

## PERSISTENCE OF HIV-1 TRANSCRIPTION IN PERIPHERAL-BLOOD MONONUCLEAR CELLS IN PATIENTS RECEIVING POTENT ANTIRETROVIRAL THERAPY

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### ABSTRACT

**Background and Methods** Although potent anti-retroviral therapy can control infection with human immunodeficiency virus type 1 (HIV-1), a long-lived reservoir of infectious virus persists in CD4+ T cells. We investigated this viral reservoir by measuring the levels of cell-associated viral DNA and messenger RNA (mRNA) that are essential for HIV-1 replication. Approximately every 6 months, we obtained samples of peripheral-blood mononuclear cells from five men with long-standing HIV-1 infection who had had undetectable levels of plasma HIV-1 RNA for 20 months or more during treatment with potent anti-retroviral drugs.

**Results** Before treatment, plasma levels of HIV-1 RNA correlated with the levels of cell-associated unintegrated HIV-1 DNA and unspliced viral mRNA. After treatment, plasma levels of HIV-1 RNA fell by more than 2.7 log to undetectable levels. The decrease in cell-associated integrated and unintegrated HIV-1 DNA and mRNA occurred in two phases. The first phase occurred during the initial 500 days of treatment and was characterized by substantial decreases in the levels of DNA and mRNA, but not to undetectable levels. The concentrations of cell-associated unintegrated viral DNA, integrated proviral DNA, and unspliced viral mRNA decreased by 1.25 to 1.46 log. The second phase occurred during the subsequent 300 days or more of treatment and was characterized by a plateau in the levels of HIV-1 DNA and unspliced mRNA. After an initial rapid decline, the ratio of unspliced to multiply spliced viral mRNA (a measure of active viral transcription) stabilized and remained greater than zero at each measurement.

**Conclusions** Despite treatment with potent antiretroviral drugs and the suppression of plasma HIV-1 RNA to undetectable levels for 20 months or more, HIV-1 transcription persists in peripheral-blood mononuclear cells. Unless the quasi-steady state levels of HIV DNA and mRNA eventually disappear with longer periods of therapy, these findings suggest that HIV-1 infection cannot be eradicated with current treatments. (N Engl J Med 1999;340:1614-22.)

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function, delays progression of disease, and prolongs survival.<sup>7,8</sup> Despite the apparent success of antiretroviral therapy in suppressing plasma HIV-1 RNA for long periods, a long-lived reservoir of infectious virus remains in CD4+ T cells and perhaps other types of cells, suggesting the need for continued long-term treatment.<sup>9-12</sup> It is important to determine the extent of this reservoir of HIV-1, whether it is being renewed, and the length of time that cells containing replication-competent HIV-1 proviral DNA remain.

Quantitative assessments of HIV-1 and HIV-1-infected cells in blood and lymphoid tissue after treatment have provided information on the kinetics of viral replication and clearance in vivo and the rapidity of the turnover of affected cells and virus.<sup>1,2,4-6,12,13</sup> Mathematical modeling suggests that the decrease in plasma HIV-1 RNA levels in response to combinations of antiretroviral drugs that block new rounds of infection occurs in two phases.<sup>13</sup> In the first phase, as drug therapy extinguishes viral replication, levels of viral RNA in plasma rapidly diminish and CD4+ T cells infected with actively replicating HIV-1 die.<sup>1,2</sup> The second phase is marked by a slower rate of decrease in plasma HIV-1 RNA, reflecting the presence of long-lived infected CD4+ T cells that contain replication-competent virus in unintegrated and integrated forms, as well as free virus associated with lymphoid-tissue follicular dendritic cells.<sup>6-13</sup>

Much of the integrated proviral DNA within CD4+ T cells is unable to replicate. Replication-competent virus, however, persists in long-lived resting memory CD4+ T cells despite one or two years of apparently effective, potent antiretroviral therapy.<sup>9-11</sup> HIV-1 may also be derived from chronically infected cells, including CD4+ T cells and possibly macrophages, that were infected before therapy was initiated and continue to survive and produce virus.<sup>5</sup> Treatment with a protease inhibitor should render most newly produced virions noninfectious. Nonetheless, some infectious particles may still be generated. Consequently, it is not known to what extent the stable viral reservoir represents infected cells that

**C**OMBINATIONS of drugs that inhibit viral reverse transcriptase and protease control infection with human immunodeficiency virus type 1 (HIV-1) in many people by reducing the levels of viral RNA in plasma and depleting the pools of virus in lymphoid tissue.<sup>1-6</sup> This sustained reduction in viral replication improves immune

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are replenished by ongoing low-level replication, chronically infected CD4+ T cells, or resting memory CD4+ T cells with replication-competent, integrated proviral DNA.<sup>10,11</sup> Therefore, we examined the characteristics of the reservoir of HIV-1 in peripheral-blood mononuclear cells of patients who were receiving potent antiretroviral therapy.

