks post-therapy initiation. In three cases, the population of wild-type episomes had been completely (179 and 270) or partially (278) replaced by drug-resistant episomes by 12 weeks post-therapy initiation. Importantly, there was no corresponding appearance of the M184V mutation in the proviral species in these patients over 48 weeks post-therapy initiation (Fig. 2). Only in one subject (169) were M184V-containing proviruses evident, and only at the last two intervals post-therapy initiation.

In three patients (013, 230, and 289), the appearance of the M184V polymorphism in episomal DNA was delayed relative to the case with the other patients, and proviral DNA remained wild type (Fig. 3). One possibility is that the level of ongoing replication in these three patients was lower. In this case, the replacement of wild-type episomes by de novo-syn-
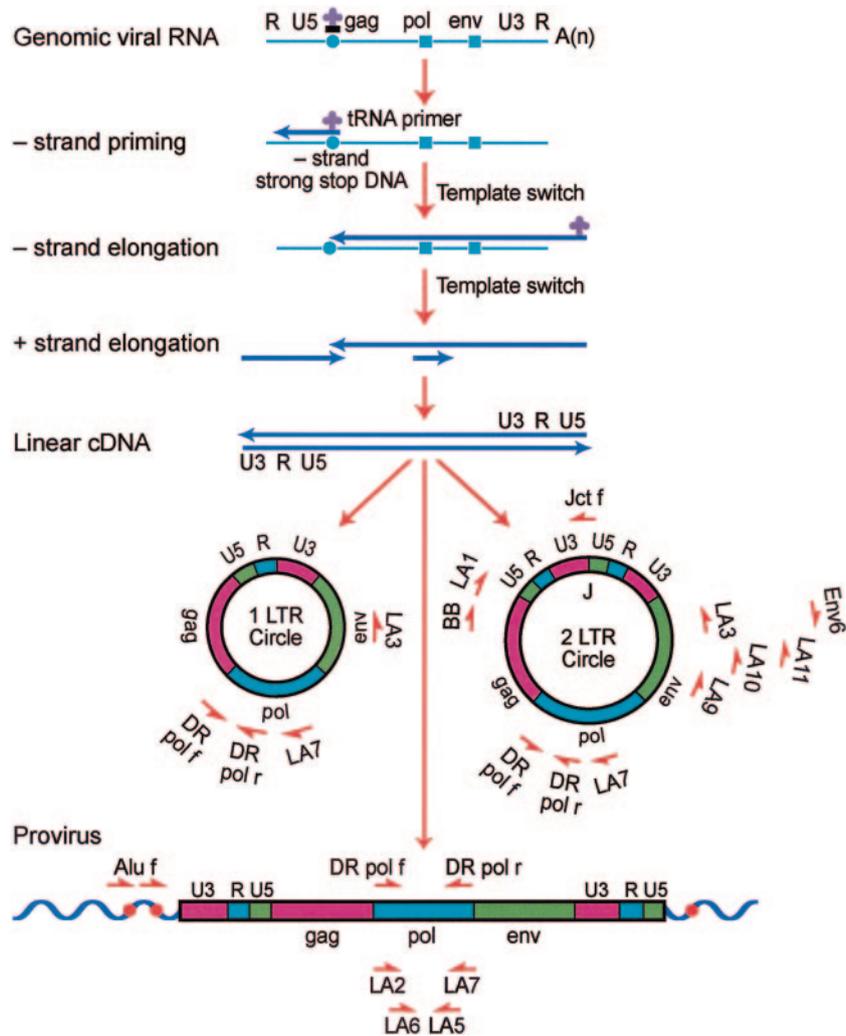
FIG. 1. Strategy for long-range PCR amplification of episomal HIV-1 cDNAs containing RT or envelope sequences. Major cDNA intermediates in reverse transcription are denoted. Thin line, viral RNA; thick line, cDNA. Primer-binding sites for initiation of minus-strand cDNA synthesis and polypurine tracks for plus- or minus-strand synthesis are indicated by open circles and squares, respectively. First-, second-, and third-round primer sets are indicated next to the specific cDNA intermediate they are designed to amplify. HIV-1 *pol* sequences were amplified using Jumpstart REDAccuTaq DNA polymerase (Sigma), following the manufacturer's protocol with the following modifications. Amplifications were done in a 50-μl reaction mixture containing 1× buffer, 0.5 mM deoxynucleoside triphosphates, 0.5 μM primers, 1.5 U of REDAccuTaq, and 10 μl (10% of total) of either episomal or chromosomal DNA purified from patient peripheral blood lympocytes. After an initial denaturation at 94°C for 2 min, first-round products were generated by cycling 25 to 30 times at 94°C for 15 s, 60°C for 30 s, and 68°C for 1 min per kilobase pair of target sequence. The first-round extension time for Alu PCR was 5 min. One or two microliters from the first round was then subjected to 20 to 30 cycles of amplification under the same reaction conditions.

