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Human Immunodeficiency Virus Type 1 Cellular RNA Load and Splicing Patterns Predict Disease Progression in a Longitudinally Studied Cohort

NELSON L. MICHAEL,[1]* THERESA MO,[2] ABDERRAZZAK MERZOUKI,[2] MICHAEL O'SHAUGHNESSY,[2] CHARLES OSTER,[3] DONALD S. BURKE,[1] ROBERT R. REDFIELD,[3] DEBORAH L. BIRX,[1] AND SHARON A. CASSOL[2]

Division of Retrovirology, Walter Reed Army Institute of Research, Rockville, Maryland[1]; Walter Reed Army Medical Center, Washington, D.C.[3]; and British Columbia Center for Excellence in HIV/AIDS, Vancouver, British Columbia, Canada[2]

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We report the results of a longitudinal study of RNA splicing patterns in 31 early-stage human immunodeficiency virus disease patients with an average follow-up time of 3 years. Eighteen patients showed no evidence for disease progression, whereas 13 patients either showed a ≥50% reduction in baseline CD4 count or developed opportunistic infections. Levels of unspliced, *tat*, *rev*, and *nef* mRNAs in peripheral blood mononuclear cells were measured by a reverse transcriptase-quantitative, competitive PCR assay. Viral RNA was detected in all patients at all time points. All 13 rapid progressors had viral RNA loads that were ≥1 log unit greater than those of the slow progressors. In addition, seven of the rapid progressors showed a reduction of more than threefold in the ratio of spliced to unspliced RNA over the 3 years of follow-up. Conversely, two slow progressors with intermediate levels of viral RNA showed no splicing shift. These results confirm earlier observations that viral RNA is uniformly expressed in early-stage patients. We further show that cellular RNA viral load is predictive of disease progression. Importantly, the shift from a predominately spliced or regulatory viral mRNA pattern to a predominately unspliced pattern both is associated with disease progression and adds predictive utility to measurement of either RNA class alone.

Expression of human immunodeficiency virus type 1 (HIV-1) RNA is characterized by a complex pattern of unspliced and spliced mRNAs. Previous work with cells acutely infected with HIV-1 shows an initial rise in the multiply spliced transcripts *tat*, *rev*, and *nef* followed by a rise in the level of both singly spliced and unspliced species (2, 10, 16, 18, 19, 27, 28, 32, 42, 49). Tat increases viral transcription from the 5′ long terminal repeat in these model systems (3, 22, 29, 52) and may facilitate elongation of initiated transcripts (15, 25, 29, 50). Rev serves to increase the steady-state level of cytoplasmic unspliced viral RNA either by directly interacting with the splicing machinery (6, 7) or by increasing the efflux of these transcripts out of the nucleoplasm, where they are susceptible to being spliced (13, 17, 21, 32). Thus, in models of acute infection, a mechanism of HIV-1 RNA metabolism that functions early in infection to express regulatory genes and then switches to a late phase of expression of both viral structural genes and genomic RNA can be demonstrated. Similar findings have been made in persistently infected cells during the switch from low-level to high-level viral production mediated by phorbol esters, cytokines, lipopolysaccharide, or other inducing agents (35, 41).

Cross-sectional studies of HIV-1 viral RNA expression in the peripheral blood mononuclear cells (PBMC) of infected individuals have uniformly demonstrated a rise in viral RNA in late-stage HIV disease (1, 4, 5, 20, 36, 46, 51). The amount of cell-free, or plasma, viral RNA has also been shown to correlate with the stage of HIV disease (4, 5, 24, 39, 45, 55). Recent data have also demonstrated the presence of significant amounts of viral RNA in the lymphoid organs of patients at all stages of HIV disease (11, 12, 37, 38). There are conflicting data on the dynamic ratio of spliced to unspliced viral RNA throughout disease progression. Our previous work with a cross-sectional cohort lacked the statistical power to address this question adequately, although a trend was seen toward a higher ratio of spliced to unspliced RNA in earlier-stage patients (36). Seshamma and colleagues also argued for a blocked state of HIV-1 viral RNA expression, characterized by a high ratio of spliced to unspliced RNA, in the early-stage asymptomatic patients represented in their cross-sectional cohort (51). This result was not confirmed by a recent study (44).

We describe here a longitudinal study of multiply spliced (*tat*, *rev*, and *nef* mRNAs) and unspliced HIV-1 RNA expression using a reverse transcriptase-quantitative, competitive PCR (RT-qcPCR) assay (34) in a cohort of 31 patients who, at study entry, were all classed as early-stage asymptomatic. During the course of follow-up, 18 of these patients showed no clinical or immunologic deterioration while the remaining 13 patients suffered a ≥50% decline in entry CD4 count and/or developed opportunistic infections. We show that HIV-1 RNA is detectable in all patients. We further show that levels both of individual multiply spliced mRNAs and of unspliced RNA are predictive of disease progression. We next show that the ratio of multiply spliced to unspliced RNA declines by fourfold over the course of study in the rapid-progressor subcohort. This allowed for a greater predictive power in the assessment of disease progression based on measurements of viral RNA expression. This work represents the first demonstration that both HIV-1 cellular RNA load and splicing patterns predict disease progression.

* Corresponding author. Mailing address: 13 Taft Ct., Suite 200, Rockville, MD 20850. Phone: (301) 762-0089. Fax: (301) 762-4177. Electronic mail address: nmichael@smtpgw.hjf.org.