## METHODS

### Study Design and Subjects

Over a period of up to 31 months, we measured the levels of cell-associated viral DNA and viral messenger RNA (mRNA) and tracked changes in the nucleotide sequences of the HIV-1 *pol* gene that occurred in concert with continued suppression of plasma HIV-1 RNA to undetectable levels in blood samples from five HIV-1-infected men. All five patients had confirmed HIV-1 infection, were enrolled in a multicenter study of the acquired immunodeficiency syndrome,<sup>14</sup> and were selected on the basis of their compliance with a potent multidrug regimen and the continued suppression of plasma HIV-1 RNA to undetectable levels (<50 RNA copies per milliliter). Plasma HIV-1 RNA levels were measured approximately every six months by a quantitative reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay (Roche Molecular Diagnostic Systems, Branchburg, N.J.). All patients provided written informed consent according to the guidelines of the human subjects–protection committee of Northwestern University.

### Analysis of Cell-Associated Unintegrated Viral DNA and Integrated Proviral DNA

Total viral DNA was measured by a quantitative PCR assay as described previously.<sup>15</sup> To assess the ability of the cellular DNA in a sample to be amplified by PCR and to ensure that samples would yield roughly equal amounts of DNA before amplification, we measured the region coding for the HLA-DQ  $\alpha$  chain using a PCR assay.<sup>15</sup> The numbers of proviral DNA molecules that were randomly integrated into cell DNA during the replication cycle were assessed by a semiquantitative nested PCR with the use of internal control standards of modified target DNA.<sup>16</sup> Covalently closed circular forms of viral DNA molecules containing either one or two long terminal repeats were measured by a semiquantitative PCR assay with the use of appropriate primers.<sup>17</sup> The number of copies was determined by comparison with a dilution series of the target DNA standard. Duplicate tubes with no target DNA were included in each assay to detect contamination. The limit of sensitivity of each DNA assay was approximately 20 copies per 10<sup>6</sup> peripheral-blood mononuclear cells.

### Analysis of Cell-Associated Unspliced and Multiply Spliced Viral mRNA

Levels of cell-associated HIV-1 mRNA were measured by an internally controlled quantitative RT-PCR assay with use of appropriate, precisely matched oligonucleotide primer pairs to identify unspliced viral mRNA and viral mRNA with multiple splices encoding Tat, Rev, and Nef, as described previously.<sup>18</sup> To adjust for the total cellular RNA and verify the integrity of the RNA in each sample, we quantified human ribosomal protein S17 mRNA by RT-PCR using appropriate primers.<sup>15</sup> Tubes with no reverse transcriptase and no target complementary DNA were included to detect contamination. The limit of sensitivity of the assay was approximately 50 copies per 10<sup>6</sup> peripheral-blood mononuclear cells.

### Sequencing of the HIV-1 *pol* Gene

We used direct sequencing of cell-associated viral DNA to assess the frequency of mutations coding for drug resistance in the reverse-transcriptase and protease regions of the HIV-1 *pol* gene.<sup>18</sup> Cell-associated viral DNA was amplified by nested PCR with a pol-F outer primer (nucleotides 52 to 73; 5'TCAGAGCAGACC-

AGAGCCAAC3') and pol-R outer primer (nucleotides 2205 to 2234; 5'ACAGCTGGCTACTATTTCTTTTGCTACTA3') and a pol-F inner primer (nucleotides 69 to 90; 5'CAACAGCCCCACCAAGAGA3') and a pol-R inner primer (nucleotides 1952 to 1976; 5'GATCTGGTTGTGCTTGAATGAT-C3'). The positions of the oligonucleotide primers are numbered according to the *pol* gene of the HXB2 isolate.<sup>19</sup> After extraction and amplification, the DNA was sequenced and analyzed with a sequencing system (Prism 377, Applied Biosystems, Foster City, Calif.) as described previously.<sup>18</sup> Our assay allowed us to detect mutations coding for drug resistance in at least 25 percent of the viral population.

### Statistical Analysis

To assess the correlation between pairs of variables for individual patients and between pairs of measurements in all patients at base line, we calculated the linear correlation coefficient for pairs of measurements obtained at different times. The data obtained during the course of therapy were log-transformed before analysis with a paired, two-sided t-test.<sup>20</sup> To calculate the rates of decrease in the amount of viral DNA and HIV-1 mRNA, we visually inspected the data points and, when appropriate, separated them into two groups: one representing the early phase of the decrease and one representing the later phase.<sup>5</sup> We estimated the rates for each patient using least-squares regression analysis, beginning with the data point obtained closest to the initiation of treatment.<sup>5</sup>

## RESULTS

### Characteristics of the Patients

The five patients ranged in age from 38 to 56 years. Four had been infected with HIV-1 for at least 10 years before the study began, and one had been infected for 2 years. The study began in February 1996 and continued until December 1998. Table 1 shows the clinical characteristics of the patients. All five patients had been receiving a potent triple-drug antiretroviral regimen that included two of four nucleoside analogues (zidovudine, stavudine, didanosine, and lamivudine) — or a non-nucleoside reverse-transcriptase inhibitor (nevirapine) in the case of Patient 2 — and a protease inhibitor (ritonavir, indinavir, or saquinavir) for 20 to 31 months (Table 1). Patient 1 had received no prior therapy. Patient 3 had received zidovudine and lamivudine, Patient 4 had received stavudine and didanosine, and Patient 5 had received zidovudine before the institution of potent antiretroviral therapy. Patient 2 had received zidovudine in the past but not immediately before beginning combination antiretroviral-drug therapy.