thesized M184V-episomes would be delayed. To evaluate whether the level of ongoing replication was lower in patients exhibiting a slow evolution to M184V episomes, we compared the genetic divergence in viral envelope sequences in 2-LTR episomal DNA from week 0 to week 24 and from week 24 to week 48 in two patients (179 and 270) where there was a rapid evolution in episomal sequences and two patients (230 and 289) where there was a delay in the appearance of M184V episomes. Six unique nucleotide sequences spanning the C2 and V3 coding regions of the envelope gene isolated from episomal cDNA at weeks 0, 24, and 48 were determined. To specifically amplify envelope sequences from 2-LTR circle genomes, primers LA9 and BB were used for eight cycles of first-round PCR specifically targeting 2-LTR circle templates

(Table 1). Ten percent of primary products were amplified for 10 cycles using 2-LTR circle-specific nested primers LA1 and LA10. The secondary products were serially diluted to determine PCR endpoints, and replicate amplifications using primers LA11 and Env6 were used to generate products that were used directly for sequencing. Nucleotide sequences corresponding to the C2 and V3 regions of Env were aligned using ClustalW (MacVector 7.1.1.), and phylogenetic relationships were generated by using the neighbor-joining method included in the MacVector software package with a Kimura-2 parameters distance matrix. Confidence limits for individual branches were estimated by bootstrap resampling of the neighbor-joining trees (1,000 replicates). Phylogenetic analysis of these sequences revealed clusters of viral sequences for each patient

TABLE 1. Primers used for long range amplification of episomal and proviral sequences encompassing RT or envelope[a]

Amplicon	Sequence(s) encompassed	Substrate	Primers	Amplification round
Episome (1- + 2-LTR circle)	LTR, Gag, Pol, Env	Extrachromosomal DNA	LA3 + LA7	1
			DR pol f + Dr pol r	2
2-LTR circle	LTR, Gag, Pol, Env	Extrachromosomal DNA	Jct f + LA7	1
			DR pol f + Dr pol r	2
Provirus	Pol	Chromosomal DNA	LA2 + LA7	1
			LA5 + LA6	2
Provirus	LTR, Gag, Pol	Chromosomal DNA	Alu f + LA7	1
			DR pol f + DR pol r	2
2-LTR circle	LTR, Env	Extrachromosomal DNA	LA9 + BB	1
			LA1 + LA10	2
			LA11 + Env 6	3

[a] Primers are numbered according to the HIV-1$_{LAI}$ consensus (K02013). LA1, 5'-G^{260}CGCTTCAGCAAGCCGAGTCCT; LA2, 5'-C^{1785}CCTCTCAGAAGCAG GAGCCGA; LA3, 5'-T^{8386}AAGATGGGTGGCAAGTGGTCA; LA5, 5'-G^{1907}ATACAGGAGCAGATGATACA; LA6, 5'-C^{3761}TCCAATTCCTTTGTGTGCTG; LA7, 5'-T^{3975}CTACTTGTCCATGCATGGCTT; LA9, 5'-A^{6015}CATGCCTGTGTACCCACAGA; LA10, 5'-C^{6428}CTGTCCAAAGGTATCCTTTGA; LA11, 5'-C^{6546} ACAGTACAATGTACACATGGA; Alu f, 5'-CTCACGCCTGTAATCCCAGCA; Jct f, 5'-AATCTCTAGCAGTACTGGAAG (spans circle junction); BB, 5'-G^{478} GATTAACTGCGAATCGTTC; Env6, 5'-T^{8179}GCGTCCCAGAAGTTCCACAA; DR pol f, 5'-T^{2064}AGGACCTACACCTGTCAACATAA; DR pol r, 5'-C^{2908}TTC TGTATGTCATTGACAGTCCA.