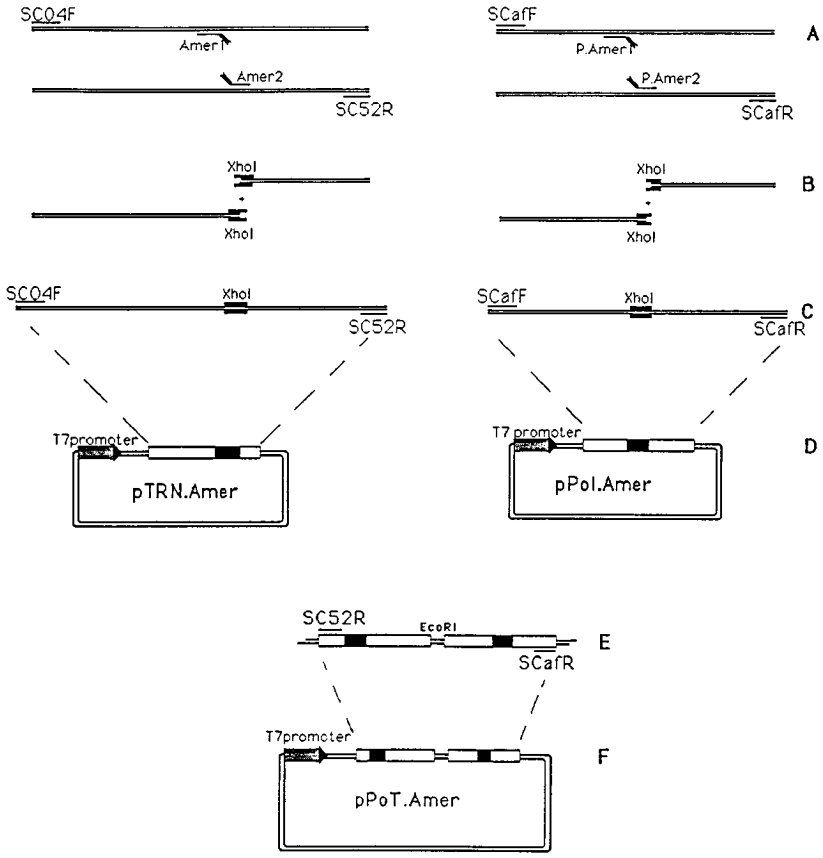

FIG. 1. Construction of the internal control template plasmid. Two individual PCR fragments, one representing an overlapping segment of *tat-rev-nef* (left panel) and a second representing *pol* sequences (right panel) were amplified from total RNA extracted from HIV-1-infected CEM cells. After purification, each fragment was reamplified in two separate reactions with SC04-Amer1 and SC52R-Amer2 primer sets for *tat-rev-nef* and SCaf.F-P.Amer1 and SCaf.R-P.Amer2 for *pol* (lines A). The internal primers, Amer1, Amer2, P.Amer1, and P.Amer2, each contained a nucleotide tail (17 to 21 nucleotides in length) and *Xho*I restriction sites at their 5′ ends. The subproducts for each template (lines B) were then digested with *Xho*I, ligated (line C), and cloned directly into the TA cloning vector pCR-II (Invitrogen, San Diego, Calif.) to create the pTRN.Amer and pPol.Amer plasmids (line D). The inserts from both of these plasmids were flanked on either side by *Eco*RI sites. Following digestion of pTRN.Amer and pPol.Amer with *Eco*RI, the released inserts were ligated together (line E). The ligation product was then amplified with SCaf.R and SC52R, and the resultant composite fragment was cloned into pCR-II to create the internal control plasmid pPoT.Amer.

MATERIALS AND METHODS

Description of cohort and patient selection criteria. All 31 patients described in this work were classed as having Walter Reed stage 1 or 2 (early-stage, asymptomatic) HIV disease at study entry (43). These patients were enrolled in phase I trials of envelope-based subunit vaccines produced in either baculovirus (MicroGeneSys, Inc.) or CHO cell expression systems (Genentech, Inc.). Slow progressors were defined as patients who demonstrated a subsequent decline of ≤35% in their CD4 counts, although half of these patients had either a stable or increased CD4 count during follow-up. Rapid progressors were defined as patients who demonstrated a decline of ≥50% in baseline CD4 count and/or developed an opportunistic infection (*Pneumocystis carinii* pneumonia or *Mycobacterium avium* complex infection). At baseline, the slow progressors and rapid progressors were indistinguishable on the basis of age, sex, CD4 count, and delayed-type hypersensitivity skin testing to recall antigens. Of the baseline PBMC samples from this cohort, 30 were drawn prior to the first vaccination. In a single patient, the baseline viral burden sample was drawn on day 5 postvaccination. None of the baseline samples were drawn from patients undergoing concurrent antiretroviral drug treatment. The follow-up samples representing the longest time from baseline were chosen for study. Baseline CD4 counts were defined as the average of three prevaccination values determined over a 6-week time frame, whereas the follow-up CD4 count was that obtained from the sample analyzed for viral burden.

Determination of HIV-1 mRNA levels. All samples were analyzed in a blinded fashion. Samples that represented a single baseline and a single follow-up point for each patient were selected. Cryopreserved PBMC samples were coded by the institution performing the clinical trials and then sent to the institution performing the RNA analyses. Only upon the completion of all quantitative RNA transcript level determinations were the code broken and the subcohort analyses performed.

The designations, locations (53), and sequences of the primers used in this study are as follows: SC04F, 5439 to 5465, CCTGGAAICATCCAGGAAGTCA GCCTA; SC02F, 5534 to 5560, AGICTTAGGCATCTCCTATGGCAGGAA; SC05F, 5558 to 5586, GAAGAAGCGGAGACAGCGACGAAGAICTC; SC52R, 8139 to 8113, TAAGTCTCTCAAGCGGTGGTAGCTGAA; SC90R-FAM, 8052 to 8028, GGATCTGTCTCTGTCTCTCTCTCCA; SCaf.F, 1896 to 1923, AAGCTCTATTAGAIACAGGAGCAGATGA; SCaf.R, 2091 to 2064, CCIAT TATGTTGACAGGIGTAGGTCCTA; SCaf.int.bR-JOE, 2079 to 2053, ACAG GTGTAGGTCCTACTAATACTGTA.

SC90R and SCaf.int.bR were labeled at their 5′ ends with a blue (FAM) and a green (JOE) fluorescent dye (Applied Biosystems, Foster City, Calif.), respectively (34). To obtain precise and differential measurement of individual transcript levels, we constructed a complex internal standard containing equimolar amounts of three overlapping (*tat*, *tat-rev*, and *tat-rev-nef*) and one tandemly arranged (*pol*) competitive template. The standard was prepared by ligation of two separate competitors in tandem, one representing a polycistronic segment of *tat-rev-nef* and the second representing a small region of the *gag-pol* message. Each of these competitors contained a small insertion. In the case of the *tat-rev-nef* template, the insert was placed in exon 5. This exon is common to all three of these regulatory transcripts. The design and construction of the competitive template are shown in Fig. 1. Since the amounts of competitor are exactly equivalent for all targets, tandem RT-qcPCR permits accurate determination of the relative ratio of different mRNA subclasses. This approach is independent of variations in RNA recovery and RT-PCR efficiency.