### Plasma HIV-1 RNA Levels and CD4+ T-Cell Counts

The mean level of plasma HIV-1 RNA before the initiation of potent antiretroviral therapy was 64,237 copies per milliliter (range, 11,720 to 54,973). After treatment, it decreased by an average ( $\pm$ SD) of more than  $2.7 \pm 0.3$  log to less than 50 copies per milliliter. The average half-life of HIV-1 RNA in plasma was  $30 \pm 18.2$  days before the levels became undetectable. Infrequent sampling of blood precluded us from measuring the length of the previously reported rapid first-phase decline in plasma HIV-1 RNA.<sup>5</sup> Nonetheless, a half-life of 30 days is in general agreement with the half-life reported for the second

**TABLE 1.** CHARACTERISTICS OF THE FIVE PATIENTS.

DRUG REGIMEN	TIME OF MEASUREMENT*	CD4+ T CELLS cells/mm <sup>3</sup>	PLASMA HIV-1 RNA† copies/ml
	mo		
<b>Patient 1</b>			
None	-2	415	11,720
Zidovudine, lamivudine, indinavir	5	493	<50
Zidovudine, lamivudine, indinavir	10	778	<50
Zidovudine, lamivudine, indinavir	16	797	<50
Zidovudine, lamivudine, indinavir	22	724	<50
<b>Patient 2</b>			
None	-1	247	24,321
Lamivudine, stavudine, nevirapine	5	158	<50
Lamivudine, stavudine, nevirapine	11	301	<50
Lamivudine, stavudine, nevirapine	17	520	<50
Lamivudine, stavudine, nevirapine	23	337	<50
<b>Patient 3</b>			
Lamivudine, zidovudine	-1	12	45,000
Lamivudine, zidovudine, ritonavir	5	131	541
Lamivudine, zidovudine, ritonavir	10	204	<50
Lamivudine, zidovudine, ritonavir	17	305	<50
Lamivudine, zidovudine, ritonavir	21	306	<50
Lamivudine, zidovudine, saquinavir	27	414	<50
Lamivudine, zidovudine, saquinavir	31	274	<50
<b>Patient 4</b>			
Didanosine, stavudine	-1	230	54,973
Lamivudine, stavudine, indinavir	5	141	<50
Lamivudine, stavudine, indinavir	11	262	<50
Lamivudine, stavudine, indinavir	17	352	<50
Lamivudine, stavudine, indinavir	23	493	<50
<b>Patient 5</b>			
Zidovudine	-5	449	22,720
Zidovudine, lamivudine, indinavir	1	601	<50
Zidovudine, lamivudine, indinavir	7	398	<50
Zidovudine, lamivudine, indinavir	13	517	<50
Zidovudine, lamivudine, indinavir	20	558	<50

\*The times indicate the duration of potent antiretroviral treatment. Negative numbers indicate measurements made before the start of this therapy.

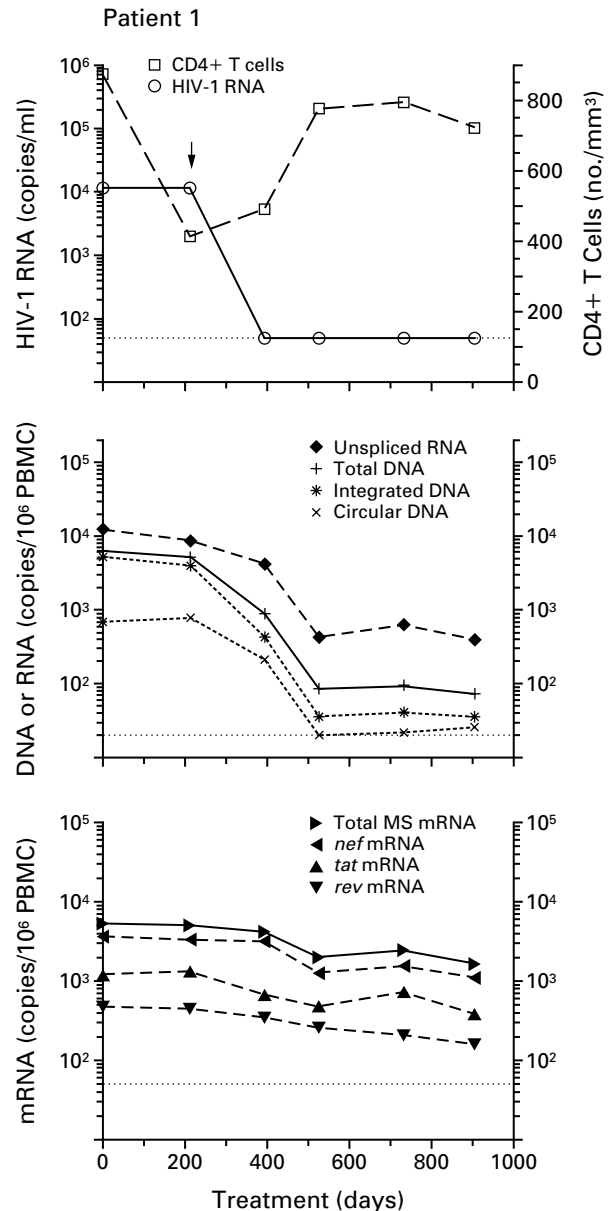
†Although the formal limit of detection is 50 copies per milliliter for the RT-PCR that we used, values ranging from 0 to 20 copies per milliliter were measured.

phase of the decline.<sup>5,21</sup> CD4+ T-cell counts ranged from 12 to 449 cells per cubic millimeter of blood before treatment and slowly rose with the continued suppression of plasma HIV-1 RNA (final range, 274 to 724 cells per cubic millimeter) (Table 1).

