Virology **302,** 393–404 (2002)
doi:10.1006/viro.2002.1621

Evolution of Total and Integrated HIV-1 DNA and Change in DNA Sequences in Patients with Sustained Plasma Virus Suppression

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Received February 25, 2002; returned to author for revision June 7, 2002; accepted June 13, 2002

Blood samples from patients with plasma HIV-1 RNA <20 copies/ml for more than 2 years were studied. Significant decreases in total and integrated HIV-1 DNA were observed during the first 15 months of suppressive therapy before the concentrations became stable. Clonal analysis of HIV-1 *pol* demonstrated that the proportions of resistance mutations in DNA sequences after 2 years were lower than those in baseline DNA and RNA sequences. The changes in the clonal composition of HIV-1 *env* populations in three patients with evidence of changes in HIV-1 *pol* populations indicated a shift from predominantly R5-like viruses to predominantly X4-like viruses in two patients and the persistence of predominantly X4-like viruses in the third. Our analyses indicate the reemergence of ancestral sequences from long-lived cells or the residual production of wild-type virus from anatomic sites with limited access to antiretroviral drugs and the preferential infection of cells expressing CXCR4.

Key Words: HIV-1 DNA; proviral DNA; HIV-1 *pol* and *env* sequences; active antiretroviral therapy.

INTRODUCTION

Treatment of human immunodeficiency virus (HIV-1) infection with highly active antiretroviral therapy (HAART), including reverse transcriptase and protease inhibitors, has led to the sustained suppression of virus RNA in the plasma of many patients. However, replication-competent virus can routinely be recovered from patients by culturing resting $CD4^+$ T lymphocytes, even from those whose plasma virus RNA has been undetectable by molecular assays for up to 3 to 4 years (Chun *et al.,* 1997b; Finzi *et al.,* 1997, 1999; Wong *et al.,* 1997). These observations and molecular techniques measuring both unintegrated and integrated HIV-1 DNA (Chun *et al.,* 1995, 1997a, 1998) have led to the suggestion that the replication-competent integrated provirus in resting $CD4^+$ T lymphocytes could be a source of reactivatable virus and therefore a major obstacle to eradication of HIV from infected individuals on HAART. Other observations also suggest that residual virus replication persists *in vivo* despite the lack of detectable HIV-1 RNA in the plasma. Studies using an ultrasensitive reverse transcriptase–polymerase chain reaction (RT–PCR) able to detect cell-free virion RNA at less than five copies/ml have shown viral RNA in the plasma of all patients of one cohort of HIV-infected individuals taking suppressive HAART (Dornadula *et al.,* 1999). Moreover, several groups have shown low levels of virus replication in selected patients with low plasma HIV-1 RNA by analyzing the cell-associated virus RNA of peripheral blood and lymphoïd tissue (Cavert *et al.,* 1997; Furtado *et al.,* 1999; Hockett *et al.,* 1999; Lafeuillade *et al.,* 1998; Natarajan *et al.,* 1999; Zhang *et al.,* 1999). Finally, HIV-1 cDNA episomes, which are labile products of virus infection and indicate recent infection, have been detected in a large percentage of infected individuals on HAART, despite sustained undetectable levels of plasma virus RNA (Sharkey *et al.,* 2000). However, these data are controversial because recent studies have suggested that HIV-1 circular DNA forms may be quite stable (Butler *et al.,* 2002; Pierson *et al.,* 2002). Extensive genetic studies of *env* and *pol* sequences (Hirsch *et al.,* 2000; Shankarappa *et al.,* 1999) have been performed in patients with actively replicating virus, but little is known about the genetic variation of HIV-1 in patients for whom the control of HIV-1 replication by HAART leads to sustained undetectable plasma HIV-1 RNA. Significant variations of HIV-1 sequences from the *env* gene were found in some patients, despite their undetectable plasma HIV RNA (Gunthard *et al.,* 1999; Martinez *et al.,* 1999; Zhang *et al.,* 1999), but the possible shift in the chemokine receptor used by HIV-1 strains has not yet been studied, nor has the evolution of resistant virus populations.

In the present longitudinal study of seven patients whose plasma HIV-1 RNA had been suppressed for more than 2 years, we investigated the evolution of genotypic drug resistance in the protease and reverse transcriptase regions of the *pol* gene from peripheral blood mononuclear cell (PBMC) DNA. We also examined the evolution of the *env* V3 region, focusing on determinants influencing the use of chemokine receptors CCR5 and

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0042-6822/02 $35.00

TABLE 1

Patient Characteristics and Effect of Treatment on the Levels of Total HIV-1 DNA and Proviral DNA in PBMC

Patient No.	Clinical stage (CDC)	CD4 count at baseline (cells/μl)	HIV-1 RNA in plasma (log copies/ml)		Total HIV-1 DNA (copies/10^6 cells)			Integrated proviral DNA (copies/10^6 cells)		
			Baseline	Months 6–24	Baseline	Month 15	Month 24	Baseline	Month 15	Month 24
1	B	1	6.5	<1.3	7,943	324	654	130	13	65
2	C	5	5.0	<1.3	3,364	130	324	324	26	13
3	A	32	3.8	<1.3	3,364	324	65	65	130	4
4	C	11	5.6	<1.3	654	654	654	65	45	64
5	C	124	5.3	<1.3	50,118	1,143	654	5,976	130	324
6	C	2	6.0	<1.3	1,143	654	654	229	130	45
10	A	259	3.2	<1.3	65	13	26	26	4	4
Median		76	5.1		3,364	324	654	130	45	45

CXCR4 for HIV entry (Xiao *et al.,* 1998). Last, we analyzed the changes in both total and integrated HIV-1 DNA over a 24-month period.