As described previously (34), RNA was extracted from patient PBMC and reverse transcribed in a single reaction with two different antisense primers, SC52R for total regulatory (*tat*, *tat-rev*, and *tat-rev-nef*) and SCaf.R for unspliced (*pol*) mRNA. In practice, replicate amounts of the cDNA were added to increasing amounts of internal standard and the two templates were coamplified for 30 cycles with outer primers specific for *tat* (SC04F and SC52R), *tat-rev* (SC02F and SC52R), *tat-rev-nef* (SC05F and SC52R), or *pol* (SCaf.F and SCaf.R). After the first round of amplification, 2 μl of PCR product was carried into a second

reaction containing the inner primers SC04F and SC90R-FAM (*tat*), SC02F and SC90R-FAM (*tat-rev*), SC05F and SC90R-FAM (*tat-rev-nef*), and SCaf.F and SCaf.int.bR-FAM (*pol*). Following amplification, products were analyzed on an Applied Biosystems 373A DNA Sequencer. The number of target molecules in the clinical specimen was calculated from a plot of the ratio of peak area of the target template to peak area of the competitive template for each amount of added competitor DNA. All copy numbers are reported for RNA equivalent to 1,400 PBMC. We have previously shown that the correlation coefficient (r^2) for standard curves ranged from 0.9814 to 0.9995 and that the intertest variation, determined by duplicate testing of six specimens on three different days, was <10% (34).

Statistical treatments. CD4 slopes were computed by assembly of all available CD4 count data for each patient and submission of these data to linear regression analysis. A minimum of six CD4 count determinations per year were available for each patient. Continuous data sets such as age, absolute CD4 count, CD4 slopes, and mRNA levels were analyzed by the Mann-Whitney treatment, given the nonparametric nature of the data. Nominal data sets, such as gender and splicing pattern versus progression (see Tables 1 and 4) comparisons, were analyzed by Fisher's exact test. All treatments were performed with StatView version 4.01 software (Abacus Concepts, Inc., Berkeley, Calif.).

RESULTS

Characteristics of the study cohort. A total of 31 early-stage asymptomatic patients were selected from two phase I trials of recombinant envelope subunit vaccines. Of these patients, 18 demonstrated a relatively stable CD4 count over follow-up and 13 demonstrated a relatively sharp decline in CD4 count and/or developed opportunistic infections (see Materials and Methods). At study entry, the baseline or prevaccination CD4 counts were statistically indistinguishable between the slow progressors and the rapid progressors, although the mean CD4 count in the rapid progressor subcohort was smaller than that of the slow progressor subcohort (Table 1). The durations of time between baseline and follow-up CD4 count determinations were similar between the two groups (Table 1). The study subcohorts were also matched for age and gender (Table 1).

The differences in the absolute CD4 counts at follow-up (labeled post in Table 1) between the slow progressors and the rapid progressors were highly statistically significant. The slow progressor subcohort also differed significantly from the rapid progressor cohort in terms of mean CD4 cell slope (absolute CD4 cell rate of change per day) and CD4 cell percent change (percent CD4 cell rate of change per year) (Table 1). The CD4 slopes are shown for each subcohort in Tables 2 and 3 and in graphic form in Fig. 2. The data are plotted on identical axes to facilitate comparison. The *y* intercept and slope for each individual were defined by linear regression analysis on multiple determinations of the absolute CD4 count. The precise terminus of each line was defined by the length of follow-up for each patient. Although most of the slow progressors (Table 2 and Fig. 2A) had either positive or very slightly negative deflections, three patients in this subcohort (patients D, GG, and W) had CD4 slopes that were indistinguishable from those of the rapid progressor cohort. Their inclusion in the slow progressor cohort was based on the criterion that the final change in CD4 count from baseline to follow-up was ≤35%. Moreover, even in these cases, the follow-up CD4 count was ≥400 cells per μl. Overall, members of the rapid progressor subcohort displayed greater negative CD4 slopes (Table 3; Fig. 2B) than did members of the slow progressor subcohort. As shown previously in Table 1, this difference was highly statistically significant.

Analysis of HIV-1 mRNA levels in the cohort. A single baseline and a single follow-up sample were chosen for each patient described in this study. After being coded at the clinical study site institution, cryopreserved PBMC samples were sent to the institution performing the RNA analyses in order to maintain a blinded study design. The data obtained are shown for the slow progressor subcohort in Table 2 and for the rapid progressor subcohort in Table 3. Each patient is identified by a letter or letters followed by the numeral 1 for baseline samples or the numeral 2 for follow-up samples. The day, relative to first vaccination, on which the sample for mRNA analysis was drawn is listed next to the patient code followed by the CD4 count. All baseline CD4 values are the means of three absolute CD4 count determinations made during the 6 weeks prior to day 1, whereas each follow-up CD4 value corresponds to the single CD4 cell count determination from that blood draw. The presence and nature of opportunistic infection during the follow-up period are noted. All mRNA quantitation data are expressed as copy number per 1,400 PBMC. Total regulatory mRNA is the arithmetic sum of the individual values for *tat*, *rev*, and *nef* mRNAs. When this number was ≤10 copies, the semiquantitative result of limiting dilution analysis was used (either <1 or <10 copies). The same is true for the measurement of *pol* mRNA (unspliced). The ratio of total regulatory to *pol* mRNAs is given next, followed by the values for the rate of change of CD4.

Table 2 is striking in that 14 of 18 of these slow progressors show very low, but detectable, levels of viral RNA expression. Three of the remaining four patients, O, S, and Z, show intermediate levels of viral RNA expression without a shift in the ratio of total regulatory to *pol* mRNAs. We will designate this ratio the splicing ratio for the remainder of the manuscript.