**Temporal Changes in Cell-Associated HIV-1 DNA and Viral mRNA**

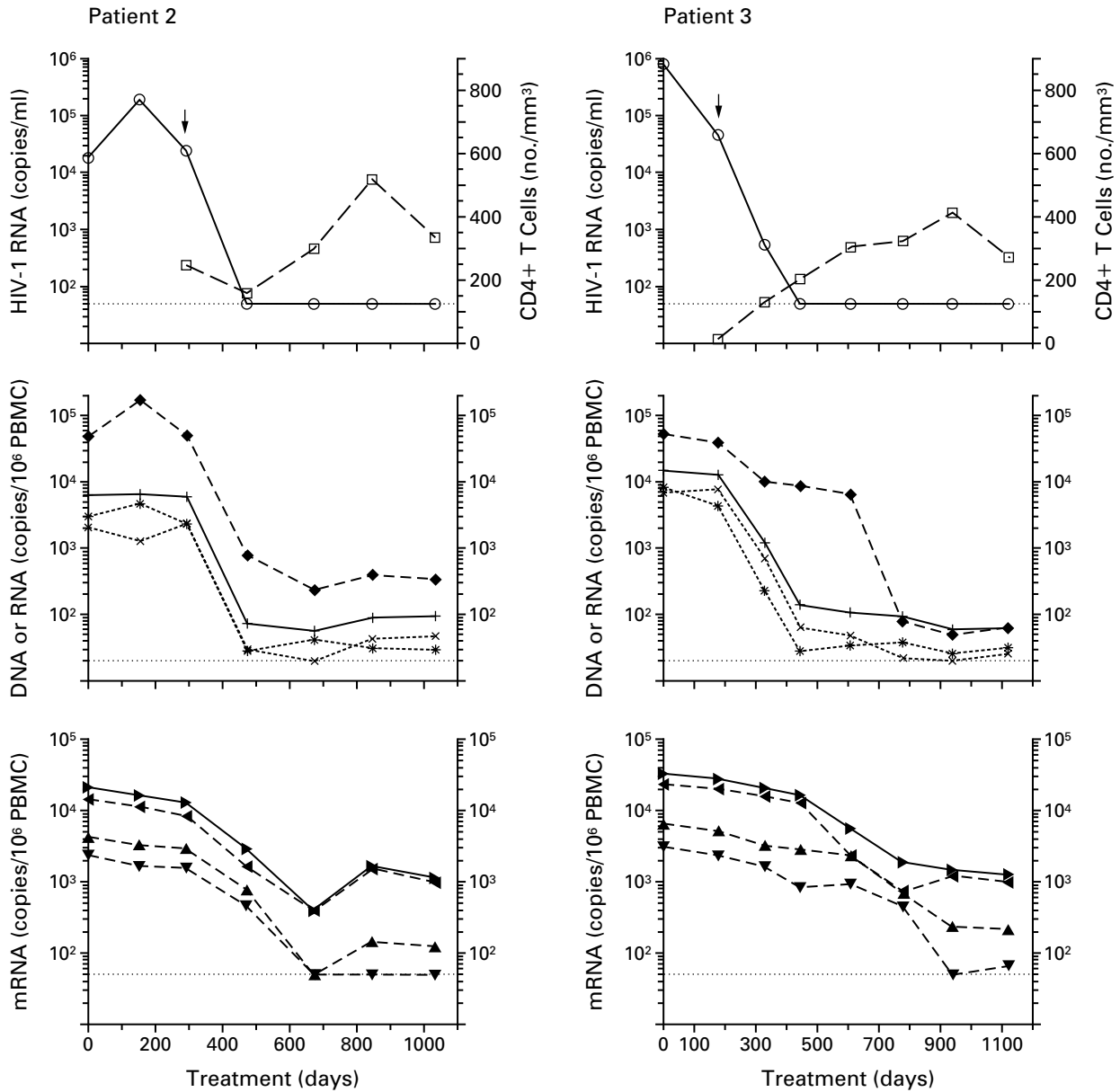
Figure 1 shows the temporal changes in the mean levels of HIV-1 RNA in plasma, CD4+ T-cell counts, and the concentrations of cell-associated viral DNA and HIV-1 mRNA in Patients 1, 2, and 3. These data are representative of the results for all the patients.

Once treatment was begun, there was a slow, two-phase decrease in the levels of integrated proviral DNA, unintegrated proviral DNA, and unspliced viral mRNA that differed in timing and extent from the previously described pattern of decrease in plasma



**Figure 1.** Effect of Treatment on the Levels of Viral DNA and mRNA in Peripheral-Blood Mononuclear Cells from Patients 1, 2, and 3.