RESULTS

Patient characteristics

All seven patients showed a rapid decline in plasma HIV-1 RNA after initiation of treatment. Five patients had fewer than 200 copies/ml after 3 months of treatment and all had fewer than 20 copies/ml after 6 months. The plasma HIV-1 RNA in all the patients remained undetectable by the ultrasensitive RT–PCR assay throughout follow-up. There was a gradual increase in the CD4 lymphocyte counts of all the patients ($P < 0.001$). The median increase in the CD4 lymphocyte count was +59 cells/μl (range, −2 to +245 cells/μl) after 6 months of treatment, +169 cells/μl (range, +43 to +405 cells/μl) after 15 months, and +226 cells/μl (range, +149 to +589 cells/μl) after 24 months.

Changes in cell-associated total HIV-1 DNA and integrated proviral DNA

The median cell-associated total number of HIV-1 DNA copies before active therapy was 3364 copies/10^6 cells (range, 65–50,118 copies/10^6 cells) (Table 1). The concentration of total HIV-1 DNA declined significantly from 3364 copies/10^6 cells to 324 copies/10^6 cells (range, 13–1143 copies/10^6 cells) after 15 months ($P < 0.001$). In contrast, there was no change in total HIV-1 DNA between months 15 and 24 (median, 654 copies/10^6 cells; range, 26–654 copies/10^6 cells).

The median number of copies of cell-associated integrated provirus before active therapy was 130 copies/10^6 cells (range, 26–5976 copies/10^6 cells). As for total HIV-1 DNA, the concentration of integrated proviral DNA dropped during the first 15 months (median integrated provirus DNA at month 15, 45 copies/10^6 cells; range, 4–130 copies/10^6 cells; $P < 0.001$), but remained stable thereafter (median integrated proviral DNA at month 24, 45 copies/10^6 cells; range, 4–324 copies/10^6 cells) (Table 1).

Changes in RT and protease *pol* regions

Genotype analysis was first performed by sequencing the first 240 codons of the RT region and the entire protease region of the *pol* gene (Table 2). Only virus species representing more than 20% of the total population can be detected using this technique. Mutations associated with resistance to zidovudine (AZT) were found in baseline samples from five patients who had been previously exposed to nucleoside RT inhibitors (patients 1, 2, 3, 5, and 6). Patients 2 and 5 had similar baseline RNA and DNA sequences, while the patterns of mutations in RNA and DNA were different for patients 1, 3, and 6. As expected, no mutations conferring resistance to protease inhibitors were found at baseline, since all the patients were protease inhibitor-naïve before active therapy. Comparison of the DNA sequences from PBMC collected 24 months after suppressive therapy with baseline RNA and DNA sequences revealed no change in the protease amino acid sequences. No new mutations associated with resistance to RT inhibitors were detected, including the M184I/V mutation for the six patients who were given lamivudine (3TC). Surprisingly, the amino acid sequences after 24 months of suppressive therapy showed mixtures of wild-type and mutant viruses (patients 2, 3, and 5) or wild-type virus (patient 6) in all patients harboring strains with AZT resistance mutations at baseline, except for one (patient 1).

The changes in HIV-1 populations from patients 2, 3, 5, and 6 were tracked by measuring the proportions of mutant and wild-type molecular clones of PCR products from plasma and PBMC samples before and after 24 months of suppressive therapy. A total of 239 amplicons were obtained and analyzed phylogenetically by the neighbor-joining method (Saitou *et al.,* 1987). Sequences from each patient clustered together, indicating the absence of sample contamination (data not shown). The

TABLE 2

Drug Resistance Genotype before and after 24 Months of Plasma HIV-1 RNA Suppression

Patient No.	Sampling time	Sample	Reverse transcriptase							Protease						
			41	67	70	184	210	215	219	30	46	48	54	82	84	90
			M	D	K	M	L	T	K	D	M	G	I	V	I	L
1	Baseline	Plasma	—	—	E	—	—	—	Q	—	—	—	—	—	—	—
		PBMC	—	N	G	—	—	Y	Q	—	—	—	—	—	—	—
	Month 24	PBMC	—	N	G	—	—	Y	Q	—	—	—	—	—	—	—
2	Baseline	Plasma	L	N	—	—	W	Y	—	—	—	—	—	—	—	—
		PBMC	L	N	—	—	W	Y	—	—	—	—	—	—	—	—
	Month 24	PBMC	—	—	E	—	—	Y/T	—	—	—	—	—	—	—	—
3	Baseline	Plasma	—	N	R	—	—	F	E	—	—	—	—	—	—	—
		PBMC	—	—	—	V	W	Y	—	—	—	—	—	—	—	—
	Month 24	PBMC	—	—	R	—	—	F/T	—	—	—	—	—	—	—	—
4	Baseline	Plasma	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		PBMC	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Month 24	PBMC	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	Baseline	Plasma	L	—	—	—	W	Y	—	—	—	—	—	—	—	—
		PBMC	L	—	—	—	W/L	Y	—	—	—	—	—	—	—	—
	Month 24	PBMC	L/M	—	—	—	—	Y/T	—	—	—	—	—	—	—	—
6	Baseline	Plasma	L	—	—	—	W	Y	—	—	—	—	—	—	—	—
		PBMC	—	—	—	—	W/L	Y	—	—	—	—	—	—	—	—
	Month 24	PBMC	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	Baseline	Plasma	—	—	—	—	—	—	—							
		PBMC	—	—	—	—	—	—	—							
	Month 24	PBMC	—	—	—	—	—	—	Q							

Note. PBMC, peripheral blood mononuclear cells.

clonal analysis is summarized in Table 3. Plasma RNA sequences were more homogeneous than PBMC DNA sequences. The proportions of mutations conferring resistance to AZT (T215Y/F, M41L, D67N, K70R, L210W, and K219Q) and to 3TC (M184I/V) in baseline RNA and DNA sequences revealed more mutations in plasma RNA ($P < 0.001$), suggesting a greater expression of resistant genotypes in infected cells before active therapy. There were fewer mutations conferring resistance to AZT and 3TC in PBMC DNA sequences after 24 months of suppressive therapy than in the baseline DNA and RNA sequences ($P < 0.001$); 94–100% of the variants in the

TABLE 3

Evolution of Quasispecies Harboring Mutations Conferring Resistance to Reverse Transcriptase Inhibitors after 24 Months of Plasma HIV-1 RNA Suppression

