JOURNAL OF VIROLOGY, Apr. 1995, p. 2092–2100 Vol. 69, No. 4
0022-538X/95/$04.00+0

Changes in the Viral mRNA Expression Pattern Correlate with a Rapid Rate of CD4$^+$ T-Cell Number Decline in Human Immunodeficiency Virus Type 1-Infected Individuals

MANOHAR R. FURTADO,[1] LAWRENCE A. KINGSLEY,[2] AND STEVEN M. WOLINSKY[3]*

Departments of Pathology[1] and Medicine,[3] Northwestern University Medical School, Chicago, Illinois 60611, and Department of Infectious Diseases and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261[2]

Received 30 August 1994/Accepted 21 December 1994

The rate of disease progression varies considerably among human immunodeficiency virus type 1 (HIV-1)-infected individuals. Several cross-sectional studies have shown an association between the stage of HIV-1 disease and the viral burden or the relative levels of viral gene expression. To study the extent of HIV-1 transcription and replication and its correlations with disease progression, we quantified serial, longitudinal samples of blood cells from 10 HIV-1-infected individuals with markedly different rates of CD4$^+$ T-cell number decline following seroconversion. After normalization for the input nucleic acid content, multiply spliced viral mRNA and unspliced viral RNA were quantified by competitive reverse transcription-PCR using oligonucleotide primers which flank the major *tat*/*rev*/*nef* splice junction and span an internal region of the *gag* open reading frame, respectively. Coamplification of internal cRNA template controls was used to normalize for variation in the efficiency of reverse transcription and in vitro enzymatic amplification. Similarly, proviral DNA was also quantified by competitive PCR performed within the linear range of amplification. Viral RNA was detected in the blood cells of each individual from all time points regardless of the rate of CD4$^+$ T-cell decline. Unspliced genomic viral RNA rapidly increased in the blood cells from HIV-1-infected individuals who had a precipitously declining CD4$^+$ T-cell number. In contrast, both unspliced and multiply spliced viral mRNAs remained relatively stable in the blood cells from HIV-1-infected individuals who have had a relatively benign clinical course. These data demonstrate that the extent of viral transcription and replication correlates with the rate of CD4$^+$ T-cell number decline and that quantifying intracellular viral RNA is of potential prognostic value.

Infection with human immunodeficiency virus type 1 (HIV-1) usually leads to a defined pattern of disease progression. After the primary infection, large numbers of infected CD4$^+$ T cells and a large amount of plasma-free virus are found in blood (9, 12, 14). The amount of circulating virus then rapidly declines as the humoral and then cellular immune surveillance mechanisms are engaged (29, 30). These early events are followed by a variable period of clinical latency that can last for several years. This latter phase of infection is associated with persistent viral replication in blood and tissue (15, 18, 41, 50), aberrations in T-cell function (40), and either a stable, slowly decreasing or rapidly declining CD4$^+$ T-cell number (11, 31, 38). The onset of disease coincides with the profound decrease in the numbers of CD4$^+$ T cells and an increase in the cell-associated and plasma-free viral burden (24–26). High levels of proviral DNA, plasma-free viral RNA, and viral mRNA in blood have been shown by a number of investigators in cross-sectional studies to correlate with the disease state (2, 3, 10, 23, 37, 42, 46, 52). In a nested case control study of HIV-1-infected men with different rates of disease progression, a rapidly declining CD4$^+$ T-cell number was associated with a high plasma-free viral burden, low levels of anti-p24 and CD8$^+$ T-cell subsets, and increased activation markers (22). A recent longitudinal study also suggested that a high level of intracellular unspliced RNA was predictive of rapid CD4$^+$ T-cell number decline and that a lack of viral mRNA was associated with clinical nonprogression (48).

* Corresponding author. Mailing address: Department of Medicine, Northwestern University Medical School, 710 N. Fairbanks, Chicago, Illinois 60611. Phone: (312) 908-5210. Fax: (312) 908-4588.

The reasons for the high degree of variability in the rates of disease progression are not clearly understood but most likely depend on parameters that modify the kinetics of viral replication. In the persistently infected U1 and ACH-2 cell lines, the transcriptional pattern demonstrates a preponderance of multiply spliced (1.8- to 2.0-kb species encoding *tat*, *rev*, and *nef*) and intermediate spliced (4.3-kb species encoding *vif*, *vpr*, *vpu*, and *env*) mRNAs and a relative lack of unspliced (9.3-kb species encoding *gag-pol*) mRNAs. Following induction of these cell lines by mitogen or phorbol ester stimulation, there is a change from the constitutive expression of multiply spliced mRNAs to the predominant expression of unspliced viral RNA (6, 36, 45). Additionally, there is an upregulation of total viral RNA transcription. These findings are consistent with a post-transcriptional regulatory mechanism for restricting productive viral infection. In contrast, the persistently infected OM-10.1 and J1.1 established cell lines express low levels of both multiply spliced and unspliced viral mRNAs in a minority of the infected cells (7). After induction by tumor necrosis factor alpha, productive infection can be achieved in the vast majority of cells. These results are more consistent with a host cell-specific mechanism for transcriptional regulation. Therefore, both host cell-specific transcriptional and posttranscriptional mechanisms may be responsible for the latent or transcriptionally active but nonproductive state (4, 5, 7, 12, 16, 37, 44, 52–56). Regardless of the mechanism by which the virus is maintained in a latent state, after the induction of active viral replication, the rate of virus production becomes a function of the numbers of productively infected cells. These data indicate that the temporal kinetics of viral transcription and the regulation of viral gene expression are critical parameters that may

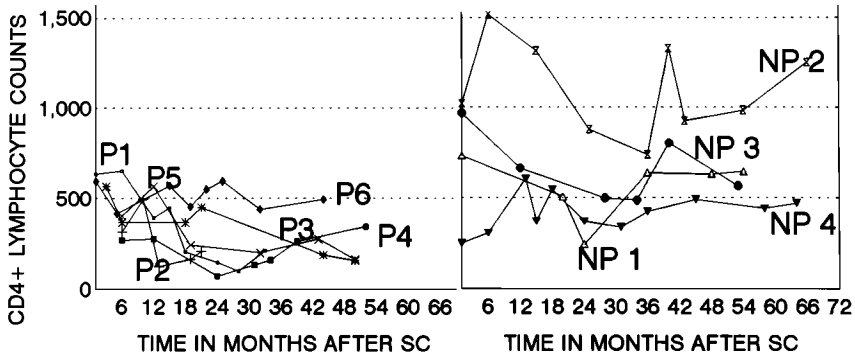

FIG. 1. Temporal changes in the $CD4^+$ T-cell counts after seroconversion (SC) in the progressor (P1 to P6) and nonprogressor (NP1 to NP4) study groups.

be intimately associated with viral pathogenesis and disease progression.

To determine the temporal dynamics of HIV-1 replication in vivo and its correlation with disease progression, we quantified viral RNA in serial longitudinal samples of blood cells from HIV-1-infected men with different rates of $CD4^+$ T-cell number decline after seroconversion. Multiply spliced viral mRNA and unspliced viral RNA were quantified by competitive reverse transcription-PCR (RT-PCR) using coamplification of a cRNA template as an internal control after normalizing for the input nucleic acid content. Proviral DNA was also quantified by PCR performed within the linear range of amplification. As a measure of viral replication in blood, both multiply spliced and unspliced viral RNAs were detected in all clinical samples regardless of the rate of $CD4^+$ T-cell number decline after seroconversion. A high proportion of unspliced mRNA predominated in the cells from HIV-1-infected men who had a precipitously declining $CD4^+$ T-cell number. These data indicate that the extent of viral transcription and replication correlates with the rate of $CD4^+$ T-cell number decline. Additionally, these data suggest a potential prognostic value for quantifying intracellular viral RNA.