For each patient, the top panel shows the CD4+ T-cell count and plasma level of HIV-1 RNA; the middle panel shows the levels of unspliced mRNA, total viral DNA, integrated proviral DNA, and circular forms of unintegrated viral DNA; and the bottom panel shows the total levels of multiply spliced (MS) viral mRNA, as well as the levels of *nef* mRNA, *tat* mRNA, and *rev* mRNA. The arrows indicate the first time points after the start of treatment with potent antiretroviral therapy at which data were recorded. The horizontal dotted lines indicate the limits of detection of the assays. PBMC denotes peripheral-blood mononuclear cells.



HIV-1 RNA.<sup>5</sup> The first phase occurred during the initial 500 days of treatment and was characterized by substantial decreases in cell-associated HIV-1 DNA and unspliced viral mRNA. In contrast, there was little change during the second phase: values remained at a quasi-steady state for an additional 300 days or more (Fig. 1).

**Detection of Cell-Associated Integrated Proviral DNA**

As shown in Table 2, before the initiation of potent antiretroviral treatment, the mean level of integrated proviral DNA was 2687 copies per 10<sup>6</sup> peripheral-blood mononuclear cells (range, 542 to 4274). After the initiation of treatment, the concen-

tration of integrated proviral DNA dropped by 1.46±0.65 log during the first phase, reflecting the loss of a population of cells presumably containing both short-lived cells infected with actively replicating virus and long-lived infected cells. The half-life of the integrated proviral DNA ranged from 29 to 108 days (mean, 53) (Table 2). The phase 1 decrease in proviral DNA was followed by stable levels in phase 2, which ranged from 28 to 49 copies per 10<sup>6</sup> peripheral-blood mononuclear cells.

**Detection of Cell-Associated Unintegrated Viral DNA**

Before treatment, the levels of cell-associated unintegrated viral DNA correlated with the plasma lev-

**TABLE 2.** BASE-LINE AND FIRST-PHASE VALUES AND HALF-LIVES OF HIV-1 DNA AND mRNA IN PERIPHERAL-BLOOD MONONUCLEAR CELLS.\*

PATIENT NO.	INTEGRATED PROVIRAL DNA			UNINTEGRATED VIRAL DNA			UNSPICED VIRAL mRNA			TOTAL MULTIPLY SPLICED VIRAL mRNA		
	BASE LINE	FIRST PHASE		BASE LINE	FIRST PHASE		BASE LINE	FIRST PHASE		BASE LINE	FIRST PHASE	
		<i>final value</i>	<i>half-life</i>		<i>final value</i>	<i>half-life</i>		<i>final value</i>	<i>half-life</i>		<i>final value</i>	<i>half-life</i>
	copies/10 <sup>6</sup> PBMC	days		copies/10 <sup>6</sup> PBMC	days		copies/10 <sup>6</sup> PBMC	days		copies/10 <sup>6</sup> PBMC	days	
1	4017	36	47	784	20	61	8,628	435	75	5,110	2516	447
2	2240	28	29	2360	30	29	50,342	235	50	12,868	395	236
3	4274	28	37	7624	63	39	38,671	<50	75	27,723	1489	183
4	2362	49	108	7324	30	76	65,231	425	100	41,452	2574	195
5	542	32	44	1672	22	28	10,724	<50	23	13,258	674	275
Mean	2687	35	53	3953	33	47	34,719	239	65	20,082	1530	267
±SD	±1516	±8.7	±31.5	±3264	±17.3	±21.1	±24,734	±190	±29.2	±14,473	±1011	±106.8

\*PBMC denotes peripheral-blood mononuclear cells.

els of HIV-1 RNA ( $r=0.98$ ,  $P<0.002$ ) and with the levels of cell-associated unspliced mRNA ( $r=0.83$ ,  $P<0.08$ ). During the first phase of the decrease, the concentration of unintegrated circular forms of viral DNA dropped by  $1.46\pm 0.68$  log (Fig. 1). The numbers of copies of unintegrated and integrated forms of viral DNA were correlated in each patient over time ( $r>0.97$ ,  $P<0.01$ ), suggesting that these forms decrease at similar rates. Indeed, during the first phase, the half-life of unintegrated forms of viral DNA ranged from 28 to 76 days (mean, 47), similar to that for integrated proviral DNA (Table 2).

#### Detection of Cell-Associated Unspliced HIV-1 mRNA

The levels of cell-associated unspliced HIV-1 mRNA correlated with the plasma levels of HIV-1 RNA at base line ( $r=0.89$ ,  $P<0.009$ ). During phase 1, the concentration of unspliced HIV-1 mRNA decreased by an average of  $1.25\pm 0.90$  log, with a half-life ranging from 23 to 100 days (mean, 65) (Table 2). Subsequently, the concentration stabilized in the absence of detectable levels of HIV-1 RNA in plasma. The levels of cell-associated unintegrated forms of viral DNA and unspliced viral mRNA were highly correlated in each patient, with correlation coefficients ranging from 0.76 to 0.98 ( $P<0.08$ ).