TABLE 1. Cohort characteristics

Cohort (n = 31)	Characteristic								
	Mean[a] CD4 count (cells/μl)	CD4 slope[b]		% ΔCD4/yr[c]		Mean follow-up (days)	Age (yr)	No. of patients of indicated sex	
		Mean	Median	Mean	Median			Male	Female
Slow progressors (n = 18)	718 ± 219 (pre), 680 ± 258 (post)	−0.044 ± 0.012	−0.033	−0.439 ± 7.434	−0.650	1,138 ± 360	28.4 ± 4.9	16	2
Rapid progressors (n = 13)	596 ± 123 (pre), 229 ± 165 (post)	−0.333 ± 0.116	−0.328	−21.5 ± 8.2	−20.9	1,084 ± 370	31.7 ± 6.8	12	1
P value[d]	0.1612 (pre), <0.0001 (post)	<0.0001	NA[e]	<0.0001	NA	0.7793	0.2221	0.4220[f]	

[a] Shown as mean ± 1 standard deviation for all mean values in the table. pre, baseline sample; post, follow-up sample.
[b] (Cells per microliter) day^{-1}.
[c] Percent CD4 cell rate of change per year.
[d] By Mann-Whitney test unless indicated otherwise.
[e] NA, not applicable.
[f] By Fisher's exact test.

Patient O showed stable, intermediate levels (between 10 and 100 copies) of total regulatory mRNAs. Unfortunately, *pol* mRNA levels were not measured for patient O because of insufficient sample amounts. Patient S showed a stable splicing ratio of approximately 3. Patient Z showed a modest 1.6-fold reduction in splicing ratio. These two patients, despite the presence of substantial amounts of viral RNA in their PBMC, have positive CD4 slopes. Patient R showed an intermediate level of viral RNA with a diminishing level of total regulatory mRNA and a rising level of *pol* mRNA, resulting in a 5.8-fold reduction of the splicing ratio from 7.5 to 1.3. This patient had an absolute CD4 count of 715 cells per μl, with a CD4 percentage of 30% on the day for which viral RNA was measured. His next CD4 count and percentage, drawn 2 months later, were 386 cells per μl and 19%, respectively. Three subsequent CD4 profiles drawn over the next 6 months from this patient reflect the higher values. Thus, he suffered a substantial but transient decrease in the number and percentage of circulating $CD4^+$ T cells after the follow-up viral RNA measurement performed on study day 711.

The data presented for the rapid-progressor subcohort in Table 3 contrast starkly with those just described for the slow-progressor subcohort. The absolute levels of HIV-1 mRNAs are generally 10 to 100 times greater than those of the slow progressors. The splicing ratios of 6 of 13 rapid progressors show a ≥3-fold reduction from baseline to follow-up values. Most of this shift could be associated with a falling level of total regulatory mRNAs and a rising level of *pol* mRNA, although in patient CC, the shift was due to an increase in *pol* mRNA that was proportionally greater than that in total regulatory mRNAs. An additional three patients, FF, I, and Q, demonstrate a reduction of ≥1.5-fold in splicing ratio over time, whereas patients BB, V, and U show a shift of <1.5-fold in splicing ratios. These latter six patients all show a temporal rise both in total regulatory mRNAs and in *pol* mRNA that is associated with a strongly negative CD4 slope and, in the case of patient FF, the development of opportunistic infection. It is remarkable that patient FF developed *P. carinii* pneumonia with an absolute CD4 count of 569 cells per μl. Patient A showed a fourfold decrease in total regulatory mRNAs. Un-

TABLE 2. Slow progressors: RNA viral burden and CD4 data

Patient code[a]	Study day[b]	No. of CD4 cells/μl	Level of RNA of indicated type[c]					Total reg/*pol*	CD4 slope	% ΔCD4/yr[d]
			tat	*rev*	*nef*	Total reg[e]	*pol*			
B1	−12	1,312	<1	<1	<10	<10	<1	NA[f]	0.00418	1.3
B2	1,527	1,386	<1	<1	<1	<1	<1	NA		
D1	−12	895	<1	<1	<1	<1	<1	NA	−0.22100	−5.7
D2	1,569	674	<1	<1	<1	<1	<10	NA		
DD1	−14	592	<1	<1	<1	<1	<1	NA	−0.12400	−4.7
DD2	866	525	<1	<1	<10	<10	<10	NA		
E1	−14	772	<1	<1	<1	<1	<1	NA	−0.00103	−8.4
E2	1,499	502	<1	<1	<1	<1	<1	NA		
EE1	−28	460	<1	<1	<1	<1	<1	NA	0.08100	3.0
EE2	892	495	<1	<1	<1	<1	<1	NA		
F1	−11	787	<1	<1	<10	<10	<10	NA	−0.02200	−8.1
F2	1,534	517	<1	<10	<10	<10	<10			
GG1	−14	1,071	<1	<1	<1	<1	<1	NA	−0.21900	−2.6
GG2	940	997	<1	<1	<1	<1	<1	NA		
H1	−19	649	<1	<1	<1	<1	<1	NA	0.09400	2.2
H2	1,646	714	<1	<1	<1	<1	<1	NA		
II1	−28	1,102	<1	<1	<10	<10	<1	NA	−0.07100	−4.6
II2	1,105	943	<1	<1	<1	<1	<1	NA		
L1	5	399	<1	<1	<10	<10	<10	NA	−0.04500	−4.2
L2	1,423	334	<1	<1	<10	<10	<10	NA		
M1	−27	388	<1	<1	<1	<1	<1	NA	−0.00549	3.6
M2	1,448	444	<1	<1	<10	<10	<1	NA		
O1	−14	756	8	14	3	25	ND[g]	NA	−0.11000	−3.5
O2	1,465	648	3	6	12	21	ND	NA		
R1	0	664	17	43	0	60	8	7.5	−0.00272	3.9
R2	711	715	2	5	12	19	15	1.3		
S1	−14	561	14	12	27	53	16	3.3	0.11500	4.9
S2	729	617	47	8	133	188	57	3.3		
T1	−28	667	<1	<1	<1	<1	<1	NA	−0.09800	−16.0
T2	758	437	<1	<1	<1	<1	<1	NA		
W1	−14	563	<1	<1	<10	<10	<10	NA	−0.18100	5.6
W2	766	630	<1	<1	<10	<10	<1	NA		
Y1	−14	745	<1	<1	<1	<1	<1	NA	−0.05700	15.6
Y2	796	1,003	<1	<1	<1	<1	<1	NA		
Z1	−28	541	30	51	117	198	53	3.7	0.07500	9.8
Z2	802	661	1	6	25	32	14	2.3		

[a] All were slow progressors; none suffered opportunistic infection.
[b] Relative to day of first immunization, defined as day 0.
[c] All RNA data are expressed as copy number per 1,400 PBMC. RNA values given as <1 or <10 were determined by limiting dilution analysis.
[d] Percent CD4 cell rate of change per year.
[e] Total reg (total regulatory mRNA), arithmetic sum of the levels of *tat*, *rev*, and *nef* mRNAs.
[f] NA, not applicable.
[g] ND, not done.

fortunately, *pol* mRNA was not measured at either time point because of insufficient sample amounts. Patient A showed a dramatic fall in CD4 count from 567 to 33 cells per μl and developed multiple opportunistic infections during follow-up.