MATERIALS AND METHODS

Study subjects. Ten participants with demonstrated HIV-1 infection, from the Chicago component of the Multicenter AIDS Cohort Study, were selected for this specific investigation. Three participants became infected between semiannual visits 1 and 2, two became infected between visits 2 and 3, and five became infected between visits 4 and 5. The time of infection was arbitrarily set at the midpoint between the last visit at which the participant was HIV-1 antibody negative and first visit at which the participant was HIV-1 antibody positive. At each visit, clinical data from an interval history and physical examination laboratory data from appropriate laboratory tests were obtained (33). Serial blood samples were separated into plasma and peripheral blood mononuclear cell (PBMC) fractions by Ficoll-Hypaque discontinuous density gradient centrifugation, aliquoted, and cryopreserved in liquid nitrogen for future analysis. Follow-up after HIV-1 infection varied between 33 and 100 months, with a mean of 84.5 months, for the four participants (nonprogressor 1 [NP1] to NP4) who did not develop clinical AIDS or have a decrease in the $CD4^+$ T-cell count to 200/μl or below and 52.2 months for the six who progressed (progressor 1 [P1] to P6) to clinical AIDS or had a decrease in $CD4^+$ T-cell count to less than 200/μl (Fig. 1). Two of the six progressors died, one of lymphoma and one of liver disease. A third participant who self-reported *Pneumocystitis carinii* pneumonia dropped out of the study. The remaining three progressors developed a $CD4^+$ T-cell count less than 200/μl but no AIDS-defining condition. Six of the ten participants, four of the progressors and two of the nonprogressors who had a relatively low but stable $CD4^+$ T-cell count that was below the median (33), received azido thymidine. The mean decreases in the $CD4^+$ T-cell count were 56/μl in the nonprogressor group ($n = 4$) and 280/μl in the progressor group ($n = 6$). These values were calculated by subtracting the mean of the $CD4^+$ T-cell counts obtained at the last three available visits from the mean of the $CD4^+$ T-cell counts at the first three visits at which the subjects were HIV-1 antibody positive.

Cells. Serial samples of cryopreserved PBMCs stored in 10% dimethyl sulfoxide–10% fetal calf serum were available for nucleic acid analysis. The 8E5/LAV established cell line (obtained from T. Folks through the AIDS Research and Reagent Program, Division of AIDS, National Institutes of Health, Bethesda, Md.), containing a single copy of integrated proviral DNA per cell, was used as the HIV-1-infected cell copy number control. Low-passage-number 8E5/LAV cells were maintained in RPMI 1640 (Gibco) containing 20% fetal bovine serum (Gibco), 2 mM L-glutamine, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) in a humidified incubator with a 5% CO_2 atmosphere. Cell copy number standards were generated by serially diluting 8E5 cells (5 to 5×10^3 cells) into uninfected H9 cells (obtained through the AIDS Research and Reagent Program, Division of AIDS, National Institutes of Health) at a final concentration of 1.5×10^5 cells per ml (21).

DNA and RNA isolation. Total cellular DNA was prepared by suspending 2×10^6 cells in 600 μl of lysis buffer (50 mM Tris-HCl [pH 8.3], 100 mM KCl, 0.1% Tween 20, and 0.1% Nonidet P-40) and then treating them with proteinase K at a final concentration of 20 μg/ml for 60 min at 37°C. The proteinase K was denatured by heating at 95°C for 10 min before the lysates were used for in vitro enzymatic amplification (20). Total RNA was prepared from 2×10^6 HIV-1-infected cells by the guanidinium isothiocyanate extraction procedure (8). The RNA preparations were treated with RQ1 DNase 1 (Promega) (49), and the DNA-free RNA preparations were used for quantitation.

Oligonucleotides. Oligonucleotides were synthesized on a model 380B DNA synthesizer (Applied Biosystems) by using phosphoramidite chemistry and purified of organic contaminants by elution through a Sephadex G-25 (Sigma) spun column. The positions of the primers and probes correspond to the nucleotide numbering system for the HIV-1 IIIB/BRU DNA sequencing alignments (39). Degenerate positions are designated as follows: Y = C or T, R = A or G, W = A or T, S = G or C, and K = G or T. The nucleotide sequences of the oligonucleotides and any additional sequences incorporated for a 5′ restriction cleavage site (lowercase letters) are as follows: MF687, 5′-GCtctagaCGACG CAGGACTCGGCTTGCT-3′ (nucleotides 87 to 707) with a 5′ *Xba*I site; MF1003, 5′-GCGctcgagCCTGTCTGAAGGGATGGTTGTAGCT-3′ (nucleotides 1003 to 978) with a *Xho*I site; MF818, 5′-CGGGGGAGAATTAGATC GATGGGA-3′ (nucleotides 818 to 841); MF1003D, 5′-GCGctcgagCCTGTCTG AAGGGATGGTTGAGTTCCCTGCTTGCCCAAACTATATGT-3′ (nucleotides 1003 to 978 joined to nucleotides 910 to 887) with an *Xho*I site; MF8760, 5′-GCGctcgagTATCTGTCCCCTCAGCTACTGCTAT-3′ (nucleotides 8760 to 8735) with a 5′ *Xho*I site; MF6056, 5′-TGCtctagaCTCATCAAGYTTCTC TAT-3′ (nucleotides 6056 to 6076) with an *Xba*I site; MF8760D, 5′-GCGctcgagT ATCTGTCCCCTCAGCTACTGCTATTCCCACCCCCTGCGTC-3′ (nucleotides 8760 to 8735 joined to nucleotides 8650 to 8635) with an *Xho*I site; MF8629, 5′-TCTGGGACGCAGGGGGTGGGAAG (nucleotides 8629 to 8651); and MF101D, 5′-GCGctcgagCTATGTCAGTTCCCCCTTGGTTCTGCAG-3′ (nucleotides 1501 to 1477 joined to nucleotides 1431 to 1398).

Plasmid constructions. To control for the efficiency of reverse transcription and in vitro enzymatic amplification, a molecularly engineered cRNA molecule, generated from a deletion-containing plasmid, was used as an exogenously added internal control (21). Deletions were generated by PCR with deletion-generating oligonucleotides within regions flanked by primer pairs MF687-MF1003, MF6056-MF8760, and SK145-SK101. To generate the MF687-MF1003 cRNA construct, plasmid pNL4-3 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health) was amplified by using oligonucleotide MF687 and a deletion-generating oligonucleotide, MF1003D, that contained 5′ sequences from MF1003 contiguous with 3′ sequences corresponding to a region 70 nucleotides upstream of MF1003. The amplification generated a DNA fragment flanked by sequences corresponding to MF687 and MF1003 but containing a 70-nucleotide deletion between nucleotides 980 and 910. Therefore, the engineered target sequence has the same primer and probe binding sequences but a deletion within the intervening sequences. Similarly, DNA from a *tat* cDNA clone (21) was amplified with primer MF6056 and a deletion-generating oligonucleotide, MF8760D, to produce an 85-nucleotide deletion between nucleotides 8735 and 8650. In both instances, the product

DNAs were inserted into pBluescript vectors (Stratagene) by using the *Xba*I site in the upstream primers and the *Xho*I site in the downstream primers. The corresponding full-length fragments were also amplified and inserted into pBluescript vectors to produce full-length cRNA. A 45-bp deletion within the region flanked by SK145-SK101 was also produced by using the deletion-generating oligonucleotide MF101D and used as an internal control during PCR DNA amplification.

In vitro transcription. The deletion plasmid constructs were linearized with *Xho*I and purified with Gene Clean II (Bio 101). A 1.5-μl aliquot of the linearized plasmid DNA was transcribed by T7 RNA polymerase by using a Riboprobe transcription kit (Promega) according to the manufacturer's recommendations. The reaction mixture was resolved by electrophoresis on a 5% polyacrylamide gel. The major cRNA transcript was distinguished by size after ethidium bromide staining, isolated, and purified from the gel matrix by procedures described previously (21, 49). Serial dilutions of the cRNA were used along with known amounts of DNA standards in a dot blot membrane hybridization assay to accurately determine the RNA copy number.