#### Detection of Cell-Associated Multiply Spliced HIV-1 mRNA

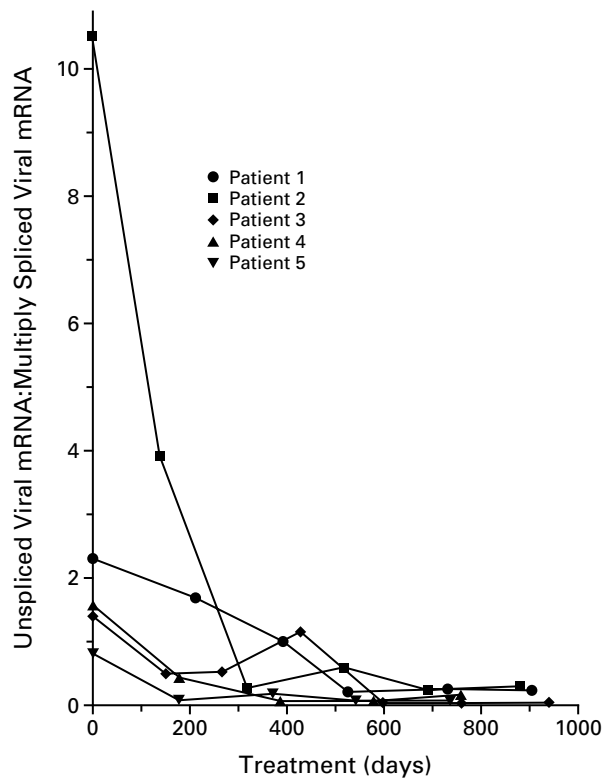
The rates of decline among individual cell-associated multiply spliced viral mRNA species were similar. As a measure of the accuracy of the measurements for *tat*, *rev*, and *nef* mRNA species, we calculated sample correlation coefficients for each measurement in individual patients over time and found correlation coefficients ranging from 0.77 to 0.99 ( $P<0.07$ ). Unlike the large reduction in unspliced HIV-1 mRNA that occurred after the initiation of treatment, there were smaller decreases in the *tat*, *rev*, and *nef* mRNA

species over time (declines of  $0.53\pm 0.37$ ,  $0.56\pm 0.58$ , and  $0.66\pm 0.35$  log, respectively) (Fig. 1). The levels of multiply spliced and unspliced HIV-1 mRNA species were correlated in individual patients over time, with correlation coefficients ranging from 0.90 to 0.98 ( $P<0.04$ ). During phase 1, the half-life of multiply spliced HIV-1 mRNA ranged from 183 to 447 days (mean, 267) (Table 2).

We used the ratio of unspliced viral mRNA to multiply spliced viral mRNA as a measure of active viral transcription and as an indicator of the proportion of cells that contained high levels of full-length viral RNA. The higher the ratio, the greater the proportion of cells that are producing unspliced HIV-1 RNA, which is indicative of replicating virus. The decline in the ratio resembled the decline in integrated forms of proviral DNA. Throughout the second phase, these ratios remained stable and greater than zero at each point (Fig. 2), despite accompanying decreases in the levels of both unspliced and multiply spliced viral mRNA species. The ratios before treatment and 20 months or more after the suppression of plasma HIV-1 RNA to undetectable levels were significantly different ( $P<0.01$ ).

#### Identification of Mutations That Confer Drug Resistance

At base line, we found a single mutation (R211K) coding for resistance to zidovudine in the *pol* gene<sup>19</sup> of the cell-associated viral DNA in Patient 3, who had previously received zidovudine monotherapy. After 31 months of therapy, three additional mutations coding for resistance to zidovudine (M41L, D67N, and L210W)<sup>18</sup> were found in the *pol* gene in Patient 3. No mutations coding for drug resistance were detected in the cell-associated viral DNA from the other patients, indicating that treatment suppressed both discernible viral replication in plasma and selection for drug-resistant variants.



**Figure 2.** Changes in the Ratio of Cell-Associated Unspliced Viral mRNA to Multiply Spliced Viral mRNA in the Five Patients.