Baseline HIV-1 mRNA levels predict disease progression. A graphic representation of the viral RNA levels described in Table 2 for the slow progressor subcohort is shown in Fig. 3A, and the viral RNA levels described in Table 3 for the rapid progressor subcohort are shown in Fig. 3B. The same *x* and *y* axes are used for both data sets to facilitate comparison between the two. The open bars represent baseline RNA values, and the closed bars represent follow-up RNA values. Measurements of <1 and <10 copies in Table 2 were arbitrarily assigned the values 0.9 and 9.9 copies, respectively, for purposes of graphic display and statistical treatment. These assignments, representing the highest level of the range for the slow progressors, would statistically favor the null hypothesis that there was no difference in transcript levels between slow and rapid progressors. The mean and 1 standard error are shown for each set of viral mRNA measurements. A statistical analysis of these data is given in the upper portion of Fig. 4. For each mRNA, the baseline mean, standard deviation, and median values are given for both the slow-progressor and rapid-progressor subcohorts. These data sets were analyzed by the Mann-Whitney nonparametric statistical treatment. The differences between baseline values of *tat*, *rev*, *nef*, total regulatory, and *pol* mRNAs between the two subcohorts were all highly statistically significant. Thus, the baseline values of three different doubly spliced and unspliced HIV-1 mRNAs, determined prior to any experimental or therapeutic intervention, were predictive of disease progression in these patients.

Disease progression is associated with a shift in HIV-1 RNA splicing patterns. As discussed previously, 9 of 13 members of the rapid progressor subcohort showed evidence for a reduction in the ratio of spliced to unspliced viral RNA during the course of study. We analyzed the changes for each of the mRNA parameters in the rapid progressors as shown in the middle section of Fig. 4 by the Mann-Whitney treatment. Only the 4.1-fold increase in *pol* mRNA and the 3.8-fold decrease in the ratio of total regulatory to *pol* mRNA were statistically significant. Both of these measurements correlated with disease progression. This suggests that the predictive power of the *pol* mRNA measurement was sufficient to carry the splicing ratio to statistical significance. All of these observations are based on an interpretation of viral RNA data per 1,400 PBMC. This ignores the substantial reduction in the $CD4^+$ T-cell count that occurred in many of the follow-up PBMC samples. Given that this cell is the major circulating reservoir of actively expressing virus (47, 48, 54), normalization of RNA values to a fixed number of CD4 cells may provide a more physiologic reflection of the state of HIV-1 expression in the peripheral blood (36). The data for the rapid-progressor subcohort given in Table 3 were normalized to 1,000 CD4 cells per μl and are shown graphically in Fig. 5. The effect of this normalization increases the follow-up values to a greater extent than the

TABLE 3. Rapid progressors: RNA viral burden and CD4 data

Patient code[a]	Study day[b]	No. of CD4 cells/μl	Opportunistic infection[c]	Level of RNA of indicated type[d]					Total reg/*pol*	CD4 slope	% ΔCD4/yr[e]
				tat	*rev*	*nef*	Total reg[f]	*pol*			
A1	−6	567	PCP and MAC	71	74	255	400	ND[g]	NA[h]	−0.39700	−28.7
A2	1,190	33		21	7	72	100	ND	NA		
BB1	−14	631		1	4	12	17	8	2.1	−0.32800	−23.2
BB2	795	306		4	17	20	41	28	1.5		
C1	−4	420	PCP and thrush	38	44	428	510	60	8.5	−0.27300	−20.9
C2	1,682	14		21	22	140	183	104	1.8		
CC1	0	598		5	15	23	43	18	2.4	−0.29900	−30.9
CC2	874	156		19	34	44	97	155	0.6		
FF1	−14	636	PCP	25	0	23	48	63	0.8	−0.30400	−4.7
FF2	799	569		112	0	186	272	526	0.5		
G1	−14	656	PCP	9	32	130	171	13	13.2	−0.33300	−19.2
G2	1,555	115		8	19	81	108	75	1.4		
I1	−46	426		112	230	0	342	28	12.2	−0.23900	−16.6
I2	1,691	90		14	19	1,611	1,644	324	5.1		
JJ1	0	857		29	13	517	559	19	29.4	−0.34200	−18.1
JJ2	1,084	397		24	62	186	272	72	3.8		
K1	0	530		83	22	599	704	80	8.8	−0.17000	−13.1
K2	1,430	258		5	9	17	31	101	0.3		
P1	−13	520		22	23	63	108	30	3.6	−0.15700	−15.7
P2	1,455	192		6	8	17	31	65	0.5		
Q1	−14	558		2	49	77	128	62	2.1	−0.46200	−24.8
Q2	794	252		39	80	176	295	248	1.2		
U1	−14	654		33	14	149	196	38	5.2	−0.47200	−32.2
U2	919	116		80	67	587	734	158	4.6		
V1	−14	774		30	51	117	198	65	3.0	−0.55400	−31.9
V2	778	238		26	52	240	318	130	2.5		

[a] All were rapid progressors.
[b] Relative to day of first immunization, defined as day 0.
[c] PCP, *Pneumocystis carinii* pneumonia; MAC, *Mycobacterium avium* complex infection.
[d] All RNA data are expressed as copy number per 1,400 PBMC.
[e] Percent CD4 cell rate of change per year.
[f] Total reg (total regulatory mRNA), arithmetic sum of the levels of *tat*, *rev*, and *nef* mRNAs.
[g] ND, not determined.
[h] NA, not applicable.