Patient No.	Sampling time	Sample	Total clones tested	Mutated clones on RT region (%)						
				41	67	70	184	210	215	219
				M	D	K	M	L	T	K
2	Baseline	Plasma	20	100	95	0	0	100	100	0
		PBMC	18	83	83	0	0	83	89	0
	Month 24	PBMC	18	11	33	0	0	11	50	39
3	Baseline	Plasma	20	0	100	100	0	0	95	95
		PBMC	22	55	45	36	36	41	59	0
	Month 24	PBMC	22	5	45	86	9	5	36	32
5	Baseline	Plasma	18	100	0	0	0	94	94	0
		PBMC	23	96	0	0	0	48	87	0
	Month 24	PBMC	23	57	0	0	0	0	61	0
6	Baseline	Plasma	21	95	0	0	0	100	100	0
		PBMC	18	17	0	6	0	67	67	0
	Month 24	PBMC	16	0	0	13	6	19	31	0

Note. PBMC, peripheral blood mononuclear cells.

baseline RNA sequences and 59–87% of the baseline DNA sequences had the major mutation T215Y, while only 31–61% of the 24-month DNA sequences had this mutation ($P < 0.001$).

Changes in the V3 *env* region

Sequences encompassing the V3 region of HIV-1 *env* were analyzed from virus in baseline plasma and PBMC and from virus in PBMC after 15 and 24 months of suppressive therapy in three patients who had changes in the RT region (patients 3, 5, and 6). A total of 227 amplicons were obtained and all the PCR products were analyzed phylogenetically by the neighbor-joining method (Saitou *et al.,* 1987). Sequences from different patients did not cluster together, indicating no sample contamination (Fig. 1). The data for each patient show virus evolution between baseline RNA and DNA sequences and month 15 and month 24 DNA sequences, although some DNA sequences on therapy were identical or very close to variants present at baseline (Fig. 1). The distribution of R5 and X4 variants over the sequential samples from each patient was estimated from the V3 amino acid sequence. It has been reported that uncharged residues at position 11 of V3 (mostly serine/glycine), negatively charged residues at residue 25 (mostly glutamic/aspartic acid), and a net charge of the V3 loop $< +5$ predicts CCR5 chemokine receptor usage (Chesebro *et al.,* 1996; Xiao *et al.,* 1998). In contrast, positively charged residues at positions 11 and 25 (mostly arginine/lysine) and a net positive charge $\geq +5$ of the V3 loop results in CXCR4 chemokine receptor usage (Chesebro *et al.,* 1996; Cho *et al.,* 1998; Xiao *et al.,* 1998). We used the net charge of V3 as the primary criterion for discriminating between X4-like and R5-like viruses. Most of the sequences identified as X4-like had at least one basic amino acid substitution at a relevant position. As shown in Fig. 2, the baseline RNA sequences were extremely homogeneous (only one mutation from the consensus form) and indicated that R5-like virus was predominant in patients 3 and 6 (100 and 95%, respectively) and X4-like virus was predominant in patient 5 (90%). The proportions of X4-like virus in baseline PBMC were 80% in patient 5, 59% in patient 3, and 37% in patient 6. Analysis of DNA sequences from PBMC collected after 15 and 24 months of suppressive therapy showed that the proportions of X4-like virus increased over time in patients 3 (68% at month 15, 94% at month 24) and 6 (74% at month 15, 100% at month 24). X4-like virus remained predominant at month 24 in patient 5 (88%), although the proportions of R5-like and X4-like clones were similar at month 15 (Fig. 2).