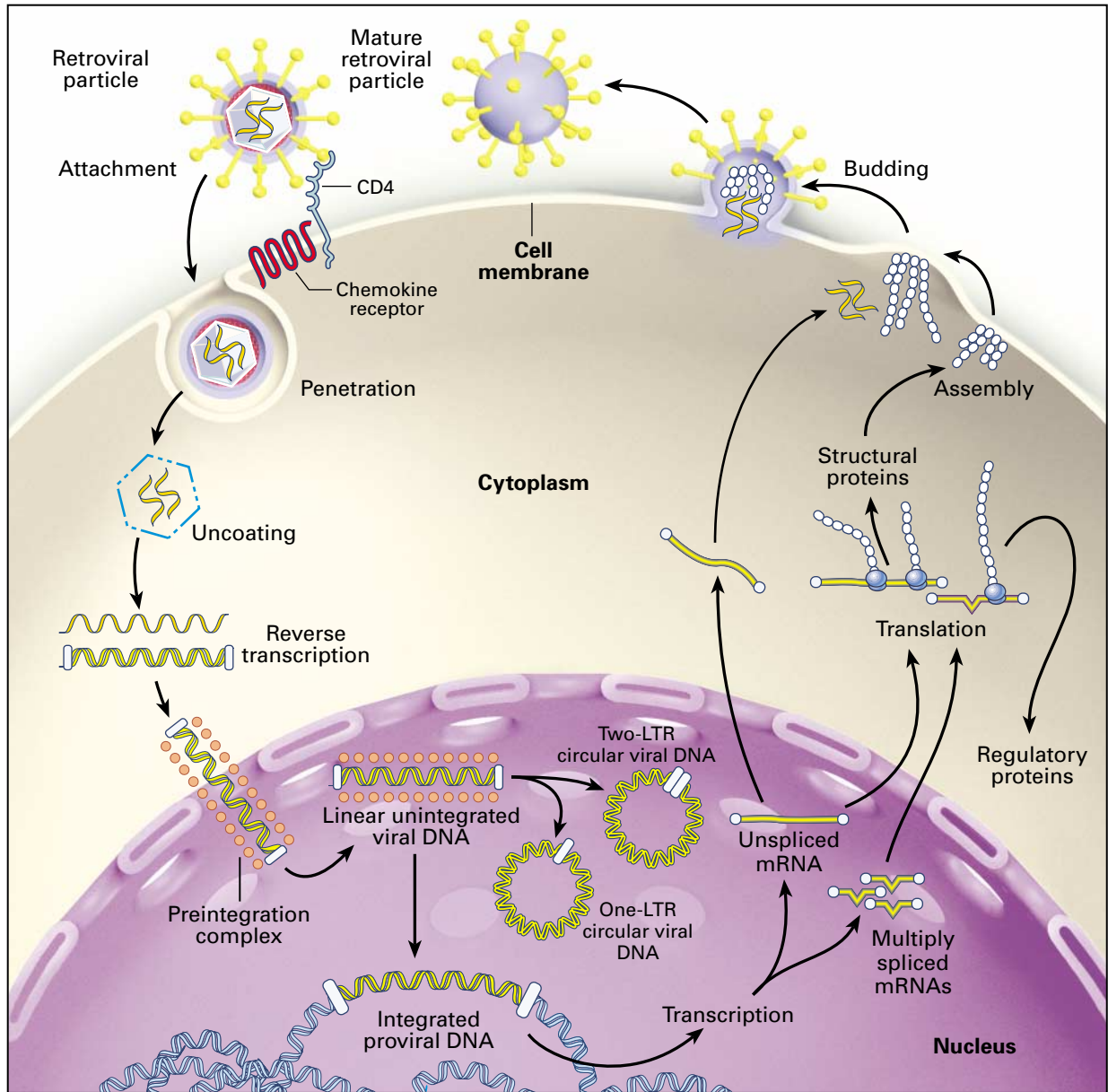
## DISCUSSION

In patients infected with HIV-1, compartments of replication-competent virus persist despite treatment with potent antiretroviral drugs that reduce plasma HIV-1 RNA to undetectable levels.<sup>9-11,16</sup> To investigate the characteristics of this viral reservoir, we studied the essential steps in the replication of HIV-1 (Fig. 3). The life cycle begins with the reverse transcription of genomic viral RNA into linear unintegrated HIV-1 DNA.<sup>22</sup> Once the viral DNA has been synthesized and incorporated into a preintegration complex, it enters the nucleus.<sup>22-24</sup> This is followed by the integration of the linear forms of viral DNA into the host-cell genome<sup>3,23,24</sup> and expression of the virus.<sup>15</sup> There are also circular forms of viral DNA in the nucleus that are byproducts of the integration process. We used quantitative PCR assays to measure cell-associated unintegrated circular forms of viral DNA, as a surrogate marker of nuclear entry, integrated proviral DNA, and viral mRNA levels. All five patients whom we studied were receiving potent antiretroviral therapy and had had undetectable levels of HIV-1 RNA in plasma for 20 months or more. They also all had evidence of viral replication in their peripheral-blood mononuclear cells, suggesting the persistent presence of reservoirs of HIV-1.

We assessed the viral reservoir by measuring changes in the concentration of cell-associated HIV-1 DNA and viral mRNA over time and found that the levels of virus decreased slowly in two phases, which differed from the previously described pattern of decrease for plasma HIV-1 RNA.<sup>5</sup> During the first phase, the concentrations of unintegrated viral DNA, integrated proviral DNA, and unspliced viral mRNA decreased by approximately 1.5 log, consistent with the hypothesized decreases in short-lived cells infected with actively replicating virus and long-lived infected cells.<sup>5</sup> The half-life of integrated proviral DNA during the first phase averaged 53 days and ranged from 29 to 108 days, a range that was similar to those for unintegrated circular forms of viral DNA and unspliced viral mRNA. These estimates represent minimal values because the cell populations would be renewed by ongoing viral replication during the first phase. The values at the upper ends of the ranges, however, are similar to the estimated life span of resting memory CD4+ T cells in people without HIV-1 infection.<sup>25</sup> We did not identify the specific phenotypes of the cells that contained HIV-1 DNA and viral mRNA. Nonetheless, on the basis of previous work,<sup>13</sup> our findings reflect the decrease in two groups of HIV-1-infected peripheral-blood mononuclear cells: short-lived cells infected with actively replicating virus, which have a half-life of one to two days,<sup>13</sup> followed by long-lived infected cells.