that were distinct from each other (Fig. 4) and from HIV clones commonly used in the laboratory (data not shown). Individual phylogenetic trees revealed that genetic distances from week 0 to week 24 and from week 24 to week 48 were

greater in patients 179 and 270, where there was rapid evolution of M184V-containing episomes, than in patients 230 and 289, where the conversion was delayed (Fig. 4; Table 2). This suggests that the delayed appearance of M184V-containing
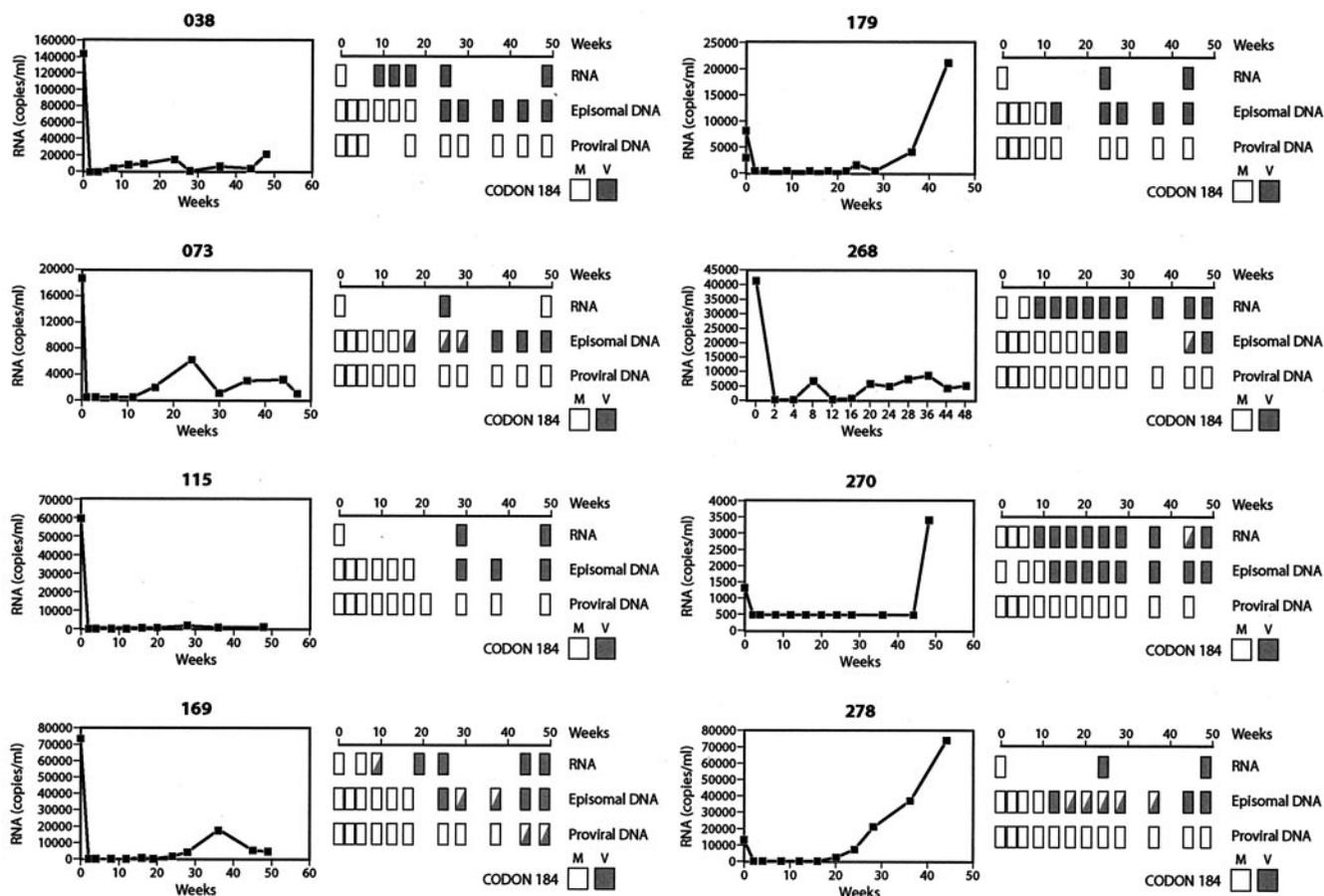