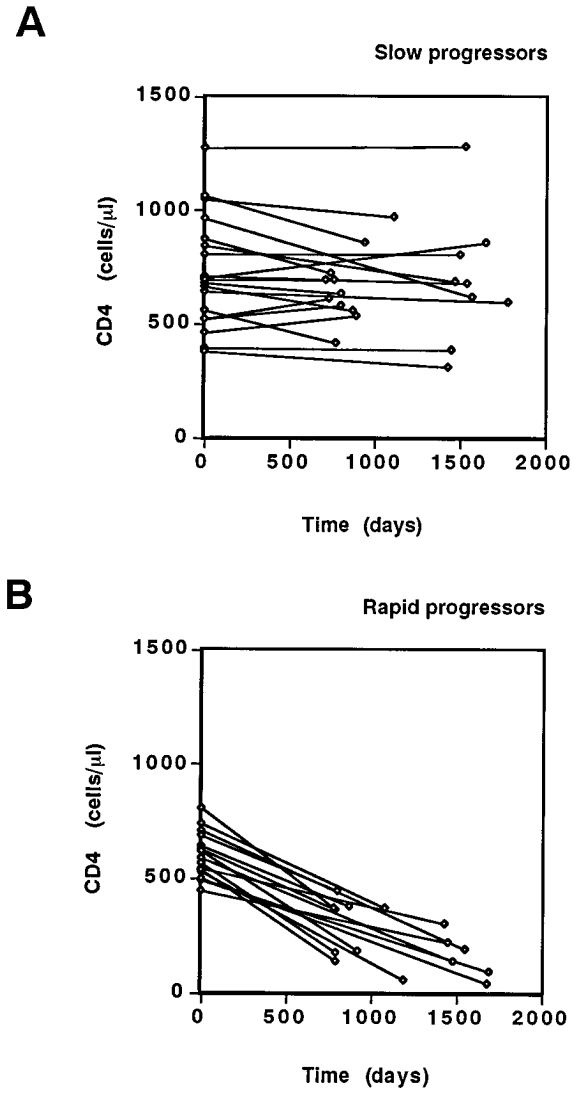

FIG. 2. Comparison of CD4 T-cell slopes for each subcohort. The CD4 T-cell slope for each patient in the study cohort is shown. The *y* intercept and slope for each individual were defined by linear regression analysis on multiple determinations of the absolute CD4 count. The precise terminus of each line was defined by the length of follow-up for each patient. (A) Slow-progressor subcohort; (B) rapid-progressor subcohort. The data for each subcohort are plotted on identical axes to facilitate comparison.

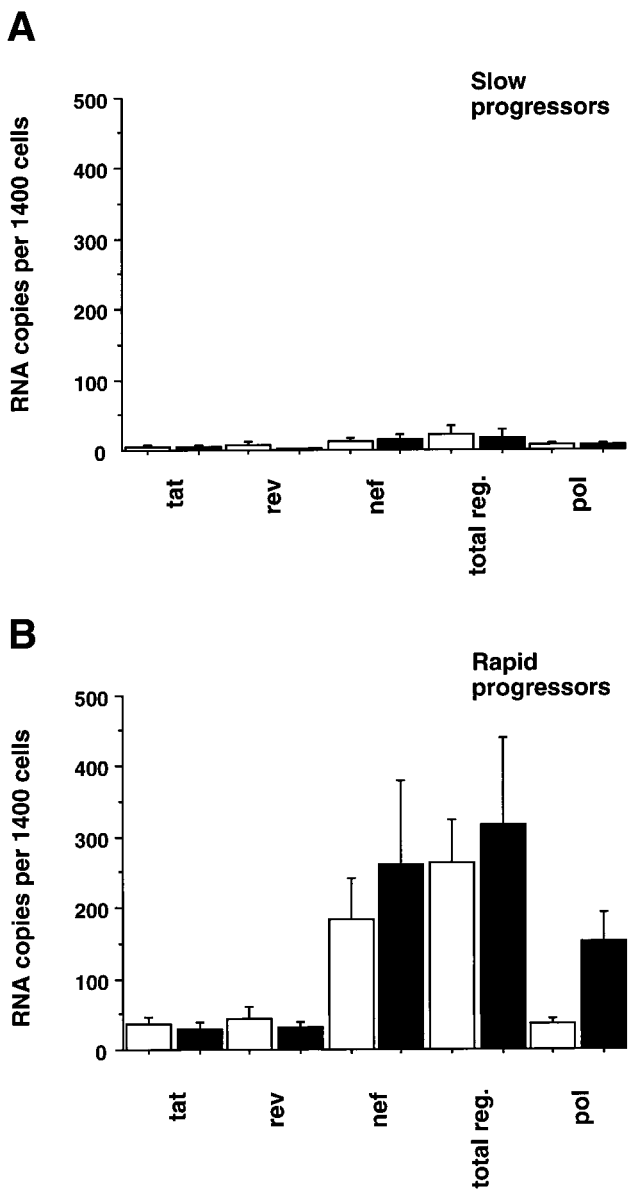

FIG. 3. A comparison of the absolute HIV-1 mRNA levels in each subcohort. The mean and 1 standard error for each mRNA measured are shown. For each mRNA, the data for baseline values are shown by open bars and the data for the follow-up values are shown by closed bars. (A) Slow-progressor subcohort; (B) rapid-progressor subcohort. The data for each subcohort are plotted on identical axes to facilitate comparison. total reg., arithmetic sum of the levels of *tat*, *rev*, and *nef* mRNAs.

baseline values. The data show that the levels of CD4-corrected *tat*, *rev*, *nef*, total regulatory, and *pol* mRNAs all rise very substantially with disease progression. As seen in the lower section of Fig. 4, only the rises in the *rev*, *nef*, and *pol* mRNAs were statistically significant. The splicing ratio remains statistically significant, since, as a ratio, it is unaffected by this multiplicative treatment. Thus, at the level of the $CD4^+$ T-cell population, these data suggest that all HIV-1 mRNA levels rise during disease progression but that the steady-state level of *pol* mRNA rises far out of proportion to that of the doubly spliced transcripts encoding Tat, Rev, and Nef. The net result is a shift in splicing patterns reflecting a greater proportion of unspliced RNA in the viral RNA pool.