After the initial decrease during the first 500 days of treatment, the levels of cell-associated HIV-1 DNA and unspliced viral mRNA reached a plateau, characterizing the second phase. Persistently infected resting memory CD4+ T cells with integrated proviral DNA and labile, unintegrated linear viral DNA represent stable<sup>10</sup> and inducible<sup>23,24</sup> viral reservoirs, respectively, that can contribute to the maintenance of the plateau. Occasional stimulation by antigens may provide an opportunity for persistently infected cells harboring replication-competent HIV-1 DNA to produce progeny virus transiently and then either die as a direct or indirect result of viral replication or become quiescent again, thus replenishing the pool of HIV-1-infected CD4+ T cells. Infectious virus produced in cells or areas of the body where medications do not reach may also have a role in sustaining this pool.<sup>26,27</sup> Both possibilities are consistent with the absence in our patients of mutations coding for drug resistance other than those that arose during previous monotherapy that failed to suppress viral replication completely.

In transformed cell lines harboring integrated proviral DNA that have been used as a model of persistent HIV-1 infection, there are low levels of incomplete viral replication that result from a block early in the life cycle of the virus.<sup>28-32</sup> Such persistently infected CD4+ T cells, which contain integrated proviral DNA that is transcriptionally active but translation-



**Figure 3.** The Essential Steps in the Life Cycle of HIV-1.

The first step is the attachment of the virus particle to receptors on the cell surface. The HIV-1 RNA genome then enters the cytoplasm as part of a nucleoprotein complex. The viral RNA genome is reverse-transcribed into a collinear DNA duplex, which has terminal duplications known as long terminal repeats (LTRs) that are not present in HIV-1 RNA. Once the viral DNA has been synthesized, the linear viral DNA molecule is incorporated into a preintegration complex that enters the nucleus. In the nucleus, unintegrated viral DNA is found in both linear and circular forms. The unintegrated circular forms of viral DNA have either one or two long terminal repeats, are byproducts of the integration process, and are found exclusively in the nucleus. The linear unintegrated viral DNA is the precursor of integrated proviral DNA, which is a stable structure that remains indefinitely in the host-cell genome and serves as a template for viral transcription. Transcription of the proviral DNA template and alternative mRNA splicing creates spliced viral mRNA species encoding the viral accessory proteins, including Tat, Rev, and Nef, and the unspliced viral mRNA encoding the viral structural proteins, including the gag-pol precursor protein. A shift in the transcriptional pattern from the expression of predominantly multiply spliced viral mRNA to predominantly unspliced viral mRNA is indicative of active viral replication. All the viral transcripts are exported into the cytoplasm, where translation and assembly and processing of the retroviral particle take place. The cycle is completed by the release of infectious retroviral particles from the cell.

ally nonproductive, may also contribute to the low, stable ratio of unspliced HIV-1 mRNA to multiply spliced HIV-1 mRNA and the stable levels of cell-associated integrated proviral DNA we found during the second phase of the viral decrease. Although our findings cannot be used to establish a half-life for the unintegrated forms of viral DNA *in vivo*, they are consistent with two hypotheses: that the unintegrated forms of viral DNA have a rather short half-life and are regenerated by constant reinfection, or that they have a long half-life and persist as long as does integrated proviral DNA in long-lived infected cells.

Mathematical modeling of HIV dynamics has suggested that at least two to three years of a completely inhibitory antiretroviral regimen would be required to eliminate virus from long-lived infected CD4+ T cells.<sup>5,21</sup> This estimate was not entirely accurate because it was based on data that did not adequately reveal the persistence of a population of cells carrying replication-competent integrated forms of proviral DNA.<sup>10,11,16</sup> This model also did not address the effects of less-than-complete suppression of viral replication. Both these factors are important in attempts to determine the duration of therapy required to eradicate the virus.<sup>33</sup>

Our data suggest that even after two or more years of complete suppression of plasma levels of HIV-1 RNA, viral transcription continues and the decrease in the levels of integrated forms of proviral DNA plateaus. Thus, the long-lived populations of persistently infected cells may not be decreasing at a simple exponential rate; this finding suggests that there are not yet sufficient data to allow estimates of the duration of therapy required to eradicate the infection. Interestingly, our finding of a quasi-steady state in the levels of the virus indicates that the rate of replenishment of these cells is approximately equal to the natural depletion rate or that the rate is so slow that it is unnoticeable. Unless this quasi-steady state eventually disappears with longer periods of therapy or can be overcome by the use of more potent therapies or alternative approaches that block the potential spread of virus within tissues, HIV-1 may never be eradicated.

In summary, persistently infected peripheral-blood mononuclear cells with integrated proviral DNA can be found in some patients who are receiving combinations of drugs that inhibit viral reverse transcriptase and protease and in whom plasma levels of HIV-1 RNA have been undetectable for 20 months or more. Our results suggest that this reservoir represents a serious impediment to the long-term goal of the eradication of HIV-1.

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