Enzymatic amplification and quantitation of proviral DNA. Lysate from approximately 1.5×10^5 cells was used for enzymatic amplification of DNA by PCR (95°C for 20 s, 55°C for 50 s, and 72°C for 60 s) in buffers (50 mM KCl, 20 mM Tris-HCl [pH 8.4], 2.5 mM $MgCl_2$, 100 μg of nuclease-free bovine serum albumin per ml, 200 μM each dATP, dCTP, dGTP, and dTTP) containing 20 pmol each of sense and antisense primers and 2 U of *Taq* polymerase (Perkin-Elmer Cetus) in a 100-μl total reaction volume (24). The HLA-DQα primer set (GH26-GH27) (47) was first used to assess the ability of the DNA in a sample to be amplified and to control for equal input DNA. The SK145-SK101 primer pair was used to amplify HIV-1 proviral DNA, and probe SK102 was used to detect and quantify the product DNA on Southern blots. One hundred copies of the linearized SK145-SK101 deletion plasmid were added to the lysate in each reaction tube to serve as an internal control. Extended copy number standards (0, 5, 50, 500, 1,000, and 5,000) comprising 8E5 cells diluted into 1.5×10^5 uninfected H9 cells were used to generate external calibration curves and to ensure that amplifications were within the linear range (data not shown) (17). The product DNAs were resolved by electrophoresis on an 8% polyacrylamide gel, transferred onto a nylon membrane by using a Trans-Blot apparatus (Bio-Rad), and detected by a 5′-end ^{32}P-labeled SK102 probe. Specific bands were excised from the membranes, and the hybridized radioactive signal was quantified by liquid scintillation counting. The counts corresponding to the full-length band were normalized to the internal control within each sample, and the copy number was determined from the external calibration curves. Each experiment was performed in duplicate, and the results were obtained as numbers of copies per 10^6 PBMCs.

Enzymatic amplification and quantitation of spliced and unspliced mRNAs. RNA was reverse transcribed by avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) for 60 min at 42°C, using 100 pmol of antisense primers in buffers containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 100 μg of nuclease-free bovine serum albumin per ml, 1 mM (each) dATP, dCTP, dGTP, and dTTP, and 25 U of RNAsin (Promega) in a 20-μl volume as described previously (20). After the addition of 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) and 100 pmol of the sense oligonucleotide primer, the cDNA was enzymatically amplified by PCR (95°C for 30 s, 55°C for 60 s, and 72°C for 150 s) in a 100-μl final total volume. To normalize for the total cellular RNA input, the cDNA was first amplified by PCR for 20 cycles, using the RP17-1–RP17-2 primer pair flanking a splice site in the human ribosomal protein S17 spliced transcript (21).

Five hundred copies of the exogenously added cRNA deletion construct were added to total cellular RNA isolated from each patient PBMC sample, reverse transcribed, and coamplified for 25 cycles by RT-PCR using MF687-MF1003 or MF6056-MF8760. To monitor for any potential contaminating DNA, controls without reverse transcriptase were run in parallel. The amplified cDNAs were resolved by electrophoresis on an 8% polyacrylamide gel, transferred onto a nylon membrane, and detected with the corresponding 5′-end ^{32}P-labeled oligonucleotide probe (MF818 or MF8629). The radioactively labeled bands were excised from the membranes, and the hybridized radioactive signal was measured by liquid scintillation counting. The ratio of the disintegrations per minute in the bands corresponding to the full-length cDNAs to those in the deletion-containing cDNA product was proportional to the endogenous mRNA content. Standard curves were generated in parallel by using cRNA generated from the full-length products to determine copy numbers and to ensure that PCR was performed within the linear range of amplification (data not shown). Each experiment was performed in duplicate, and the results were obtained as numbers of copies per 10^6 PBMCs.

Statistical analysis. For graphic presentation of laboratory covariates, each participant's data were used to fit a linear, quadratic, or cubic regression line. For comparison of and statistical interference between progressors and nonprogressors, the nonparametric Wilcoxon rank sum test was used and the two-tailed *P* values were derived. For the levels of HIV-1 proviral DNA, unspliced RNA, and spliced RNA and the unspliced/spliced RNA ratio, observed datum points at HIV-1 seroconversion and at 24 months following seroconversion, the last observed value, and the mean of all the values were used in the nonparametric analysis. The viral nucleic acid content was adjusted to that per 1,000 $CD4^+$ cells and used in these analyses. Scatter plots were also generated, and nonlinear regression analysis was performed to demonstrate the relative strengths of correlation between $CD4^+$ T-cell number and the levels and ratios of viral nucleic acids. Data analysis performed after representing the viral nucleic acid copy numbers relative to 1,000 $CD4^+$ T cells did not significantly alter the correlation profiles (data not shown).

RESULTS

Quantitation of HIV-1 nucleic acids. To quantify proviral DNA, a conserved 141-nucleotide fragment in the *gag* region was amplified by competitive PCR with SK145 and SK101. Product DNA was detected by hybridization with a radioactively end-labeled SK102 internal probe (47). Multiply spliced mRNAs were amplified by competitive RT-PCR, using MF6056 (located in the first exon of *tat*/*rev*) and MF8760 (located in the second exon of *tat*/*rev*), and used for amplification of all spliced mRNA species (21). These primers flank the major *tat*/*rev*/*nef* splice junction at nucleotide 6083 and amplify a 363-bp cDNA fragment by using multiply spliced mRNAs as templates. The product of MF6056 and MF8760 from unspliced mRNA would be greater than 2.7 kbp in length and could not be amplified under the PCR conditions that we used. MF687 and MF1003 flank the major splice donor site at nucleotide 745 just upstream of the *gag* start codon. Since all of the multiply spliced and singly spliced transcripts use the splice donor at nucleotide 745 (21, 45, 51), these primers generate a 316-bp fragment from the unspliced mRNA species, exclusively.

Initially, we confirmed that the full-length and deletion template transcripts amplified with equal efficiencies as described previously (20). Copy number standards containing 0 to 10,000 copies of full-length transcripts were mixed with 500 copies of control cRNA molecules and used to generate standard curves. Amplification was within the linear range if numbers of target sequences remained below 8,000 copies; 28 cycles of amplification were used (data not shown). Similarly, linearity was observed for fewer than 5,000 copies of proviral DNA containing 100 copies of the internal control plasmid that was amplified for 25 cycles (data not shown).

Total RNA and DNA were isolated from 10 cryopreserved patient PBMC samples from serial time points after seroconversion. Appropriate internal standards were added to control for variance in the efficiency of reverse transcription and PCR amplification. Amplified proviral DNA, unspliced RNA, and spliced RNAs were quantified and adjusted to a value per 100 $CD4^+$ T cells as described above. Both spliced and unspliced RNAs were detected in each sample from all patients, regardless of the rate of disease progression. Figure 2 shows autoradiograms of serial amplified intracellular unspliced and spliced mRNAs from a representative nonprogressor (NP1) and appropriate internal controls. Figure 3 illustrates the temporal changes in the adjusted levels of proviral DNA, unspliced RNA, spliced RNA, and total RNA following seroconversion in the two groups.