Assessment of HIV-1 RNA splicing patterns adds predictive utility to quantitative measures of viral load. The 31 patients in this cohort were divided into their constituent subcohorts of 18 slow progressors and 13 rapid progressors. They were then assigned to one of the four viral RNA expression patterns given in the top portion of Table 4. Seven rapid progressors and one slow progressor showed a shift of ≥3-fold in viral RNA splicing ratios. This slow progressor, patient R, was the patient who subsequently showed a transient drop in his CD4 count at the next follow-up visit. Six rapid progressors and one slow progressor showed increases in viral RNA with splicing shifts of <3-fold. Two slow progressors, but none of the rapid progressors, showed intermediate levels of viral RNA (between 10 and 100 copies), but without either a quantitative increase or a reduction in splicing ratio of >3-fold. Finally, 14

TOTAL COHORT, PRE VALUES

	tat		rev		nef		total reg.		pol	
	SP	RP	SP	RP	SP	RP	SP	RP	SP	RP
Mean	4.6	35.4	7.4	43.9	11.4	184.1	22.1	263.5	6.6	37.2
SD	8.0	33.8	15.0	59.8	27.2	203.7	47.3	220.4	12.5	25.9
Median	1.0	29.0	1.0	23.0	1.0	117.0	6.5	196.0	1.0	30.0
Mann-Whitney	0.0001		0.0009		0.0002		<0.0001		0.0004	

RAPID PROGRESSORS, PRE VS. POST (NON-CD4 CORRECTED DATA)

	tat		rev		nef		total reg.		pol		reg/pol	
	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post
Mean	35.4	29.2	43.9	30.5	184.1	259.8	263.4	317.4	37.2	152.8	7.60	1.98
SD	33.8	32.0	59.8	26.2	203.6	433.5	220.4	441.2	25.9	142.2	8.00	1.67
Median	29.0	21.0	23.0	19.0	117.0	140.0	196.0	183.0	30.0	104.0	4.40	1.45
Mann-Whitney	0.4885		0.8174		0.7778		0.6815		0.0017		0.0079	

RAPID PROGRESSORS, PRE VS. POST (CD4 CORRECTED DATA)

	tat		rev		nef		total reg.		pol		reg/pol	
	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post
Mean	66.7	289.1	87.0	290.8	317.9	2988.5	471.6	3564.8	65.7	1345.7	7.60	1.98
SD	75.5	425.0	141.6	413.0	378.5	5300.0	432.6	5728.1	49.4	2047.4	8.00	1.67
Median	39.3	121.8	44.2	211.1	151.2	698.4	260.7	939.1	58.1	652.2	4.40	1.45
Mann-Whitney	0.0545		0.0210		0.0378		0.0612		0.0005		0.0079	

FIG. 4. Comparative analysis of HIV-1 RNA measurements. CD4 corrected data were computed by normalization of the raw data to 1,000 CD4 cells. SD, standard deviation; Mann-Whitney, *P* value derived from this statistical treatment; SP, slow progressors; RP, rapid progressors; pre, baseline sample; post, follow-up sample.

slow progressors but none of the rapid progressors showed very low levels of viral RNA without quantitative changes. The middle two groups represent nine patients (29% of the cohort) with substantial amounts of viral RNA without large splicing shifts that could not be placed in the high-RNA and high-splicing shift group or the low-RNA group. Six of the seven patients that showed increased amounts of viral RNA without splicing shifts and both of the patients that showed no RNA increase or splicing shift were appropriately sorted according to disease progression. This allowed us to condense the original four groups into the following two: high RNA or shift and low RNA or intermediate without shift. As seen in the lower portion of Table 4, all 13 of the rapid progressors sorted into the high RNA or shift category and 16 of 18 of the slow progressors sorted into the low RNA or intermediate without shift category. These categories were highly predictive of disease progression on the basis of both quantitative levels of viral RNA and viral RNA splicing patterns ($P < 0.0001$, Fisher's exact test).

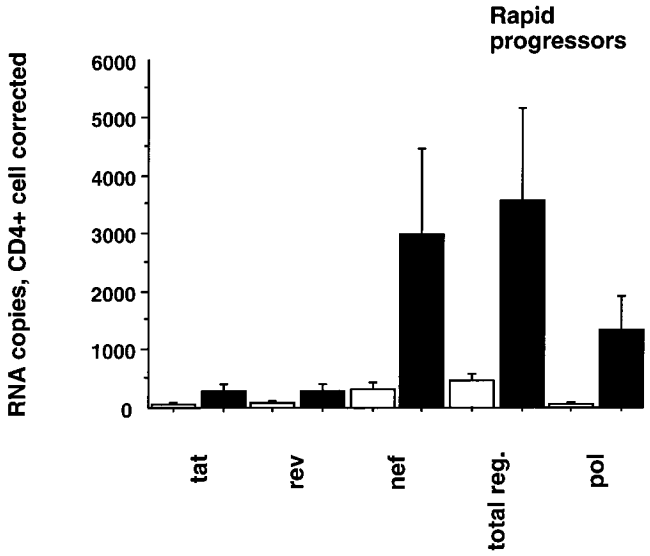

FIG. 5. CD4 cell-adjusted HIV-1 mRNA levels for the rapid-progressor subcohort. The mean and 1 standard error for each mRNA measured are shown. For each mRNA, the data for the baseline values are shown by open bars and the data for the follow-up values are shown by closed bars. The data for this subcohort, given in Table 3, were normalized for 1,000 $CD4^+$ T cells and plotted in a fashion similar to that of Fig. 3B. total reg., arithmetic sum of the levels of *tat*, *rev*, and *nef* mRNAs.

DISCUSSION

Longitudinal studies are critical to the dissection of the molecular pathogenesis of HIV disease as well as to the validation of viral burden as a surrogate marker of disease progression. This experimental design is also inherently more robust for the interpretation of temporal changes in RNA expression, as the baseline values for all patients can be used as

TABLE 4. Quantitative and qualitative HIV-1 RNA patterns and disease progression

HIV-1 RNA expression pattern	No. of patients in indicated cohort showing pattern	
	Rapid progressors	Slow progressors
Original classification		
Splicing shift ≥ 3	7	1[a]
RNA increase with splicing shift < 3	6	1
No RNA increase with splicing shift < 3	0	2
Low RNA, no changes	0	14
Condensed classification[b]		
High RNA or shift	13	2
Low RNA or intermediate without shift	0	16

[a] This patient had a transient 46% decline in CD4 count at the next 2-month follow-up visit.
[b] $P < 0.0001$ by Fisher's exact test.

a reference for subsequent transcript levels. In the present study, we have asked whether HIV-1 viral RNA measurements are predictive of disease progression. We have used the terms slow progressor and rapid progressor in this study to distinguish between those patients who had a stable clinical course and those who had a decline of ≥50% in CD4 cell count and/or developed opportunistic infection. It should be emphasized that approximately half of the slow-progressor subcohort in this study would not meet the rigorous definition of long-term nonprogressors in that their CD4 slopes were negative and the length of follow-up was less than the required 5 years in many cases (14). Even so, the criteria for disease progression employed in this study were still useful for discrimination between patients with markedly different clinical courses.