FIG. 2. Evolution of codon 184 of HIV-1 RT in episomal and proviral cDNAs in patients on ZDV-3TC therapy. For each patient, plasma viral RNA loads were monitored after the initiation (week 0) of dual therapy. In each case, an M-to-V substitution at codon 184, which confers resistance to 3TC, was evident at week 24 post-therapy initiation. At various intervals following initiation of ZDV-3TC therapy, the amino acid identity for codon 184 was determined in plasma viral RNA, episomal DNA, and proviral DNA. Boxes indicate the sampling intervals. A solid box denotes the exclusive presence of the M184V mutation in sequence electropherograms of PCR products at that sampling interval. Partially filled boxes denote the presence of both wild-type and M184V genotypes at that sampling interval.
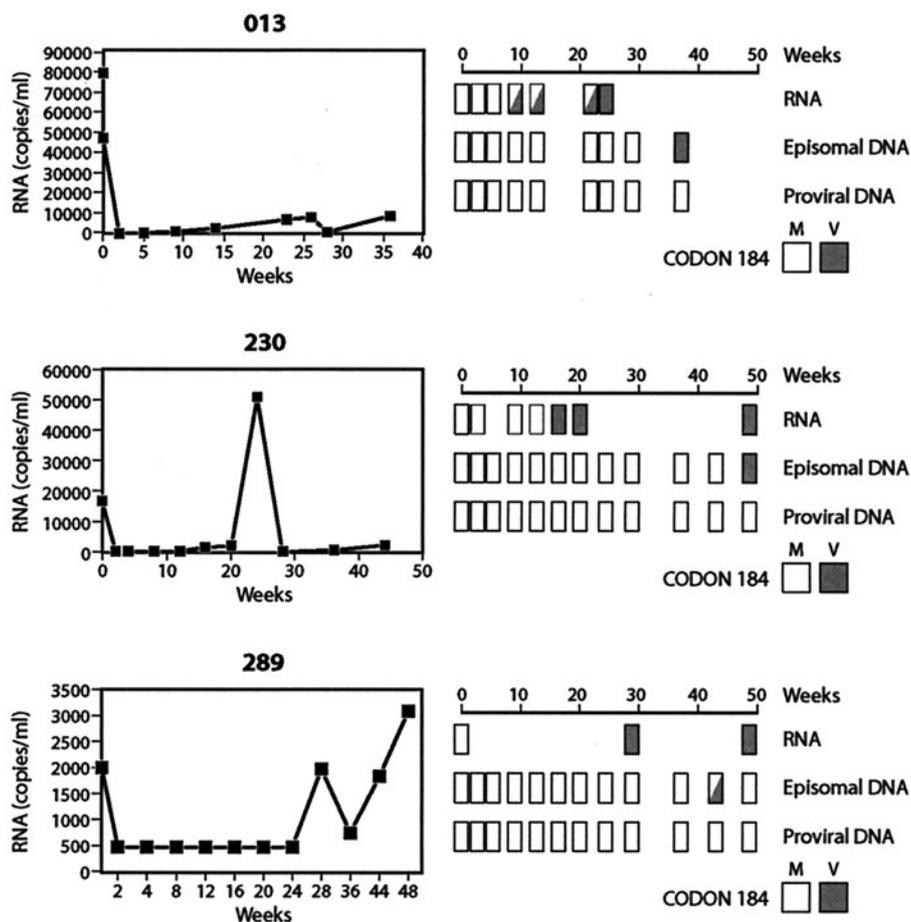
FIG. 3. Delayed emergence of M184V mutations in episomal cDNA in patients on dual nucleoside therapy. Symbols are as described in the legend for Fig. 2.

episomes in patients 230 and 289 was due to lower levels of ongoing replication and hence slower replacement of wild-type episomes with M184V-containing episomes.

Because of incomplete viral suppression and rapid clearance of virions (30), wild-type viruses in plasma are rapidly replaced by drug-resistant variants within weeks of initiating suboptimal therapy. In this study, we tracked the emergence of drug resistance mutations in episomal and proviral DNA in patients on such suboptimal therapies. We demonstrate that in most cases, the wild-type episomal cDNA population is replenished by a drug-resistant episomal population even though proviruses remain wild type. One possible explanation is that episomes are labile and, during ongoing replication, episomes decay and are replaced as a result of ongoing infection. An alternative explanation is that episomes are intrinsically stable. In the latter scenario, the replacement of wild-type episomes with drug-resistant episomes could be explained by turnover of episome-harboring cells or their redistribution to tissue compartments. We feel this explanation is unlikely, since one would expect a similar evolution of drug resistance mutations in the proviral species. Our finding that drug resistance mutations evolve in episomes but not proviruses strongly argues for an intrinsic instability of episomal cDNAs rather than death or redistribution of the infected cell itself. As episomes are syn-