Changes in proviral DNA levels during disease progression. Proviral DNA was detected in all PBMC samples from both nonprogressors and rapid progressors during all stages of disease. The proviral DNA content in most of the study participants was below an unadjusted value of 500 copies per 10^6 PBMCs throughout the study period. Figures 3a and b show the temporal changes in the adjusted levels of proviral DNA after seroconversion. Although there was a trend toward a higher proviral burden among the progressors over time, this could be attributed to an increased proviral burden in the PBMCs of two of six men in the progressor group. The Wilcoxon rank sum test showed that the differences in the proviral DNA content between the two groups was not statistically significant at all time points examined ($P = 0.91$ at serocon-

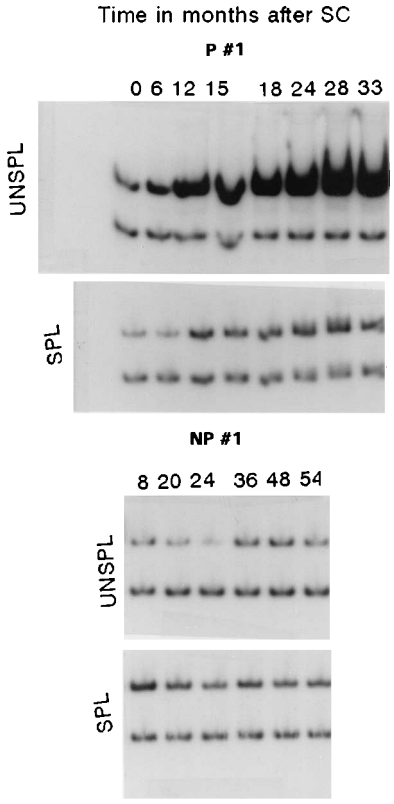

FIG. 2. Levels of HIV-1 unspliced (UNSPL) and spliced (SPL) transcripts in the PBMCs from a representative progressor (P1) and a representative nonprogressor (NP1). The time in months after seroconversion is indicated above each lane. The more slowly moving band corresponds to products amplified by RT-PCR from endogenous HIV-1 RNA in infected cells. The lower, faster-moving band corresponds to 500 copies of deletion cRNA template molecules added into the reaction mixture to serve as an internal control for the fidelity of reverse transcription and enzymatic amplification.

version, $P = 0.35$ at 23 months after seroconversion, and $P = 0.91$ late in infection). Scatter plots of $CD4^+$ T-cell counts against proviral DNA content over calendar time also indicated a very poor inverse correlation ($r^2 = 0.067$) (Fig. 4).

Changes in viral RNA levels during disease progression. Total cell RNA isolated from longitudinal samples of PBMCs and the appropriate molecular construct were reverse transcribed and coamplified by competitive RT-PCR with MF6056-MF8762 and MF687-MF1003. The human ribosomal protein S17 gene was amplified by PCR with RP17-1–RP17-2 to verify the integrity of the RNA in each sample at each study visit. Human ribosomal protein S17 mRNA was detected in all 77 PBMC samples tested. Both unspliced genomic mRNA and multiply spliced viral mRNAs were also detected at the preseroconversion visit and at each subsequent interval visit. Figure 3 illustrates the temporal changes in the adjusted levels of unspliced viral RNA (Fig. 3c and d) and spliced mRNA (Fig. 3e and f) after seroconversion. For both the progressors and the nonprogressors, the levels of HIV-1 multiply spliced mRNAs remained relatively stable throughout the course of infection. Therefore, regardless of the rate of HIV-1 disease progression, the temporal dynamics of HIV-1 transcription remained relatively constant for each individual. The levels of total viral mRNA, however, differed significantly between the progressors and the nonprogressors. For the nonprogressors, the full-length-mRNA levels remained relatively constant throughout the time course studied (Fig. 3d). In contrast, for the progressors, the increase in the full-length RNA level correlated inversely with the precipitous decline in the $CD4^+$ T-cell number (Fig. 3c). The level of total viral RNA, a measure of unpackaged or particle-associated genomic RNA, was an indicator of the extent of viral transcription. Although the total viral RNA levels differed between the two study groups (Fig. 3g and h), active viral gene expression was detected in all stages of HIV-1 infection regardless of the $CD4^+$ T-cell number or the rate of decline.

The ratio of unspliced to multiply spliced viral mRNA was calculated as a measure of the rate of active viral replication and an indicator of the fraction of cells that harbor high levels of HIV-1 genomic RNA. A high ratio of unspliced to multiply spliced viral mRNA is indicative of a high level of intracellular genomic RNA synthesis. In contrast, a low ratio is consistent with the predominant syntheses of multiply spliced mRNA species relative to unspliced viral RNA in a relatively large fraction of the infected cells. Figure 5 shows the ratios of unspliced to multiply spliced viral mRNA over calendar time following seroconversion. In general, an increased ratio of unspliced to multiply spliced viral mRNA correlated with the rate of $CD4^+$ T-cell decline. Five of six members of the progressor group (P1 to P5) had high ratios of unspliced to spliced viral RNA early after seroconversion, compatible with the high level of viral replication observed in late-stage infection in vitro (27). One patient in the progressor group (P6) had a relatively low ratio of unspliced to spliced viral RNA early after seroconversion but had elevated values 25 months later (Fig. 5). The nonprogressors exhibited much lower unspliced mRNA-to-spliced RNA ratios during the first 3 years after seroconversion compared with the progressor group during the same time period (Fig. 5). Despite a relatively stable $CD4^+$ T-cell count, one subject (NP4) had a comparatively high unspliced-to-spliced RNA ratio. Another nonprogressing patient (NP3), although classified as a nonprogressor by virtue of a relatively stable $CD4^+$ T-cell number, had a ratio of unspliced to multiply spliced viral mRNA over calendar time after seroconversion commensurate with that of the progressors. Clinical materials and laboratory data from subsequent time points were unavailable for analysis. The Wilcoxon test indicated that the difference in the ratio of unspliced to spliced RNA between the two groups was significant at later time points ($P = 0.17$ at seroconversion, $P = 0.04$ at 24 months postseroconversion, and $P = 0.01$ late after seroconversion).

Changes in levels of viral RNA relative to DNA over time. The RNA/DNA ratio was calculated as an indicator of the mean transcriptional activity (3). Figure 5 shows that within each group, the values for this ratio were within similar ranges with the exception of one progressor (P2), who had very low values. Examination of the temporal changes in the viral nucleic acid ratios indicates that the ratio of total viral RNA to DNA in each group also paralleled the ratio of unspliced to spliced RNA. The distinction between the progressor and nonprogressor groups was more pronounced for the ratios of unspliced to spliced RNA than for the ratio of RNA to DNA. There was no significant difference between the progressor and nonprogressor groups for the RNA/DNA ratios alone ($P = 0.61$ at seroconversion, $P = 0.48$ at 24 months postseroconversion, and $P = 0.11$ late after seroconversion). Scatter plots also indicated a more significant correlation between the temporal change in the $CD4^+$ T-cell number and the ratio of unspliced to spliced RNA ($r^2 = 0.55$) than the RNA/DNA ratio ($r^2 = 0.033$) (Fig. 6).