We feel that the data presented in this study further validate PBMC-based measurements of HIV-1 viral RNA as a surrogate marker for disease progression. Since the baseline measurements presented in this work were drawn prior to any intervention, they represent a natural history data set. First, these data confirm earlier work by our group and others that HIV-1 viral RNA is uniformly expressed in early-stage, asymptomatic patients (1, 4, 5, 11, 12, 20, 24, 36–39, 45, 46, 51, 55). Second, these baseline measurements were predictive of disease progression. The follow-up viral RNA determinations in this study were all drawn after experimental intervention with vaccine products of unknown clinical impact and, in many cases, with Food and Drug Administration-approved antiretroviral drugs. Although it is possible that the rises in *tat*, *rev*, and *pol* mRNAs and the attendant shift in the balance of spliced to unspliced viral RNAs seen with disease progression in this study may be valid only in those patients undergoing concurrent intervention with HIV-1 envelope subunit vaccine products and antiretroviral drugs, we feel that this is unlikely. Ultimately, only additional studies of this type will be able to address the generalizability of the findings presented in the present study.

Recently, two smaller, longitudinal studies of HIV-1 RNA expression in PBMC have demonstrated a correlation between viral load and disease progression (20, 44). One of these two longitudinal studies (an examination of 10 patients) did not address the issue of RNA splicing patterns and disease progression (20). In the second study, Saksela and colleagues studied 18 patients in the New York Blood Center cohort using a quantitative, noncompetitive RT-PCR assay (44). This study showed that increased viral RNA load predicted disease progression in the seven patients that developed a CD4 cell decline during the course of the study. None of the 11 nonprogressors in that study were found to express large amounts of multiply spliced RNA but small amounts of unspliced RNA. In contrast, 3 of the 18 slow progressors in our study showed such a pattern previously referred to as blocked (Table 2, patients R, S, and Z) (51). It is formally possible that the RNA data for these patients represent a differential efficiency of amplification for the regulatory mRNAs over *pol* mRNA, but we feel that this is unlikely. Patient R showed a transient drop in CD4 count and a splicing ratio shift after the follow-up viral RNA measurements. This observation was possibly reflective of a transient burst of viral RNA expression. It is possible that the unusual viral RNA expression pattern in these three patients is an intermediate between the pattern of very low-level expression and the pattern of high-level expression with viral RNA splicing shifts. Thus, it will be important to compare the clinical courses of these three patients with those of the remaining 14 members of the slow-progressor cohort to see if they progress more rapidly. Saksela et al. (44) identified seven progressors in their study. Of these, two (patients A and B) showed a splicing ratio shift similar to that which we observed in 9 of 13 of our rapid progressors. It is difficult to assess the remaining five patients in their study, as only a semiquantitative summary of their quantitative results was shown. Taken together, the data from these studies strongly associate PBMC viral RNA levels with disease progression and confirm that the blocked viral RNA expression pattern described by Seshamma et al. is an identifiable in vivo expression pattern (51).

It should be noted that contamination of the cell-associated viral RNA pool by the rising level of cell-free viral RNA previously reported for late-stage patients (4, 39) could have influenced this ratio, although we have no direct experimental evidence for this. Conversely, this previously reported rising level of cell-free RNA could have been influenced by cross-contamination by cellular nucleic acids.

It is presently unclear what proportion of HIV-1-infected cells not producing virions express no viral RNA, short promoter-proximal RNAs, or only multiply spliced mRNAs. The following discussion, therefore, is based on a population analysis of HIV-infected cells. Previous work by our group and by Pomerantz and his colleagues demonstrated a shift in the balance of spliced to unspliced viral RNA that accompanied induction of high-level virion production in persistently infected cell lines (35, 41). On the basis of *rev* complementation studies of *rev*-deficient proviruses, Pomerantz has speculated that this switch may be mediated by a Rev threshold effect in HIV-1-infected cells that produce little or no virus (40). Once a certain stoichiometry of Rev protein to Rev response element (RRE)-containing mRNAs is attained, the stabilization of these transcripts could support high-level virion production (40). The finding that multiple copies of Rev protein are necessary for Rev-RRE interactions to result in the Rev phenotype support this hypothesis (8, 30, 31). Only after reaching a threshold level of Rev protein would the balance of transcripts in the rising level of total viral RNA favor the proportional enrichment for unspliced mRNA. Rev would provide for a greater level of unspliced mRNA since Rev serves to increase the nuclear efflux of such RRE-containing viral RNAs (2, 6, 7, 13, 17, 21, 32). Once in the cytoplasm, unspliced mRNA is segregated away from the splicing machinery of the nucleoplasm, which is capable of processing it into spliced, subgenomic viral mRNAs. This would provide for a greater level of cytoplasmic unspliced RNA both to serve as mRNA for the translation of *gag* and *pol* proteins and to be available for

packaging into virions as genomic RNA. Both processes are crucial to the high-level production of virus seen in late-stage HIV disease (1, 4, 5, 9, 11, 12, 20, 23, 24, 26, 33, 36–39, 44–46, 51, 55).

Most of this view of HIV RNA expression has been derived from work with cell culture models. We believe that we have now demonstrated evidence that supports this view of HIV expression in natural infection. We have shown that at the level of $CD4^+$ T cells, disease progression is associated with a rise in all HIV-1 transcripts measured, but especially in the mRNAs encoding Tat, Rev, and the Gag-Pol proteins. We have further shown that this is accompanied in many cases by a shift in the balance of HIV-1 RNA from spliced to unspliced mRNA. This is likely the end result of a collapse of effective host immunoregulation of HIV expression which favors the existence of HIV-1-infected cells producing high levels of virus. We believe that taken together, these data provide direct support for a model for HIV pathogenesis based on an increasing level of expression of HIV-1 throughout disease progression.

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