thesized and degraded, they are replenished by episomes synthesized de novo during ongoing viral replication. In patients where there was a delay in the appearance of M184V episomes, we also documented significantly lower rates of genetic divergence. Therefore, the rate with which the episomal cDNA pool evolves can be expected to vary between patients depending upon the ongoing level of viral replication. Because antiretroviral therapy impacts selection pressure, evolution rate cannot be a direct measure of replication. Nevertheless, the evolution of episomal cDNA can be used to determine relative levels of viral replication in patients on similar regimens. We feel that lower levels of ongoing viral replication provide a better explanation for the slower conversion to M184V episomes observed in three patients than intrinsic differences in stability of episomal cDNA in different cell types. Viral replication has been documented in both resting and activated CD4^{+} T cells (46). While we cannot rule out the possibility that episomal cDNA stability may be influenced by cell cycle status, we feel it unlikely that significant differences in the distribution of episomal cDNA in resting and activated CD4^{+} T cells would explain the markedly different rates with which M184V episomes emerged in the patients studied here.

Our observation that wild-type proviruses are maintained, despite the emergence of drug resistance mutations in plasma
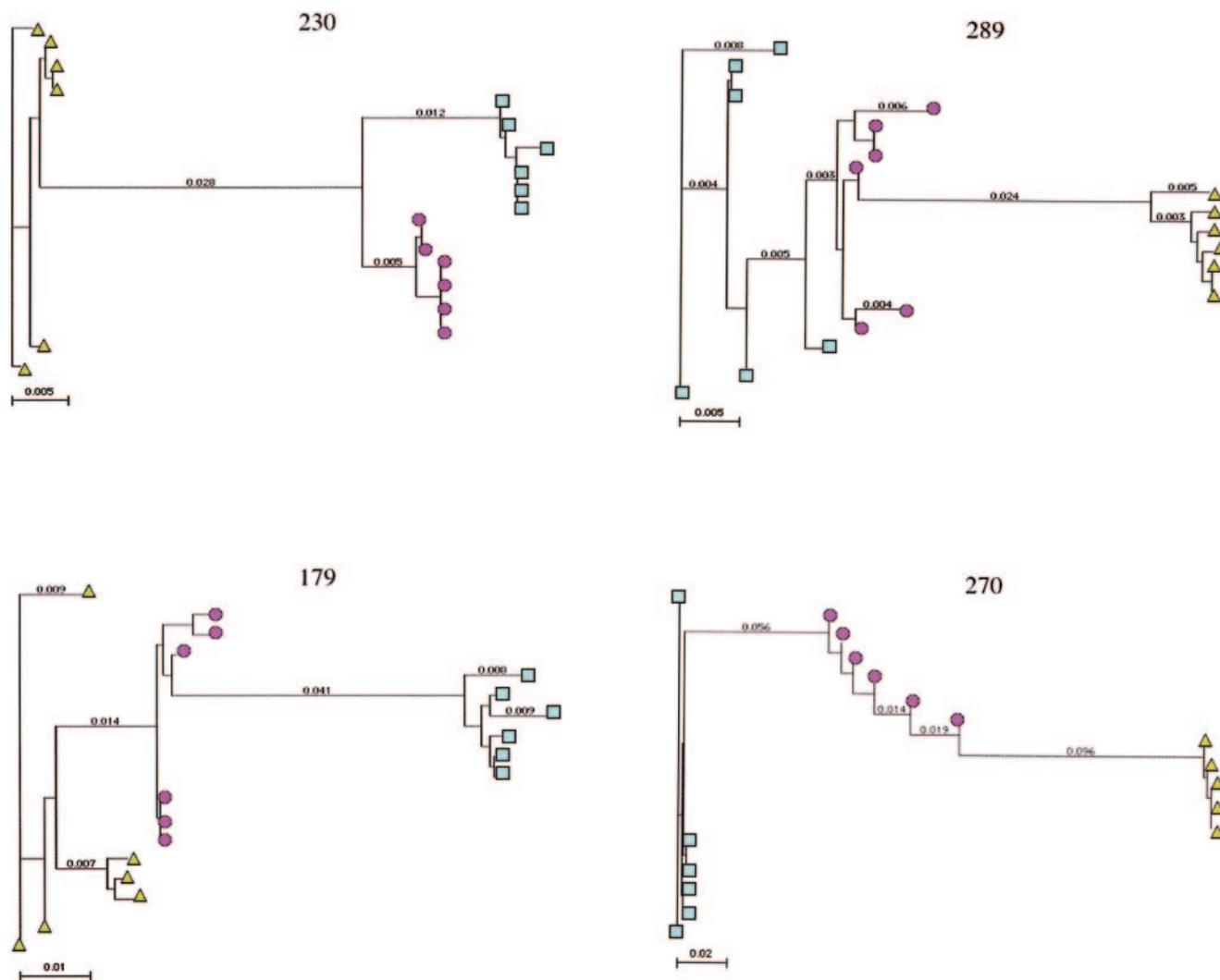
FIG. 4. Phylogenetic relationships in episomal (2-LTR circle) envelope genes in patients with rapid and delayed evolution of M184V polymorphisms in episomal cDNA. Nested PCR was used to specifically amplify envelope sequences from 2-LTR circles, using primers detailed in Table 1. Nucleotide sequences corresponding to the C2 and V3 regions of the envelope glycoprotein were aligned by using Clustal W (MacVector 7.1.1), and the phylogenetic relationships were generated by using the neighbor joining method included in the Mac Vector software package with a Kimura-2 parameters distance matrix. Six unique nucleotide sequences spanning C2 and V3 were determined at weeks 0 (circles), 24 (squares), and 48 (triangles) of the study for two patients (179 and 270) with rapid emergence of M184V episomes and two patients (230 and 289) with delayed emergence of the M184V polymorphism in episomal cDNA. Median genetic distances for early and late converters were significantly different (P < 0.03) in a Mann-Whitney nonparametric analysis.