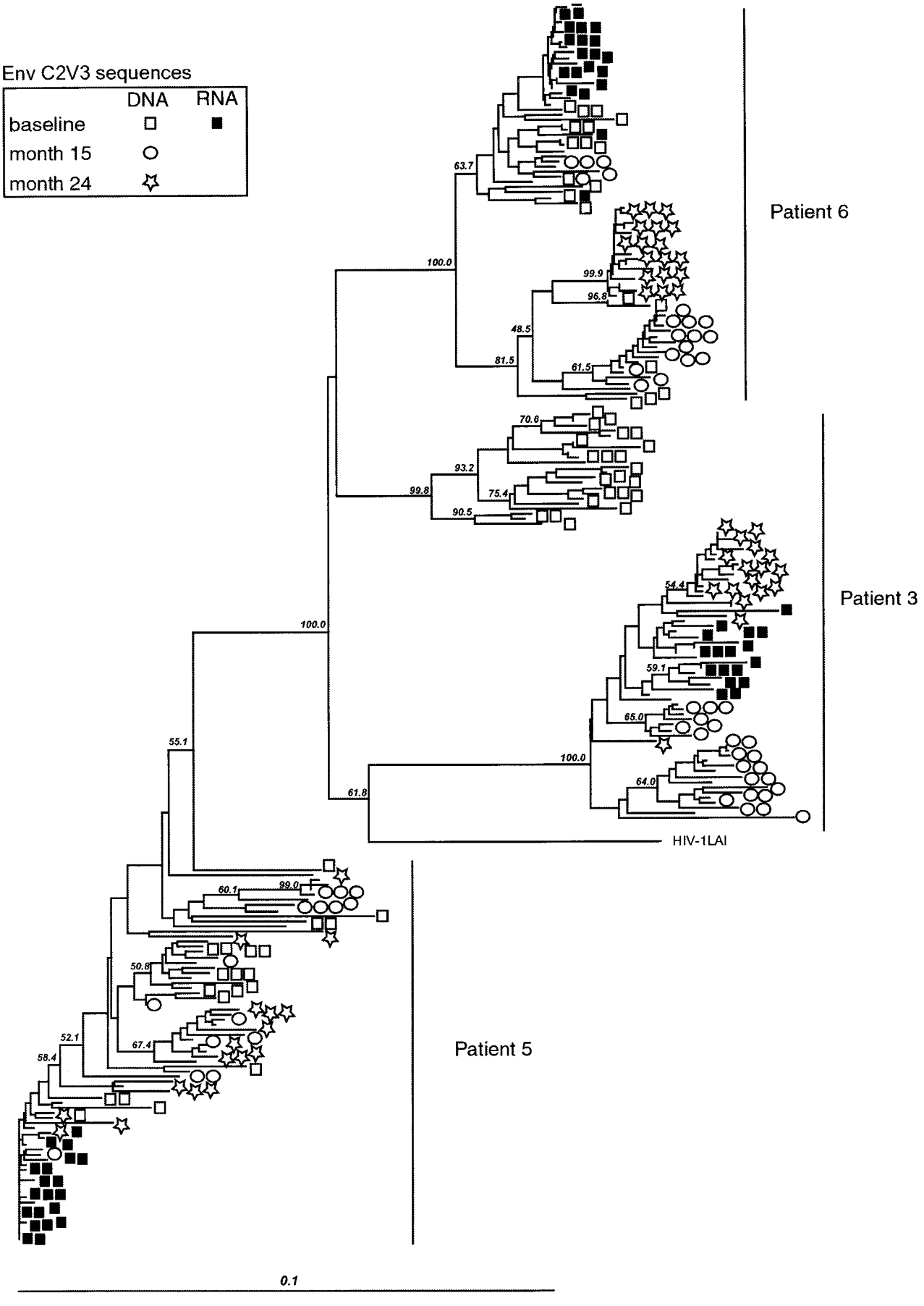
DISCUSSION

This longitudinal study of patients given suppressive therapy for more than 2 years in which total and integrated HIV-1 DNA were measured and *pol* and *env* quasispecies characterized demonstrates a shift from a drug-resistant genotype to a predominantly wild-type genotype and a shift from a mainly R5-like virus to a mainly X4-like virus.

One important issue for assessing virus populations based on PCR products is the use of a sufficient number of templates (Liu *et al.,* 1996). The determination of the distribution of genetic variants that make up the quasispecies at each time point has not been biased in our study for several reasons: (i) taking into account baseline plasma HIV-1 RNA concentrations (3.8–6.0 log copies/ml), the extraction recovery, and the reverse transcription step, a large number of target molecules from each sample were subjected to PCR, (ii) over 300–600 HIV-1 DNA copies/10^6 cells were used for clonal analysis of all the samples but one (patient 3, month 24); therefore, at least 50–100 target molecules were subjected to PCR, indicating that the average number of distinct clones is near the number of clones selected, (iii) a similar distribution of variants was obtained on clonal analysis done on distinct DNA aliquots for selected patients (data not shown), (iv) sequences obtained by direct sequencing precisely matched consensus sequences at near all codons of *pol* and *env* fragments (data not shown).

Despite a significant decrease in cell-associated total HIV DNA of 1 log during the first 15 months of suppressive therapy, the concentration of HIV DNA remained stable between months 15 and 24 in our patients. The median half-life of HIV-1 DNA decay during the first 15 months of active therapy was previously estimated at 40 weeks (Izopet *et al.,* 1998a). The change in integrated HIV DNA over time was similar, with a decrease of 0.5 log after 15 months, but no further decrease thereafter. The actual stability of total and integrated HIV-1 DNA after 15 months was confirmed by measuring these parameters on other cell samples that were available for three patients (data not shown). These results are in agreement with those recently reported by Furtado *et al.* (1999) showing that the evolution of total and integrated HIV DNA in subjects on potent antiretroviral therapy was

FIG. 1. Neighbor-joining phylogenetic tree depicting HIV-1 *env* sequences found in patients 3, 5, and 6. The entire PCR product was used for phylogenetic analyses. All sequences are shown with LAI (GenBank Accession No. K02013) as the outgroup. RNA sequences derived from baseline plasma are represented by solid squares. DNA sequences derived from peripheral blood mononuclear cells are indicated by open symbols: open squares (baseline), open circles (month 15), and open stars (month 24). The genetic relatedness of two different sequences in the phylogenetic tree is represented by the horizontal distance that separates them, with the length of the bar at the bottom denoting a sequence divergence of 0.10. Bootstrap values are expressed as percentages for each relevant branch and represent the percentage of occurrence of that branch per 1000 bootstrap replicates.


Env C2V3 sequences
DNA
RNA
baseline
month 15
month 24
Patient 6
Patient 3
Patient 5
HIV-1LAI
63.7
100.0
99.9
96.8
48.5
81.5
61.5
70.6
93.2
99.8
75.4
90.5
54.4
100.0
59.1
65.0
55.1
100.0
64.0
61.8
60.1
99.0
50.8
52.1
67.4
58.4
0.1

biphasic, with a substantial decrease during the initial 500 days and a subsequent plateau. Because the technique used for measuring integrated proviral DNA could be less efficient than the technique used for measuring total HIV-1 DNA due to the length of the amplified product, it is difficult to estimate accurately the proportions of both forms. However, the data show that the ratios of proviral DNA to total HIV-1 DNA at months 15 and 24 were similar to baseline ratios. This point provides indirect evidence of the persistence of unintegrated DNA suggesting the occurrence of recent infections. Linear DNA incorporated into the preintegration complex is unstable (Zack *et al.,* 1990), while the circular unintegrated DNA found exclusively in the nucleus is a labile product of the integration process (Sharkey *et al.,* 2000). But this latter point needs to be reevaluated (Butler *et al.,* 2002; Pierson *et al.,* 2002). The stability of integrated DNA after 15 months suggests that the intrinsic decay rate of the latent reservoir, i.e., resting $CD4^+$ T lymphocytes harboring replication-competent virus, is balanced by an equal rate of *de novo* infection regenerating the latent reservoir. Ramratnam *et al.* (2000) showed that the decay of the latent reservoir of replication-competent HIV was inversely correlated with the extent of residual virus replication. This could also partly explain differences in the reported half-life of replication-competent HIV in the pool of latently infected, resting $CD4^+$ T cells, which ranges from 44 months (Finzi *et al.,* 1999) to 6 months in patients with minimal evidence of residual virus replication (Zhang *et al.,* 1999). Our data are consistent with new projections of viral decay that take into account the virus production by cells infected after the initiation of active therapy (Grossman *et al.,* 1999).