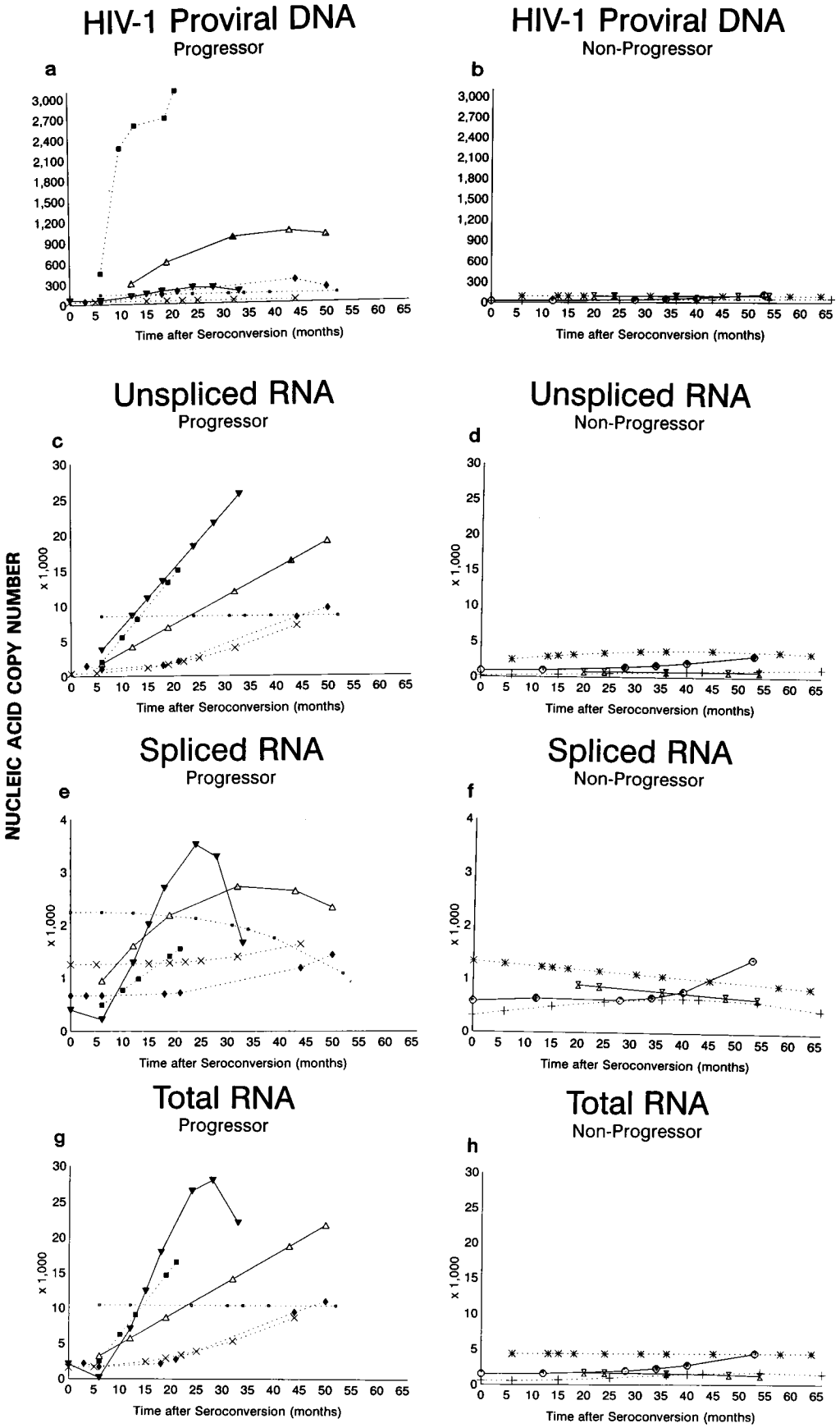

FIG. 3. Comparison of changes in the levels of HIV-1 proviral DNA, unspliced RNA, spliced mRNA, and total RNA during the natural course of infection. Shown are nucleic acid contents for the progressors (P1 [▼], P2 [■], P3 [◆], P4 [□], P5 [△], and P6 [×]) and the nonprogressors (NP1 [⧖], NP2 [+], NP3 [○], and NP4 [*]).

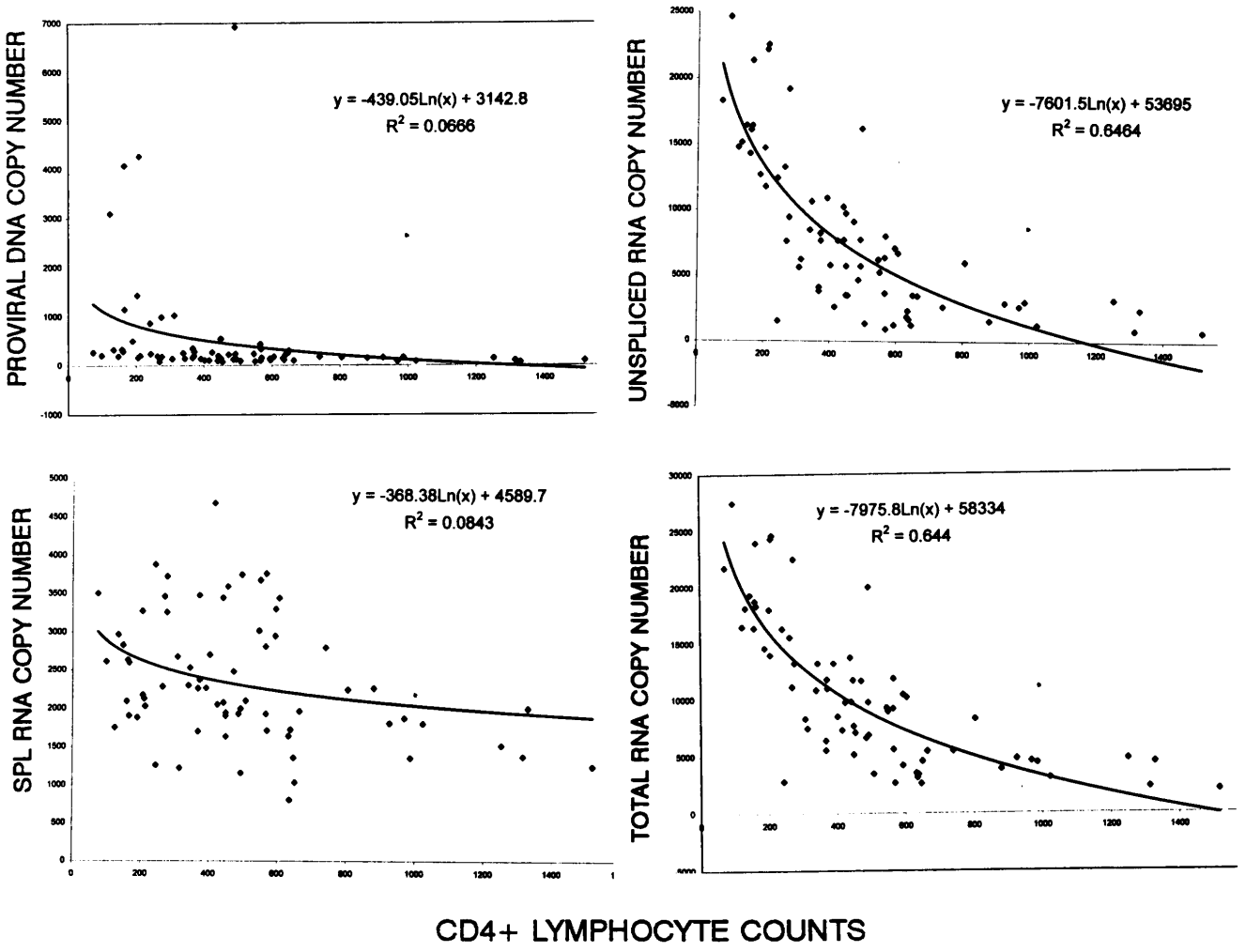

FIG. 4. Scatter plots showing trends in the viral nucleic acid levels in relation to the absolute $CD4^+$ T-cell counts. Nonlinear regression analysis was executed with a confidence interval of 95%, and the regression line was superimposed on the datum points. The equation defining the relationship between the viral nucleic acid levels (ordinate) and the absolute $CD4^+$ T-cell count (abscissa) and the r^2 values are indicated.

DISCUSSION

In this study, we quantified the levels of viral mRNA and proviral DNA in serial blood samples from 10 HIV-1-infected individuals. Six of these ten seroconverters had $CD4^+$ T-cell numbers which gradually declined to less than 200/μl, or an AIDS-defining condition. The remaining four participants had stable $CD4^+$ T-cell numbers and remained disease free throughout the study. For both the progressors and the nonprogressors, we found evidence for viral replication at the preseroconversion visit and at each subsequent visit throughout the course of infection. While the levels of multiply spliced viral mRNAs remained relatively stable, qualitative differences in the amount of unspliced viral RNA were observed for these two groups. Furthermore, the amount of unspliced viral RNA correlated inversely with the $CD4^+$ T-cell number. Subjects with the highest rate of $CD4^+$ T-cell number decrease over time had the largest increase in the amount of unspliced viral RNA. Therefore, the temporal association between the increased amount of unspliced viral RNA and the decreased number of $CD4^+$ T cells suggests a significant association between the extent of viral transcription and the rate of disease progression. These data are consistent with models of viral pathogenesis that equate clinical quiescence and retroviral persistence with a reduced level of viral expression and increased viral replication with subsequent disease progression (37, 52).

Our results confirm and extend earlier cross-sectional studies that also found productive viral infection at all stages of disease and an association between the relative levels of viral mRNAs and the HIV-1 disease state (1, 2, 10, 28, 36, 52). In a previous study of asymptomatic infected individuals who either ultimately progressed or failed to progress to clinical disease, a change in the transcriptional activity was found to precede the decline in the $CD4^+$ T-cell number at the inflection point (48). Since we did not examine the blood of patients who matched this clinical profile, we could not confirm this finding. As in this previous study, however, each of the six participants who did progress could be discriminated from those who did not by their mRNA splicing profiles at the time of infection. In contrast to this previous study, subjects in both the progressor and nonprogressor groups established a pattern of mRNA splicing shortly after the primary infection that persisted over time.