viral RNA and in episomal cDNA, is not unexpected. Previous studies examining total cell-associated viral DNA have shown that viral DNA levels decay slowly and emergence of drug resistance mutations is greatly delayed relative to results with

TABLE 2. Longitudinal genetic changes in *env* of early versus delayed M184V converters

Time interval	Genetic distance[a]			
	Late M184V conversion		Early M184V conversion	
Patient	230	289	179	270
Weeks 0–24	0.020	0.013	0.052	0.085
Weeks 24–48	0.045	0.039	0.081	0.184

[a] Kimura-2 parameters distance, taking into account the sum of transitions and transversions, with a ratio of transitions to transversions of 1.

plasma viral RNA (1, 17, 34, 37, 42). In one study, emergence of the M184V mutation was compared in genomic viral RNA and viral DNA; however, there was no attempt to distinguish the emergence of the mutations in the different species of viral DNA (37). To our knowledge, this is the first study to independently characterize the emergence of drug resistance mutations in proviral and in episomal cDNA. Our results would suggest that during ongoing viral replication, only a small percentage of the proviruses are a result of recent infection events, and that the majority of proviruses are nondynamic and are archival because they are either defective or latent. By comparison, our data provide evidence that episomal cDNAs are labile and, as such, are surrogates of ongoing viral replication.

The ability to monitor the extent of HIV-1 replication in aviremic patients on suppressive therapies has been hampered

by a lack of convenient markers of ongoing viral replication. The validity of episomal cDNAs as surrogates of ongoing viral replication has been questioned by studies demonstrating intrinsic stability of episomal cDNA in short-term in vitro experiments (4, 31). In one study, in experiments extending over 10 days, the authors reported a decay in 2-LTR circles and concluded that the decay was a result of dilution through cell division rather than degradation of episomal DNA (31). In another study, no significant decay in 2-LTR circle forms in a $CD4^+$-T-cell line was noted over a 70-h time period under single-cycle infection conditions (4). In contrast, other studies have shown that episomal viral cDNA is labile in primary cells and $CD4^+$-T-cell lines (29, 35, 39) and in vivo (27, 35). Regardless of the differences in these studies, the in vitro experiments are limited in that they track circle stability in experiments extending over a period of days. In patients on highly active antiretroviral regimens, plasma viral RNA is suppressed to below the level of detection for months to years. Therefore, short-term in vitro studies on episomal DNA stability do not provide an accurate assessment of the dynamics of episomal viral cDNA turnover in patients on long-term suppressive therapies. Our data provide direct evidence for the in vivo evolution and turnover of episomal viral cDNA. As such, we feel it is unlikely that episomes detected in patients on highly suppressive therapies, who have been aviremic for extended intervals, are archival and were originally formed during the viremic phases of the patient's infection history. Monitoring levels of episomal DNA or cDNAs offers one approach for gauging the level of ongoing viral replication in these individuals. Our data argue that the ability to detect episomal viral cDNA in aviremic patients on highly suppressive therapies (35) provides evidence of ongoing viral replication in these individuals.

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