Several studies have used population sequencing of viral isolates recovered from sensitive cultures to show that drug resistance mutations do not emerge in patients with prolonged plasma virus suppression (Finzi *et al.,* 1997; Gunthard *et al.,* 1998; Wong *et al.,* 1997). This was also observed in our study by sequencing multiple molecular clones from the PBMC of several patients exposed to lamivudine, which rapidly selects the M184I/V mutation, even when there is very little viral replication (Descamps *et al.,* 2000). However, the month-24 PBMC from one patient (patient 6) had 1 clone of 16 with the M184V mutation, suggesting that there was a period when replication was more rapid in this patient, but which was not identified because of infrequent sampling. A similar observation was reported by Martinez *et al.* (1999), although their patient had plasma RNA concentrations of 20–200 copies/ml. One of our patients given only a two-drug regimen showed no major mutations conferring drug resistance after 24 months, suggesting that a durable antiviral effect can be obtained with a regimen of modest antiviral potency in some individuals provided the target of therapy (fewer than 20 copies RNA/ml) is achieved. Surprisingly, clonal analysis of sequential DNA sequences from PBMC of four patients harboring resistant viral populations before active therapy showed the emergence of wild-type virus at critical codons for resistance after 24 months. The proportions of wild-type virus in PBMC increased substantially in all the patients studied. Because the DNA sequences from PBMC may reflect earlier steps in viral production and selection, our results are compatible with the selection of long-lived cells populations harboring wild-type drug-sensitive virus (Hermankova *et al.,* 2001). Another possibility is the ongoing production of wild-type virus in anatomical sites to which antiretroviral drugs have limited access and/or proximal activation and transmission involving cells chronically and latently infected with wild-type virus (Grossman *et al.,* 1998). The precise location of this viral production remains undefined but many sites, such as the central nervous system, the genital organs, and the thymus, are possible candidates (Schrager *et al.,* 1998). Low drug concentrations at these sites could generate resistant variants, but their poor fitness could preclude the emergence of a population of resistant virus.

We looked for changes in the *env* sequences in three patients in which there was evidence of changes in the *pol* gene during prolonged plasma HIV RNA suppression. The chemokine receptor usage of virus populations in sequential samples was determined by sequencing multiple molecular clones and analyzing the distribution of R5- and X4-like viruses. The HIV variants were separated according to the net charge of the V3 loop and the critical amino acid residues at positions 11 and 25 on either side of the conserved tip of the V3 loop, which are major determinants of coreceptor usage. Although there may be discrepancies between genotyping and phenotyping for some strains, there seems to be relatively good agreement between the two methods (Briggs *et al.,* 2000; Hung *et al.,* 1999; Xiao *et al.,* 1998). Our genetic analysis showed a shift from mainly R5-like viruses to mainly X4-like viruses in two patients and the persistence of predominantly X4-like viruses in the third patient. Chun *et al.* (1997b) also detected syncytium-inducing (SI) virus in resting $CD4^+$ T cells induced *in vitro* in three of nine patients who had undetectable plasma viremia on HAART.

The R5 to X4 shift that we observed may be due to the preferential implication of X4 viruses in the latent reservoir. The predominant forms in the PBMC of patient 6 during plasma HIV RNA suppression were identical or closely related to a minor variant present in baseline PBMC but not in baseline plasma. Replication-competent HIV-1 provirus has been detected within naïve $CD45RA^+$/$CD62L^+$ $CD4^+$ T cells (Ostrowski *et al.,* 1999). The proviral loads in memory and naïve $CD4^+$ T cell subsets were similar in the patients who harbored X4 viruses, while the proviral load in naïve $CD4^+$ T cells was very low in the patients who harbored R5 viruses (Ostrowski

Sample	No. of clones / total tested	Amino acid sequence of the V3 domain (11 25)	Net charge	R5 or X4-like genotype
Patient 3				
Baseline DNA	Consensus	C T R P N N N T R K R I S M G P G R V Y Y T T G D I V G D I R Q A H C	+4	R5
	6/22	- - - - - - - - - - - - - L - - - - - - - - - - G - - - - - - - - - -	+5	X4
	5/22	- - - - - - - - - - - V -	+4	R5
	4/22	- - - - - - - - - - - V - - - - - - - - - - - - - - - - - - K - - -	+5	X4
	3/22	- I - - S - - - - - S - P I - - - - A F - A - - - - I - - - - - - - -	+3	R5
	1/22	- - - - - - D - - - - - V P - - - - - - - - - - - - - - - - - - K - - -	+4	R5
	3/22	- - - - - - - - - - - - - T - - - - - - - - - - G - - - - - - - - - -	+5	X4
Baseline RNA	Consensus	- - - - - - - - - - S - H I - - - - A F - A - * - - I - N - - - - - -	+4	R5
	13/17	- - - - - - - - - - S - H I - - - - A F - A - * - - I - N - - - - - -	+4	R5
	2/17	- - S - - - - - - - S - H I - - - - A F - A - * - - I - N - - - - - -	+3	R5
	1/17	- - S - - - - - - - S - H I - - - - A F - A - * - - I - N - G - - - -	+2	R5
	1/17	- - - L - - - - - - S - H I - - - - A F - A - * - - I - N - - - - - -	+4	R5
Month 15 DNA	Consensus	- - - - - K K I I R - - H I - - - - A F - A - * - - R - N - - - - - -	+7	X4
	9/22	- - - - - K K I I R - - H I - - - - A F - A - * - - R - T - - - - - -	+7	X4
	6/22	- - - - - - - - - - S - H I - - - - A F - A - * - - I - N - - - - - -	+4	R5
	4/22	- - - - - K K I I R - - H I - - - - A F - A - * - - R - N - - - P - -	+7	X4
	1/22	- - - - - - - - - - S - H I - - - - A F - A - * - - I - N - - - - - -	+4	R5
	1/22	- - - - - K K I I R - - H I - - - - A F - A - * - - R - N - - - P - Y	+7	X4
	1/22	- - - - - K K I I R - - H I - - - - A F - A - * - V R - N - - - P - -	+7	X4
Month 24 DNA	Consensus	- - - - - - - - - - S - H I R - R - A F - A - * - - I - N - - - - - -	+6	X4
	14/17	- - - - - - - - - - S - H I R - R - A F - A - * - - I - N - - - - - -	+6	X4
	1/17	- - - - - - - - - - S - H I R - R - A F - A - * - - I - D - - - - - -	+5	X4
	1/17	- - - - - - - - - - S - H I R - R - A F - A - * - V I - N - - - - - -	+6	X4
	1/17	- - - - - - - - - - S - H I - - - - A F - V - * - - I - N - - - - - -	+4	R5