The extent of viral replication can be governed by the numbers of persistently infected cells or the numbers of virions produced per productively infected cell. The reservoir of per-

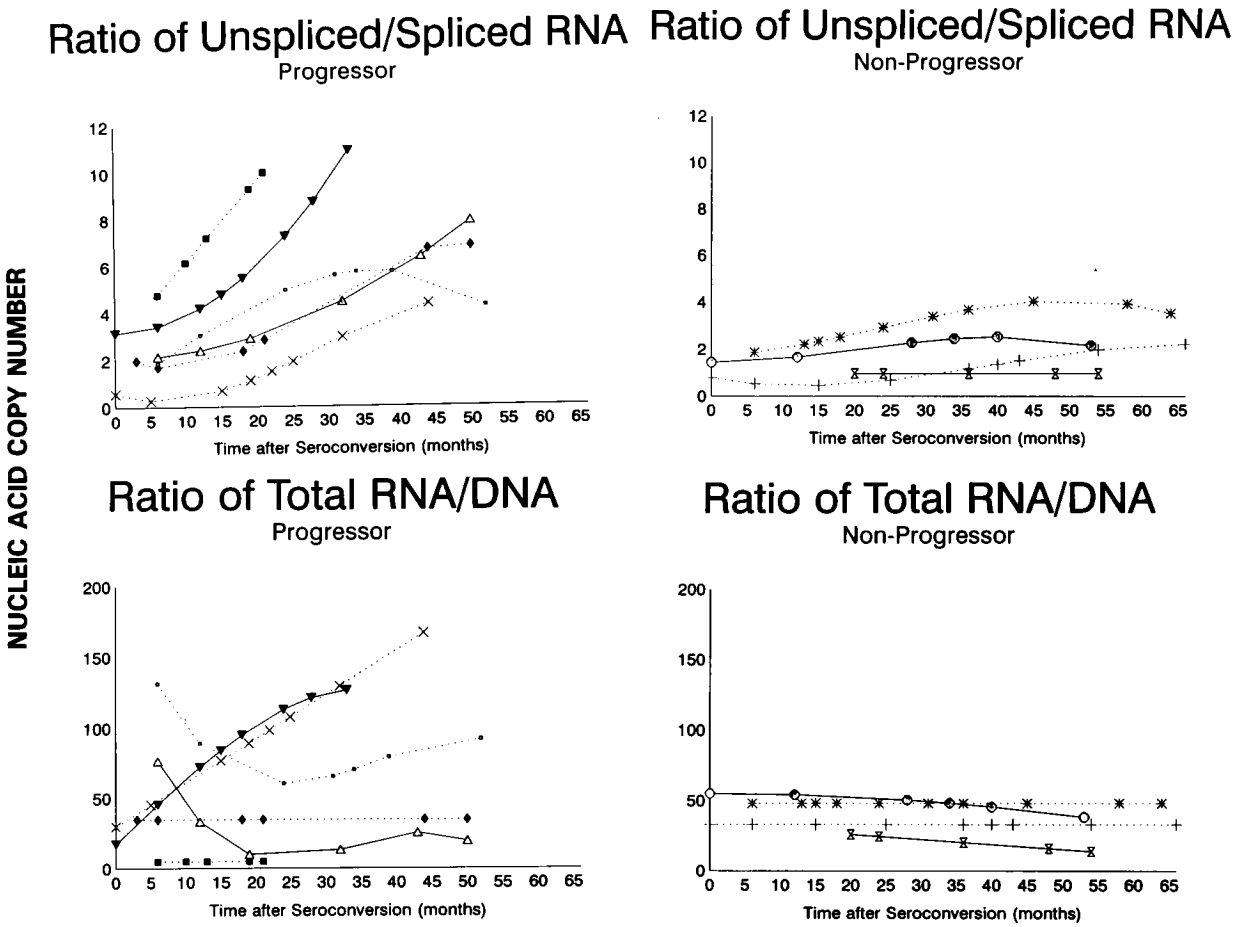

FIG. 5. Comparison of changes in the ratio of HIV-1 unspliced to spliced RNA and total RNA/proviral DNA ratio during the course of infection. Shown are values for the progressors (P1 [▼], P2 [■], P3 [◆], P4 [□], P5 [△], and P6 [×]) and the nonprogressors (NP1 [⧖], NP2 [+], NP3 [○], and NP4 [∗]).

sistently infected cells represents a dynamic equilibrium between the cell populations that are productively infected, are latently infected, or contain defective virus. Modification of the relative numbers of persistently infected cells would, at a relatively constant rate of replication, increase virus production. Alternatively, at the single-cell level, viral expression in a single productively infected cell is a function of either an increased proviral burden that provides a larger number of templates for transcription or upregulation of the rate of endogenous transcription. To discriminate between these two alternative mechanisms for increasing viral transcription, we compared the levels of proviral DNA with the relative contribution of the multiply spliced and unspliced species to the total RNA content. While all of the immunological progressors had escalating ratios of multiply spliced to unspliced viral mRNAs, the intracellular proviral DNA content was stable in five individuals but temporally increased in one, suggesting that in this limited data set, both posttranscriptional control mechanisms may occur in vivo.

Within the infected cell, postintegration viral persistence can be achieved by posttranscriptional regulatory control of retroviral gene expression by both cellular and viral transcriptional factors (13, 16, 53, 55, 56). *rev*-dependent viral determinants and differential utilization of splice acceptor sites are potential mechanisms which regulate viral gene expression (17, 43). *rev* interacts with a *cis*-acting *rev*-responsive element within the *env* coding region to regulate the expression of the intermediate spliced and unspliced mRNAs (35). If *rev* is expressed at a subthreshold level required for functional multimerization (34), then a predominant multiply spliced mRNA pattern may result. Alternatively, multiple inhibitory sequence elements in the *gag*, *pol*, and *env* coding regions may act as RNA recognition sites for cellular factors to downregulate expression of these viral genes (51). *rev* may modulate the action of these cellular factors and subsequently change the mRNA splicing pattern. Differential utilization of splice acceptor sites mediated by changes in the levels of cellular splicing factors has been observed for both adenovirus and simian virus 40 infections (19, 32, 57). Therefore, the comparably low levels of viral transcription in persistently infected cells may be a consequence of several independent mechanisms. Regardless of the precise mechanism by which postintegration viral persistence is achieved, it is ultimately the transcriptional activity and replicative capacity of the virus that become critical determinants of viral pathogenesis.

To assess the magnitude of viral replication, we calculated the ratio of unspliced to multiply spliced viral mRNA. This ratio is also a relative indicator of the fraction of cells that harbor high levels of HIV-1 genomic RNA. While a high ratio of unspliced to multiply spliced viral mRNA is indicative of a high level of intracellular genomic RNA synthesis, a low ratio is consistent with the predominant syntheses of multiply spliced mRNA species relative to unspliced viral RNA in a relatively large fraction of the infected cells. Five of the six