FIG. 2. Predicted protein sequences of the V3 loop of virus from three patients with 24-month plasma HIV RNA suppression. All letter designations for amino acids conform to the standard International Union of Pure and Applied Chemistry code. The consensus and clonal sequences are given for each subject and source. The V3 loops were aligned with reference to the consensus baseline DNA sequence for each patient. Dashes denote an amino acid that is identical to an amino acid in the consensus baseline DNA sequence. Asterisks denote a gap inserted to maintain alignment. Replacements are indicated by the appropriate code letter. Residues at positions 11 and 25 have been boxed to highlight the substitutions noted. The total amino acid charge of the V3 loop was calculated by subtracting the number of negatively charged amino acids (D and E) from the number of positively charged ones (K and R) using DNAid 1.8 software (Frederic Dardel, Palaiseau, France).

et al., 1999). It has also been reported that SI variants are equally distributed between $CD45RO^+$ cells and naïve $CD45RA^+$ cells, in contrast to NSI variants that are mainly present in memory $CD45RO^+$ cells (Blaak *et al.,* 2000). However, a recent study showed that most of the viruses in latently infected $CD4^+$ resting T cells used CCR5 for entry, although viruses using CXCR4 were also detected (Pierson *et al.,* 2000). An alternative explanation of the R5 to X4 shift could be the preferential residual replication of X4 viruses. The surface densities of CCR5 and CXCR4 on $CD4^+$ T cells differs. More CXCR4 is found on naïve T cells, while CCR5 predominates on memory and activated T cells (Connor *et al.,* 1997; Feng *et al.,* 1996; Simmons *et al.,* 1996). Thymocytes express CXCR4 during most stages of maturation, although few thymocytes express CCR5 (Taylor *et al.,* 2001). The preferential infection of naïve T cells or thymocytes by X4 virus during prolonged plasma HIV RNA suppression is compatible with the two distinct phases of immune restoration on potent antiretroviral therapy: simple redistribution of memory lymphocytes from lymphoïd tissues to the circulation followed by a slower increase in largely naïve $CD4^+$ cells. This probably reflects continued thymic production or extrathymic cell division that is no longer counterbalanced by cell sequestration and destruction by HIV (Autran *et al.,* 1997; Pakker *et al.,* 1998). Alternatively, the emergence of X4 viruses might be linked to negative selective forces acting against R5 viruses, such as chemokines (Trumpfheller *et al.,* 1998), neutralizing antibodies (Burton *et al.,* 1997), or antiretroviral drugs (van 't Wout *et al.,* 1996) in the microenvironment of virus production.

In summary, we have shown a stability of total and integrated HIV-1 DNA after 15 months of suppressive therapy, a shift in the virus populations from drug-resistant variants, and the emergence of X4-like viruses. Our findings suggest the reemergence of ancestral sequences or the residual production of wild-type virus and the preferential infection of cells expressing CXCR4. Phenotypic assays should be useful for better characterizing HIV-1 from cell reservoirs. The pathogenicity of the emergent X4 viruses in patients on HAART is unclear (Holt-

Sample	No. of clones / total tested	Amino acid sequence of the V3 domain (11 … 25)	Net charge	R5 or X4-like genotype
Patient 5				
Baseline DNA	Consensus	C T R P N N N T R R **G** I H I G P G R A W Y A T D **R** I I G N I R Q A H C	+5	X4
	8/20	- - - - - - - - - - **-** - - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	2/20	- - - - - - - - - - **S** - - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	2/20	- - - - - - - - - - **S** - R - - - - - - - - - - - - **-** - - - - - - - - - -	+6	X4
	1/20	- - - - - - H - - K **S** - - - - - - - - - - - - G **E** - - - D - - - - - -	+3	R5
	1/20	- - - - - - - - - - **S** - - - - - - - - - - - - G **E** - - - - - - - - - -	+4	R5
	1/20	- I - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	1/20	- - - - - - - - - - **-** - - - E - - - - - - - - - **-** - - - - - - - - - -	+4	R5
	1/20	- - - - - - - - - - **-** - - - - - - - - - - - - G **-** - - - - - - - - - -	+6	X4
	1/20	- - - - - - - - - - **-** - - - - - - - - - - - - - **K** - - - - - - - - - -	+5	X4
	1/20	- - - - - - - - - - **S** - - - - - - - - - - - - G **Q** - - - - - - - - - -	+5	X4
	1/20	- - - - - - - - - K **S** - - - - - - - - - - - - G **E** - - - D - - - - - -	+3	R5
Baseline RNA	Consensus	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	18/20	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	1/20	- - - - - - D - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - - -	+4	R5
	1/20	- - - - - - - - - - **-** - - - - - - - N - - - - **-** - - - - - - - - - -	+4	R5
Month 15 DNA	Consensus	- - - - - - - - - - **S** - - - - - - - - - - - - G **D** - - - - - - - - - -	+4	R5
	4/15	- - - - - - - - - - **S** - - - - - - - - - - - - G **D** - - - D - - - - - -	+3	R5
	3/15	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	3/15	- - - - - - - - - - **S** - - - - - - - - - - - - G **E** - - - - - - - - - -	+4	R5
	2/15	- - - - - - - - - - **-** - - - - - - - - - - - - - **K** - - - - - - - - - -	+5	X4
	1/15	- - - - - - - - - - **S** - - - - - - - - - - - - - **K** - L - - - - - - - -	+5	X4
	1/15	- - - - - - - - - - **-** - - - - - - - - - - - - G **D** - - - D - - - - - -	+3	R5
	1/15	- - - - - - - - - - **S** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
Month 24 DNA	Consensus	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	6/17	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	4/17	- - - - - - - - - - **S** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	3/17	- - - - - - - - - - **-** - - - - - - - - - - - - - **K** - - - - - - - - - -	+5	X4
	1/17	- - K - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - - -	+5	X4
	1/17	- - - - - - - - - - **-** - - - - - E - - - - - - - **-** - - - - - - - - - -	+4	R5
	1/17	- - - - - - - - - - **-** - - - - - - - - - - - - G **K** - - - - - - - - - -	+6	X4
	1/17	- - - - - - - - - - **S** - - - - - - - - - - - - G **D** - - - D - - - - - -	+3	R5