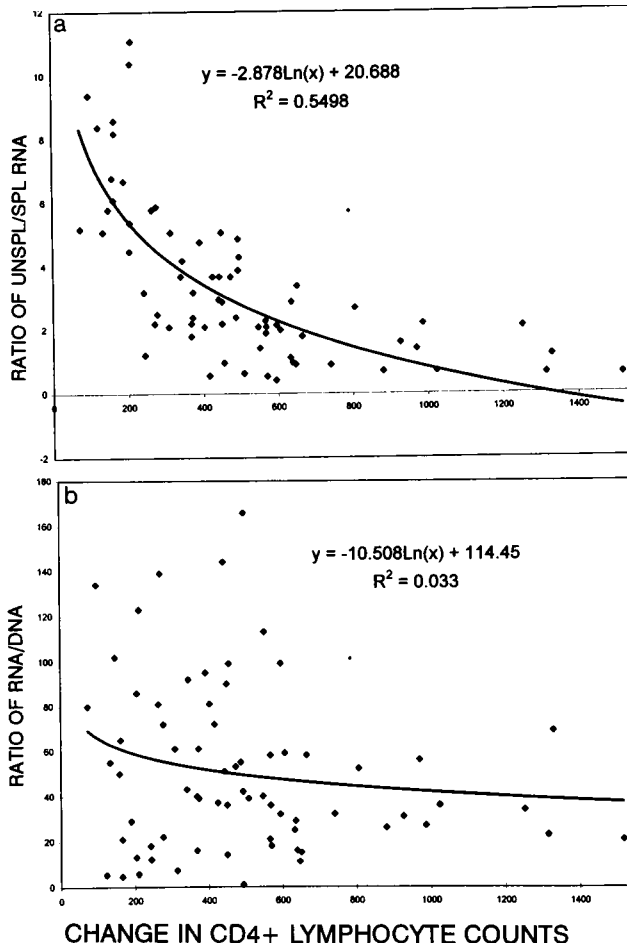

FIG. 6. Scatter plots showing trends in the unspliced/spliced (UNSPL/SPL) (a) and RNA/DNA (b) ratios of HIV-1 nucleic acids in relation to the absolute $CD4^+$ lymphocyte counts. Nonlinear regression analysis was executed with a confidence interval of 95%, and the regression line was superimposed on the data. The equations defining the relationship between the viral nucleic acid ratios (y) and the $CD4^+$ T-cell counts (x) and the r^2 values are given.

subjects in the progressor group had high ratios of unspliced to multiply spliced viral mRNA following seroconversion. The single subject in the progressor group who had a relatively low ratio after seroconversion had an increasing ratio coincident with disease progression. Among the nonprogressors, there was a general trend toward a lower, relatively stable ratio. Although two of the nonprogressors had ratios of unspliced to multiply spliced viral mRNA commensurate with the ratios for the progressor group, prospective clinical data and additional sequential samples needed to define clinical outcome for these individuals were lacking. Previous in vitro studies have shown a temporal shift in the transcriptional pattern from the expression of multiply spliced viral mRNAs to the predominance of unspliced viral mRNAs coinciding with the productive infection (37, 52). Although the trends in the ratio of unspliced to multiply spliced viral mRNA are compatible with a posttranscriptional block to replication, the observed differences in the relative abundance of the different mRNA species in the progressors are more consistent with de novo viral replication. Comparisons with plasma viral RNA levels and quantitative analyses at the single-cell level using PCR-driven in situ hybridization will be required to further elucidate and differentiate these potential mechanisms for increased viral replication.

Our data also suggest that defining the viral transcriptional activity may be an important surrogate marker for assessing disease progression, antiretroviral efficacy, and the ability of a prophylactic vaccine to ameliorate disease. Changes in the splicing pattern of HIV-1 RNA have been observed following the administration of antiretroviral therapy (21). Although transient, these changes may be useful for understanding the kinetics of replication and assessing antiviral activity in vivo once efficacious therapeutic agents do become available. The HIV-1 RNA splicing pattern is also an important parameter for determining whether a prophylactic vaccine can ameliorate disease in an infected vaccine recipient. If the natural history of infection is modified and this can be determined rapidly, then defining the HIV-1 RNA splicing pattern will be extremely valuable for monitoring outcome of vaccine trials. These and other data (42) indicate that the HIV-1 RNA burden in blood cells has predictive value and biological relevance to the disease process.

ACKNOWLEDGMENTS

This work was supported by grants and contracts from the Public Health Service (the Multicenter AIDS Cohort Study to S.M.W. [AI 32535 and AI 45218] and to L.A.K. [AI 72631]) and by a research grant from the Northwestern Memorial Foundation to M.R.F.

We thank John Phair and Kathy Rundell for helpful discussions.

REFERENCES

1. **Arens, M., T. Joseph, S. Nag, J. P. Miller, W. G. Powderly, and L. Ratner.** 1993. Alterations in spliced and unspliced HIV-1 specific RNA detection in peripheral blood mononuclear cells of individuals with varying $CD4^+$ lymphocyte counts. AIDS Res. Hum. Retroviruses **9:**1257–1263.
2. **Bagnarelli, P., S. Menzo, A. Valenza, A. Manzin, M. Giacca, F. Ancarani, G. Scalise, P. E. Varaldo, and M. Clementi.** 1992. Molecular profile of human immunodeficiency virus type 1 infection in symptomless patients and in patients with AIDS. J. Virol. **66:**7328–7335.
3. **Bagnarelli, P., A. Valenza, S. Menzo, A. Manzin, G. Scalise, P. E. Varaldo, and M. Clementi.** 1994. Dynamics of molecular parameters of human immunodeficiency virus type 1 activity in vivo. J. Virol. **68:**2495–2502.
4. **Bednarik, D. P., and T. M. Folks.** 1992. Mechanisms of HIV-1 latency. AIDS **6:**3–16.
5. **Bukrinsky, M. I., T. L. Stanwick, M. P. Dempsey, and M. Stevenson.** 1991. Quiescent T-lymphocytes as an inducible virus reservoir in HIV-1 infection. Science **254:**423–427.
6. **Butera, S. T., B. D. Roberts, and T. M. Folks.** 1993. Regulation of HIV-1 expression by cytokine networks in a $CD4^+$ model of chronic infection. J. Immunol. **150:**625–634.
7. **Butera, S. T., B. D. Roberts, L. Lam, T. Hodge, and T. M. Folks.** 1994. Human immunodeficiency virus type 1 RNA expression by four chronically infected cell lines indicates multiple mechanisms of latency. J. Virol. **68:** 2726–2730.
8. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
9. **Clark, S. J., M. S. Saag, W. Don Decker, S. Campbell-Hill, J. L. Roberson, P. J. Veldkamp, J. C. Kappes, B. H. Hahn, and G. M. Shaw.** 1991. High titers of cytopathic virus in plasma from patients with symptomatic primary HIV-1 infection. N. Engl. J. Med. **324:**954–960.
10. **Clementi, M., A. Manzin, P. Bagnarelli, S. Menzo, P. E. Varaldo, and G. Carioni.** 1992. Molecular profile of human immunodeficiency virus type 1 infection in symptomless patients and in patients with AIDS. J. Virol. **66:** 7328–7335.
11. **Connor, R. I., H. Mohri, C. Yunzhen, and D. D. Ho.** 1993. Increased viral burden and cytopathicity correlate temporally with $CD4^+$ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. J. Virol. **67:**1772–1777.
12. **Coombs, R. W., A. C. Collie, J. P. Allain, B. Nikora, M. Leuther, G. Gjersel, and L. Corey.** 1989. Plasma viremia in human immunodeficiency virus infection. N. Engl. J. Med. **321:**1626–1631.
13. **Cullen, B. R., and W. C. Green.** 1989. Regulatory pathways governing HIV-1 replication. Cell **58:**423–426.
14. **Daar, E. S., T. Mougdil, R. D. Meyer, and D. D. Ho.** 1991. Transient high levels of viremia in patients with primary immunodeficiency virus type 1 infection. N. Engl. J. Med. **324:**961–964.