FIG. 2—*Continued*

kamp *et al.,* 2000). Further longitudinal studies are needed to determine whether the emergence of X4 virus influences the T cell turnover or simply reflects a change in cell dynamics in patients on potent antiretroviral therapy.

MATERIALS AND METHODS

Patients

Subjects were selected from a well-characterized cohort of HIV-1-infected patients treated at the Department of Infectious Diseases of Toulouse University Hospital (Toulouse, France) (Izopet *et al.,* 1998a). Informed consent was obtained from all the subjects. Each had had continued suppression of plasma HIV RNA for more than 2 years, as assessed at 3-month intervals by the ultrasensitive Amplicor HIV-1 Monitor RT–PCR assay (Roche Diagnostics, Meylan, France). The subjects were four men and three women, with a mean age of 35 years, who were classified as U.S. Centers for Disease Control and Prevention (CDC) class A ($n = 2$), B ($n = 1$), or C ($n = 4$) (Table 1). The median baseline CD4 count was 76 cells/μl (range, 1–259 cells/μl) and the median baseline plasma RNA was 5.1 copies/ml (range, 3.2–6.5 copies/ml). Six patients (patients 1–6) were placed on a first triple-drug regimen consisting of zidovudine (AZT) + lamivudine (3TC) + indinavir (IDV) after multiple failures of nucleoside reverse transcriptase inhibitor-based therapies. The remaining patient (patient 10) was given a first line AZT + zalcitabine (ddC) regimen.

$CD4^+$ T lymphocyte count

Peripheral blood $CD4^+$ T lymphocytes were counted by flow cytometry (Epics Profile; Coulter, Hialeah, Florida) using commercially available monoclonal antibodies (Beckton–Dickinson, Mountain View, California).

Plasma HIV-1 RNA

The HIV-1 RNA in the plasma was measured using the ultrasensitive Amplicor HIV-1 Monitor RT–PCR assay (Roche Diagnostics) according to the manufacturer's in-

Sample	No. of clones / total tested	Amino acid sequence of the V3 domain	Net charge	R5 or X4-like genotype
Patient 6				
Baseline DNA	**Consensus**	C T R P S N N T R K **S** I H I G P G R A F Y T T G **A** I T G D I R Q A H C	+ 4	R5
	11/19	- - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - -	+ 4	R5
	4/19	- - - - H S I - - Q **R** - - - - - - - - - H - - K - **-** K - - - - - - -	+ 6	X4
	1/19	- - - - - - I - - Q **R** - - - - - - - - - H - - R T - K - G - - - - - -	+ 7	X4
	1/19	- - - - - - - - - - **-** - - - - - - - - - - - - - R - - - - - - - - - -	+ 5	X4
	1/19	- - - - - - - - - - **-** - - - - - - - - - - - - - - - **-** - - - - - G - - - -	+ 3	R5
	1/19	- - - - H S I - - Q **R** - - - - - - - - - H - - R T - K - G - K - - - -	+ 7	X4
Baseline RNA	**Consensus**	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - -	+ 4	R5
	18/21	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - -	+ 4	R5
	1/21	- A - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - -	+ 4	R5
	1/21	- - - - - D - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - -	+ 3	R5
	1/21	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - R - - -	+ 5	X4
Month 15 DNA	**Consensus**	- - - - H S I - - Q **R** - - - - - - - - - H - - R T - K - G - K - - - -	+ 7	X4
	13/19	- - - - H S I - - Q **R** - - - - - - - - - H - - R T - K - G - K - - - -	+ 7	X4
	4/19	- - - - - - - - - - **-** - - - - - - - - - - - - - **-** - - - - - - - - -	+ 4	R5
	1/19	- - - - - - - - - - **-** - - - - - - G - - - - - - **-** - - - - - - - - -	+ 3	R5
	1/19	- - - - H S I - G Q **-** - - - - - - - - - H - - R T - K - G - K - - - -	+ 5	X4
Month 24 DNA	**Consensus**	- - - - H S I - Q R **-** - - - - - - - - - H - - K **-** - - - S - - - - - -	+ 6	X4
	16/18	- - - - H S I - Q R **-** - - - - - - - - - H - - K **-** - - - S - - - - - -	+ 6	X4
	1/18	- - G - H S I - Q R **-** - - - - - - - - - H - - K **-** - - - S - - - - - -	+ 5	X4
	1/18	- - - - - G I - Q R **-** - - - - - - - - - H - - K **-** - - - S - - - - - -	+ 6	X4

FIG. 2—*Continued*

structions. The lower limit of detection of the assay was 20 copies/ml.

Cell-associated total and integrated HIV-1 DNA

Total and integrated HIV-1 DNA were measured by end point dilution PCR. Serial fivefold dilutions were prepared from a lysate of 1×10^6 PBMC and duplicate aliquots of each dilution were amplified and then detected. Total HIV-1 DNA was measured using *gag* primers (Amplicor HIV-1, Roche Diagnostics) as previously described (Izopet *et al.,* 1998b). Integrated HIV-1 DNA was measured by an Alu–PCR method and nested primers (Chun *et al.,* 1997a). The external primers were Alu–long terminal repeats (LTR) 5′ from conserved sequences of human Alu and Alu–LTR 3′ from conserved HIV-1 LTR sequences. The internal primers, NI-2 5′ and NI-2 3′, allowed amplification of a portion of the LTR region of HIV-1 DNA. The copy numbers of total and integrated DNA were determined from the limiting dilution PCR data by the method of maximum likelihood (Myers *et al.,* 1994).

Sequencing the HIV-1 *pol* and *env* genes

Nucleic acid isolation, cDNA synthesis, and PCR amplification of DNA and cDNA were performed as previously described (Izopet *et al.,* 1998a; Pasquier *et al.,* 1998). All the appropriate measures to prevent contamination, such the aliquoting of all reagents and the physical separation of sample processing and post-PCR handling steps, were strictly applied (Kwok *et al.,* 1989). The first 240 codons of the reverse transcriptase region was amplified using RT1 (sense primer, 5′-GGAAACCAAAA-ATGATAGGGGGAATTGGAGG-3′; nucleotides (nt) 1959 to 1989 of the HIV_{LAI} genome) and RT2 (antisense primer, 5′-TCTACTTGTCCATGCATGGCTTC-3′; nt 3975–3953) as outer primers and RT3 (sense primer 5′-ATTTTCCCATT-AGTCCTATT-3′; nt 2127–2146) and RT4 (antisense primer, 5′-ATGTCATTGACAGTCCAGCT-3′; nt 2901–2882) as inner primers. The entire protease region was amplified using PR1 (sense primer, 5′-AGAGCTTCAGGTTTGGGG-3′; nt 1753–1770) and PR2 (antisense primer, 5′-GC-CATCCATTCCTGGCTT-3′; nt 2185–2168) as outer primers and PR3 (sense primer, 5′-GAAGCAGGAGCCGATAG-ACA-3′; nt 1794–1812) and PR4 (antisense primer, 5′-ACTGGTACAGTTTCAATA-3′; nt 2160–2143) as inner primers. A region of 667 nucleotides encoding the gp120 V3 loop was amplified using E1 (sense primer, 5′-TTAG-GCATCTCCTATGGCAGGAAGCGG-3′; 5956–5985 of the HIV_{HXB2} genome) and E2 (antisense primer, 5′-AGTGCT-TCCTGCTGCTCCCAAGAACCCAAG-3′; 7810–7781) as outer primers and E3 (sense primer, 5′-CTGTTAAATG-GCAGTCTAGC-3′; 7001–7020) and E4 (antisense primer, 5′-CACTTCTCCAATTGTCCCTCA-3′; 7661–7647) as inner primers. The limit of detection for the nested primer pairs that flank the reverse transcriptase, protease, and *env* regions was five copies of template DNA. The amplification products were sequenced in the sense and antisense directions by the dideoxy chain-termination method (ABI PRISM Ready Reaction AmpliTaq Fs, Dye Deoxy Terminators, Applied Biosystems, Paris, France)

on an ABI 377 automated DNA sequencer (Applied Biosystems).

Plasmid cloning

The PCR products from several samples amplified with inner primers were purified with QIAamp columns (Qiagen, Courtaboeuf, France) and cloned with the PCRII TA cloning kit (Invitrogen BV, Leek, The Netherlands). Recombinant plasmids were used to transform competent *Escherichia coli* cells according to the manufacturer's protocol, and transformants were grown on ampicillin plates; 17–23 clones from PCR products of each region were selected. Plasmid DNAs containing *env* or *pol* inserts were prepared and sequenced on both strands with dye-labeled universal and reverse M13 primers (ABI PRISM, Dye Primer Cycle Sequencing Ready Reaction Kit, Applied Biosystems) on an ABI 377 automated DNA sequencer (Applied Biosystems). The rate of misincorporation generated by the above protocol was evaluated by sequencing 20 clones from two different PCR experiments on LAV-8E5 cells containing one copy of HIV-1 provirus per cell. The misincorporation rate was 0.047% (1/2,086), corresponding to three point mutations.

Analysis of sequence data

Electropherogram data were analyzed using the Sequence Navigator program (Applied Biosystems). Multiple alignments were done with CLUSTALW version 1.7 (Thompson *et al.,* 1996). Sequences from the entire PCR products were compared to each other and to control sequences by phylogenetic analysis and distance mapping (Saitou *et al.,* 1987) to exclude any possibility of sample contamination or mix-up. Phylogenetic distances of *env* sequences from three patients (patients 3, 5, and 6) were calculated with the two-parameter Kimura algorithm applying a transition/transversion ratio of 2.0 (DNADIST from PHYLIP). Dendograms were created by the neighbor-joining method with the CLUSTALW program and tree diagrams were plotted with the TREEVIEW program. Bootstrap analysis consisting of 1000 replicates was performed with the CLUSTALW program. The frequency with which the node occurred is indicated at each relevant branch point. Bootstrapping was performed with the CLUSTALW program. Charges were calculated from the peptide sequence of the V3 loop region by subtracting the number of negatively charged amino acids (D and E) from the number of positively charged amino acids (K and R) using DNAid 1.8 software (Frederic Dardel, Palaiseau, France).

Nucleotide sequence Accession numbers

All the sequences reported here have been submitted to GenBank and were given Accession Nos. AJ225914, AJ225916, AJ225917, AJ225921, AJ225925, AJ225928-AJ225931, AJ225945, AJ225946, AJ225948, AJ225951, AJ225952, AY029809–AY030047, and AF368527–AF368753.

Statistical analysis

Quantitative variables were analyzed by the Wilcoxon test and by the Friedman test for repeated measures. Qualitative variables were analyzed by χ^2 test or Fisher's exact probability test. A value of $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

We thank Quentin Sattentau for critical review. We also thank Eliane Coutanceau for secretarial assistance.

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