15. **Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tener-Racz, and T. Haase.** 1993. Massive covert infection of helper T-lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature (London) **362:**359–362.
16. **Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46:**807–817.
17. **Felber, B. K., M. Hadzoupoulou-Cladaras, D. Cladaras, T. Copeland, and G. N. Pavlakis.** 1989. The rev protein of human immunodeficiency virus type 1 affects the stability and transport of viral mRNA. Proc. Natl. Acad. Sci. USA **86:**1495–1499.
18. **Fox, C. H., K. Tenner-Racz, P. Racz, A. Firpo, P. Pizzo, and A. S. Fauci.** 1991. Lymphoid germinal centers are reservoirs of human immunodeficiency virus type 1 RNA. J. Infect. Dis. **164:**1051–1057.
19. **Fu, X. D.** 1993. Specific commitment of different pre-mRNAs to splicing by single SR proteins. Nature (London) **365:**82–85.
20. **Furtado, M. R., R. Balachandran, R. Gupta, and S. M. Wolinsky.** 1991. Analysis of alternatively spliced human immunodeficiency virus type 1 mRNA species, one of which encodes a novel *tat-env* fusion protein. Virology **185:**258–270.
21. **Furtado, M. R., R. Murphy, and S. M. Wolinsky.** 1993. Quantification of human immunodeficiency virus type 1 tat mRNA as a marker for assessing the efficacy of antiretroviral therapy. J. Infect. Dis. **167:**213–216.
22. **Giorgi, J. V., Z. Liu, L. E. Hultin, W. S. Cumberland, K. Hennessey, and R. Detels.** 1993. Elevated levels of $CD38^+$ $CD8^+$ T-cells in HIV infection add to the prognostic value of low $CD4^+$ T-cell levels: results of 8 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. J. Acquired Immune Defic. Syndr. **5:**904–912.
23. **Graziosi, C., G. Pantaleo, L. Butini, J. F. Demarest, M. S. Saag, G. M. Shaw, and A. S. Fauci.** 1993. Kinetics of human immunodeficiency virus type 1 (HIV-1) DNA and RNA synthesis during primary HIV-1 infection. Proc. Natl. Acad. Sci. USA **90:**6405–6409.
24. **Ho, D. D., T. Moudgil, and M. Alam.** 1989. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. N. Engl. J. Med. **321:**1621–1625.
25. **Holodniy, M., D. A. Katzenstein, and S. Sengupta.** 1991. Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. J. Infect. Dis. **164:**862–866.
26. **Hsia, K., and J. A. Spector.** 1991. Human immunodeficiency virus DNA is present in a high percentage of CD4+ lymphocytes of seropositive individuals. J. Infect. Dis. **164:**470–475.
27. **Kim, S., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**584–590.
28. **Klotman, M. E., S. Kim, A. Buchbinder, A. DeRossi, D. Baltimore, and F. Wong-Staal.** 1991. Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes. Proc. Natl. Acad. Sci. USA **88:**5011–5015.
29. **Koup, R. A., J. L. Sullivan, P. H. Levine, F. Brewster, A. Mahr, G. Mazzara, S. McKenzie, and D. Panicalli.** 1989. Antigenic specificity of antibody-dependent cell-mediated cytotoxicity directed against human immunodeficiency virus in antibody-positive sera. J. Virol. **63:**584–590.
30. **Levy, J. A.** 1993. Pathogenesis of human immunodeficiency virus infection. Microbiol. Rev. **57:**183–289.
31. **Lifson, A. R., G. W. Rutherford, and H. W. Jaffe.** 1988. The natural history of human immunodeficiency virus infection. J. Infect. Dis. **158:**1360–1367.
32. **Lou, Y., and G. G. Carmichael.** 1991. Splice site choice in a complex transcription unit containing multiple inefficient polyadenylation signals. Mol. Cell. Biol. **11:**5291–5300.
33. **MacDonell, K. B., J. S. Chmiel, M. S. Poggensee, S. Wu, and J. P. Phair.** 1990. Predicting progression to AIDS: combined usefulness of CD4 lymphocyte counts and p24 antigenemia. Am. J. Med. **89:**706–710.
34. **Malim, M. H., and B. R. Cullen.** 1991. HIV-1 structural gene expression requires the binding of multiple *rev* monomers to the viral RRE: implications for HIV-1 latency. Cell **65:**241–248.
35. **Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 *rev* transactivator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338:**254–257.
36. **Michaels, N. L., P. Morrow, J. Mosca, M. Vahey, D. S. Burke, and R. R. Redfield.** 1991. Induction of human immunodeficiency virus type 1 expression in chronically infected cells is associated primarily with a shift in RNA splicing patterns. J. Virol. **65:**1291–1303.
37. **Michaels, N. L., M. Vahey, D. S. Burke, and R. R. Redfield.** 1992. Viral DNA and mRNA expression correlate with the state of human immunodeficiency virus type 1 infection in humans: evidence for viral replication in all stages of HIV disease. J. Virol. **66:**310–316.
38. **Munoz, A. M., C. Wang, S. Bass, J. M. Taylor, L. A. Kingsley, J. S. Chmiel, and B. F. Polk.** 1989. Acquired immunodeficiency syndrome (AIDS)-free time after human immunodeficiency virus type 1 (HIV-1) seroconversion in homosexual men. Multicenter AIDS Cohort Study Group. Am. J. Epidemiol. **130:**530–539.
39. **Myers, G.** 1989. Human retroviruses and AIDS: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
40. **Pantaleo, G., C. Graziosi, and A. S. Fauci.** 1993. The immunopathogenesis of human immunodeficiency virus infection. N. Engl. J. Med. **328:**327–335.
41. **Patterson, B. K., M. Till, P. Otto, C. Goolsby, M. R. Furtado, L. J. McBride, and S. M. Wolinsky.** 1993. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science **260:**976–979.
42. **Piatek, M., Jr., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K.-C. Luk, B. H. Hahn, G. M. Shaw, and J. D. Lifson.** 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science **259:**1749–1754.
43. **Pomerantz, R. J., T. Seshamme, and D. Trono.** 1992. Efficient replication of human immunodeficiency virus type 1 requires a threshold level of Rev: potential implications for latency. J. Virol. **66:**1809–1813.
44. **Pomerantz, R. J., D. Trono, M. B. Feinberg, and D. Baltimore.** 1990. Cells productively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: a molecular model for latency. Cell **61:**1271–1276.
45. **Purcell, D. F. J., and M. A. Martin.** 1993. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. J. Virol. **67:**6365–6378.
46. **Saag, M. S., M. J. Crain, M. D. Decker, S. Campbell-Hill, S. Robinson, W. E. Brown, M. Leuther, R. J. Whitley, B. H. Hahn, and G. M. Shaw.** 1991. High level viremia in adults and children infected with human immunodeficiency virus: relation to the disease stage and CD4+ lymphocyte levels. J. Exp. Med. **172:**1055–1063.
47. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Thorn, K. B. Mullis, and H. A. Ehrlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
48. **Saksela, K., C. Stevens, P. Rubenstein, and D. Baltimore.** 1994. Human immunodeficiency virus type 1 mRNA expression in peripheral blood cells predicts disease progression independently of the numbers of $CD4^+$ lymphocytes. Proc. Natl. Acad. Sci. USA **91:**1104–1108.
49. **Sambrook, J., E. F. Fritsch, and R. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
50. **Schnittman, S. M., J. J. Greenhouse, H. C. Lane, P. F. Pierce, and A. S. Fauci.** 1991. Frequent detection of HIV-1 specific mRNAs in infected individuals suggests ongoing active viral expression in all stages of the disease. AIDS Res. Hum. Retroviruses **7:**361–367.
51. **Schwartz, G. N., M. Campbell, G. Nasioulas, J. Harrison, B. K. Felber, and G. N. Pavlakis.** 1992. Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent *gag* expression. J. Virol. **66:**7176–7182.
52. **Seshamma, T., O. Bagasra, D. Trono, D. Baltimore, and R. J. Pomerantz.** 1992. Blocked early stage latency in peripheral blood cells of certain individuals infected with human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **89:**10663–10667.
53. **Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica.** 1990. HIV-1 replication is controlled at the level of T-cell activation and proviral integration. EMBO **9:**1551–1560.
54. **Trono, D., and D. Baltimore.** 1990. A human cell factor is essential for HIV-1 *rev* action. EMBO J. **9:**4155–4160.
55. **Vaishnav, Y. N., M. Vaishnav, and F. Wong-Staal.** 1991. Identification and characterization of a nuclear factor that specifically binds to the rev response element (RRE) of human immunodeficiency virus type 1 (HIV-1). New Biol. **3:**142–150.
56. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. Haislip, and L. S. Y. Chen.** 1990. HIV-1 entry into quiescent lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell **61:**213–222.
57. **Zahler, A. M., K. M. Neugebauer, W. S. Lane, and M. B. Roth.** 1992. Distinct functions of SR proteins in alternative pre-mRNA splicing. Science **260:**219–222.